SAF-2, a Splice Variant of SAF-1, Acts as a Negative Regulator of Transcription*

Bimal K. Ray‡, Ryan Murphy, Papiya Ray, and Alpana Ray

From the Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri 65211

Serum amyloid A-activating factor-1 (SAF-1), a Cys$_2$His$_2$-type zinc finger transcription factor, regulates inflammation-induced expression of serum amyloid A protein that is linked to the pathogenesis of reactive amyloidosis, rheumatoid arthritis, and atherosclerosis. Here we report the identification of a novel splice variant, SAF-2, of the SAF family bearing strong sequence similarity to SAF-1. The N-terminal 426 amino acids of both SAF-1 and SAF-2 are identical containing two polyalanine tracts, one proline-rich domain, and six zinc fingers. However, the C terminus of SAF-2 containing two additional zinc fingers is different from SAF-1, which indicates the capability of different biochemical function. We show that SAF-2 interacts more avidly with the SAF-binding element, but its transactivation potential is much lower than SAF-1. Furthermore, co-expression of SAF-2 markedly suppresses SAF-1-regulated promoter function. Finally, we show that the level of SAF-2 protein is reduced during many inflammatory conditions, whereas the SAF-1 protein level remains unchanged. Together, these data suggest that the relative abundance of SAF-2 plays a critical role in the fine tuned regulation of inflammation-responsive genes that are controlled by SAF-1.

Persistent high levels of serum amyloid A (SAA)$^1$ protein is linked to various pathophysiological conditions, including amyloidosis, rheumatoid arthritis, and atherosclerosis ($1,2$). Aberrant transcriptional induction of SAA in response to inflammation is regulated by a group of transcription factors in which SAF-1 plays a major role ($3,4$). Consequently, mutation of the SAF-1 DNA-binding element of the SAA gene reduces its transcription by as much as 80% in several nonhepatic cells ($4$). Many inflammatory agents including LPS, PMA, and cytokines like IL-1 and IL-6, which trigger SAA overexpression, induce both the DNA binding and transactivation potential of SAF-1 ($3-7$). These studies showed that SAF-1 could play a critical role in all SAA-linked pathological conditions. Recently, SAF-1 is shown to be involved in the regulation of the γ-fibrinogen gene, whose abnormal expression is associated with myocardial infarction and stroke ($8$). Human and mouse homologs of SAF-1, called MAZ ($9$) and Pur-1 ($10$) respectively, have been identified as a regulator of expressions of c-myc ($9$), insulin ($10$), serotonin 1A receptor ($11$), CD4 ($12$), PNMT ($13$), and CLC-K1 ($14$) genes. All of these observations suggest that the SAF-1/MAZ/Pur-1 transcription factor is involved in controlling expression of genes associated with diverse cellular processes. Its activation in different tissues in response to diverse physiological conditions apparently determines its ability to regulate expression of different genes.

Critical unanswered questions are how transcriptional properties of SAF-1 are regulated. In general, activities of many transcription factors are regulated either by controlling expression of the genes coding these factors at the transcriptional level or by modification of the proteins at post-translational level, most often by phosphorylation ($15,16$). Some transcription factors are regulated via interaction of another protein that has a regulatory role, such as that seen in the case of NF-$k$B/IkB association ($17$). Alternative splicing is another important mechanism for regulating activity of a transcription factor in which the same gene can be used to generate splice variants with different functional activities (reviewed in $18-20$). Wilm's tumor gene product, a Cys$_2$His$_2$ zinc finger containing transcription factor ($21$), cAMP-responsive element-binding protein modulator ($22$), and signal transducer and activator of transcription, STAT3 ($23$), represent a few examples of many known transcription factors that contain multiple splice variants with distinct functional properties.

Here we describe alternative splicing of SAF transcripts that yields a novel splice variant, designated as SAF-2, with an additional exon that is normally embedded within a large intron. Insertion of this exon in SAF-2 mRNA results in the formation of SAF-2 protein containing a C-terminal region that is different from SAF-1. Compared with SAF-1, SAF-2 protein has higher DNA binding but reduced transactivation ability. Furthermore, overexpression of SAF-2 suppresses SAF-1-regulated promoter function. Interestingly, during inflammation, the level of SAF-2 protein is considerably reduced, whereas the SAF-1 protein level remains unchanged. These unique properties of SAF-2 strongly support the notion that SAF-2 plays a critical role in fine-tuning the regulation of all SAF-1-controlled genes.

EXPERIMENTAL PROCEDURES

Isolation of a Splice Variant—HeLa cDNA library in λgt-11 (a gift from M. Blanar) was screened with a rabbit cDNA probe of SAF-1 (3). Five positive clones were selected and subcloned into the plasmid pTZ19U and sequenced. Human genomic DNA library in A5ML3 (Clontech) was screened with the SAF-1 cDNA probe, and three independent positive clones were selected. Regions of the phase DNA spanning the SAF-1 gene were sequenced.

Received for publication, June 25, 2002, and in revised form, September 20, 2002
Published, JBC Papers in Press, September 20, 2002, DOI 10.1074/jbc.M206299200

‡ To whom correspondence should be addressed: Dept. of Veterinary Pathobiology, University of Missouri, Columbia, MO 65211. Tel.: 573-884-4641; Fax: 573-884-5414; E-mail: rayb@missouri.edu.

* This work was supported in part by National Institutes of Health Grant R01 DK49205 and funds from the College of Veterinary Medicine, University of Missouri. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/Eli Data Bank with accession number(s) AF489858.

The abbreviations used are: SAA, serum amyloid A; SAF, SAA-activating factor; MAZ, Myc-associated zinc finger protein; CAT, chloramphenicol acetyltransferase; MAP kinase, mitogen-activated protein kinase; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; EMSA, electrophoretic mobility shift assay; IL, interleukin; RT, reverse transcriptase; ESE, exonic splicing enhancer; RPA, RNase protection assay.

46822
This paper is available on line at http://www.jbc.org

Printed in U.S.A.
**Plasmids Constructs**—The CAT reporter plasmid, wtSAF-CAT, was constructed by ligating three tandem copies of the wild-type CAT-binding element, bp −254 to −226 of the SAA promoter (3) into pBLCAT2 (24). Reporter plasmid, 0.6 SAA-CAT3, was constructed by ligating rabbit SAA genomic DNA sequences from −605 to +63, a KpnI + DraI fragment, into the pBLCAT3 vector. The pCMVFLAG-SAF-1 was constructed by inserting a FLAG tag sequence in-frame at the N terminus of full-length SAF-1 cDNA and further subcloning in the pcDNA3 vector (Invitrogen). The pCMVHis-SAF-2 expression plasmid was constructed by inserting full-length SAF-2 cDNA into pcDNA3, I/His vector (Invitrogen). For RNAse protection assay, a SucII-EcoRI fragment of SAF-2 cDNA spanning 164 bp of exon V + 305 bp of exon V was cloned into pGEM3Z (Promega).

**Cell Culture and Transfection**—HeLa (human epithelial carcinoma) and THP-1 (human monocyte/macrophage) cells were obtained from the American Type Culture Collection. HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing a high concentration of glucose (4.5 g/liter) and supplemented with 7% fetal calf serum. THP-1 cells were grown in RPMI 1640 containing 10% fetal calf serum. HeLa cells were transiently transfected by the calcium phosphate method (26) using a mixture of reporter chloramphenicol acetyltransferase (CAT) plasmid, 0.5 μg of pSV-beta-gal plasmid (Promega) as a control for measuring transfection efficiency, and carrier DNA so that the total amount of DNA in each transfection assay remained constant. Twenty four hours after transfection, cells were harvested, and extractions were prepared for the determination of CAT activity following the methods described previously (3, 4).

**RNA Isolation and Northern Blot**—Total RNA was isolated by using guanidium thiocyanate (27) from THP-1 and HeLa cells, both untreated and those treated with either LPS (10 μg/ml), PMA (100 ng/ml), or a combination of TNF-α (10 units/ml) and IL-12 (200 units/ml) (28). Rats were injected subcutaneously with a single dose of turpentine (1 ml/kg of body weight), casein (3 ml of 10% solution/kg of body weight), or silver nitrate (1 ml of 2% solution/kg of body weight). Twenty four hours after the injection, animals were sacrificed, and tissues were collected for RNA isolation. Poly(A)+ RNA was isolated by using oligo(dT)-Sepharose. For Northern analysis 50 μg of total RNA of each sample was fractionated in a 1% agarose gel containing 2.2% formaldehyde and transferred onto a nylon membrane. The blot was hybridized to an SAF-2-specific probe that contained the 223-bp unique sequence probe (29). Two complementary oligonucleotides, 5′-GGCTCTCCT-GTCACCCCAAGCCCC-3′ and 3′-CGAGGAGGGGGCTCTGGG- GG-5′ were annealed to prepare the double-stranded SAF-binding element. For DNA binding assays, bacterially expressed SAF-1 and SAF-2 proteins were phosphorylated by MAP kinase following the method described earlier (30). In some assays, anti-SAF-1 antiserum was added in the reaction mixture during a preincubation period of 30 min on ice. A non-radioactive competitor double-stranded oligonucleotide that contains the SAF-binding sequence element of SAA promoter, −254 to −226 (3), was added in some assay mixtures to assess the specificity of the DNA-protein complexes.

**Western Immunoblot Assay**—Proteins were separated by SDS-11% PAGE and transferred onto nitrocellulose membrane. The immunoblotting was performed as described earlier (3) with either anti-SAF-1, anti-SAF-2, anti-FLAG (Sigma), or anti-His tag (Santa Cruz Biotechnology) antibodies as indicated in the figure legends. Bands were detected by using a chemiluminescence detection system (Amersham Biosciences).

**RESULTS**

**Structurally Altered Form of SAF-1**—Alternative splicing is a common and economic mechanism of gene regulation in which multiple, functionally distinct protein isoforms are generated from a single gene. Furthermore, relative abundance of different isoforms may provide additional levels of regulation. To test whether the expressions of SAF-regulated genes are governed by this mechanism, we searched for distinct members of the SAF family. Screening of a human Agt-11 HeLa cDNA expression library with SAF-1 cDNA resulted in the isolation of several full-length SAF cDNA clones that were slightly different in size. Sequence analysis showed that two cDNA clones were identical to MAZ (9), the human homolog of rabbit SAF-1. One other full-length cDNA clone, termed SAF-2, contained identical sequences at the N-terminal end but had additional 254 positions in the terminal region. These transcripts were created by alternative splicing of SAF-1 and SAF-2. SAF-2 proteins were phosphorylated by MAP kinase following the method described earlier (3) with either anti-SAF-1, anti-SAF-2, anti-FLAG (Sigma), or anti-His tag (Santa Cruz Biotechnology) antibodies as indicated in the figure legends. Bands were detected by using a chemiluminescence detection system (Amersham Biosciences).

**Common Origin of SAF-1 and SAF-2**—Sequence homology between SAF-1 and SAF-2, except for an insertion of 223 nucleotides in the SAF-2 mRNA, indicated the possibility of a common origin of these two transcripts. To investigate whether these transcripts could arise from a single gene by alternative splicing, we searched for the genomic DNA representing this gene. The gene coding for SAF-1 was isolated from the human genomic library and was characterized by sequencing. The gene for SAF-1 contained five exons and four introns (Fig. 2A); the intron-exon boundaries were in accordance with the consensus splice donor and acceptor sequences. The genomic sequence of SAF-1 matched with the published sequence of human MAZ (31) genomic sequence. While examining the 1333-nucleotide-long sequence of the fourth intron, we detected the presence of 223 nucleotides that were identical to the unique sequences present in the SAF-2 cDNA (Fig. 2B). Consensus splice donor and acceptor sequences plus a potential polypyrimidine tract for PTB binding, which are normally found in vertebrate precursor mRNA splice sites, also flank this 223-nucleotide-long region. This result indicated that alternative splicing within the fourth intronic region may have resulted in the generation of SAF-2 mRNA containing an additional exon.
FIG. 1. Analysis of a structurally altered form of SAF. Nucleic acid sequence of a novel form of SAF cDNA, designated as SAF-2, from HeLa cells is shown. The initiator ATG codon is depicted as white box. Comparative analysis of SAF-2 sequence with that of SAF-1, a homolog of rabbit SAF-1, is shown. Dots indicate common sequence between SAF-1 and SAF-2. The unique coding triplets of SAF-1 and SAF-2 are shown by underlines and overlines, respectively. Polyalanine, polyproline, and zinc finger domains are indicated. Termination codons of SAF-1 and SAF-2 are indicated by shaded and black boxes.
the interaction of polypyrimidine tract-binding protein. Overlined sequences.

The cDNA sequence. Five exons are defined by the exons was determined by DNA sequence analysis and comparison with the cDNA sequence. The terminal TGA codons of SAF-1 and SAF-2 are shown by arrows. B, DNA sequence of the alternate exon V is shown by the shaded area along with the flanking intronic sequences. Overlined sequence represents a potential polypyrimidine tract-binding protein that is absent in the SAF-1 mRNA. To verify, we performed RT-PCR analysis using two primers flanking the 223-nucleotide putative exon, as described in Fig. 3A and HeLa cell mRNA. Agarose gel analysis revealed two products of 205 and 428 bp in size. (lane 1). The 205-bp band co-migrated with the PCR product derived from SAF-1 cDNA (lane 2) and the 428-bp band of lane 1 co-migrated with the PCR product of SAF-2 cDNA (lane 3) following amplification with the same two primers. These results verified in vivo existence of SAF-2 mRNA in HeLa cells. Although RT-PCR in general is considered not to be quantitative, it was interesting to note that the intensity of the 428-bp band was less than the 205-bp band indicating that the level of SAF-2 mRNA might be lower than SAF-1. To estimate the relative abundance of SAF-1 and SAF-2, we designed an RNase protection probe that distinguishes between the protected fragments generated by SAF-2 (469 nucleotides) and SAF-1 (305 nucleotides). A schematic describing the riboprobe, analysis. A 20-mer oligonucleotide, specific for exon V, was used as a primer in reverse transcription (RT) reaction. For PCR, U4, a 22-mer oligonucleotide specific for exon IV, and D2, a 20-mer oligonucleotide specific for exon V, were used. RT-PCR products were fractionated in an agarose gel and are identified by arrows (lane 1). PCR analysis of the SAF-1 and SAF-2 cDNAs with U4 and D2 primers generated respective cDNA-specific products (lanes 2 and 3). SAF-1-specific PCR product co-migrates with 205-bp size product of total RNA RT-PCR, whereas SAF-2-specific PCR product co-migrates with 428-bp size RT-PCR product. B, schematic of the RNase protection analysis for simultaneous detection of SAF-1- and SAF-2-specific transcript accumulation. Antisense RNA probe was prepared as described under "Experimental Procedures." Predicted sizes of the protected fragments are shown in parentheses. C, RNase-protected bands were detected by autoradiography. Lane 1 contains T7 RNA polymerase-transcribed 32P-labeled probe without any treatment. Lanes 2 and 3 contain protected RNA following incubation of the 32P-labeled probe with HeLa cell RNA and yeast tRNA, respectively. In lane 4, 32P-labeled sense-strand RNA prepared by transcription with SP6 RNA polymerase was incubated with HeLa RNA. Due to the presence of alternative flanking sequences of the polylinker region of pGEM3Z plasmid vector, the untreated probe in lane 1 migrates slower than the SAF-2 mRNA protected band in lane 2.

Fig. 2. Organization of exons and introns in SAF-1 genomic DNA. A, human SAF-1 genomic DNA spanning the entire exonic sequences was located within a 5-kb DNA fragment. Arrangement of the exons was determined by DNA sequence analysis and comparison with the cDNA sequence. Five exons are defined by the stippled boxes with the lengths of exons and introns marked. Intron 4 is marked by a bracket, and an alternative exon within this intron is designated as exon V that is present in SAF-2. The termination TGA codons of SAF-1 and SAF-2 are shown by arrows. B, DNA sequence of the alternate exon V is shown by the shaded area along with the flanking intronic sequences. Overlined sequence represents a potential polypyrimidine tract-binding protein that is absent in the SAF-1 mRNA. To verify, we performed RT-PCR analysis using two primers flanking the 223-nucleotide putative exon, as described in Fig. 3A and HeLa cell mRNA. Agarose gel analysis revealed two products of 205 and 428 bp in size. (lane 1). The 205-bp band co-migrated with the PCR product derived from SAF-1 cDNA (lane 2) and the 428-bp band of lane 1 co-migrated with the PCR product of SAF-2 cDNA (lane 3) following amplification with the same two primers. These results verified in vivo existence of SAF-2 mRNA in HeLa cells. Although RT-PCR in general is considered not to be quantitative, it was interesting to note that the intensity of the 428-bp band was less than the 205-bp band indicating that the level of SAF-2 mRNA might be lower than SAF-1. To estimate the relative abundance of SAF-1 and SAF-2, we designed an RNase protection probe that distinguishes between the protected fragments generated by SAF-2 (469 nucleotides) and SAF-1 (305 nucleotides). A schematic describing the riboprobe, analysis. A 20-mer oligonucleotide, specific for exon V, was used as a primer in reverse transcription (RT) reaction. For PCR, U4, a 22-mer oligonucleotide specific for exon IV, and D2, a 20-mer oligonucleotide specific for exon V, were used. RT-PCR products were fractionated in an agarose gel and are identified by arrows (lane 1). PCR analysis of the SAF-1 and SAF-2 cDNAs with U4 and D2 primers generated respective cDNA-specific products (lanes 2 and 3). SAF-1-specific PCR product co-migrates with 205-bp size product of total RNA RT-PCR, whereas SAF-2-specific PCR product co-migrates with 428-bp size RT-PCR product. B, schematic of the RNase protection analysis for simultaneous detection of SAF-1- and SAF-2-specific transcript accumulation. Antisense RNA probe was prepared as described under "Experimental Procedures." Predicted sizes of the protected fragments are shown in parentheses. C, RNase-protected bands were detected by autoradiography. Lane 1 contains T7 RNA polymerase-transcribed 32P-labeled probe without any treatment. Lanes 2 and 3 contain protected RNA following incubation of the 32P-labeled probe with HeLa cell RNA and yeast tRNA, respectively. In lane 4, 32P-labeled sense-strand RNA prepared by transcription with SP6 RNA polymerase was incubated with HeLa RNA. Due to the presence of alternative flanking sequences of the polylinker region of pGEM3Z plasmid vector, the untreated probe in lane 1 migrates slower than the SAF-2 mRNA protected band in lane 2.

SAF-1 and SAF-2 mRNAs Produce Distinct Proteins—The open reading frames of SAF-1 and SAF-2 code for proteins with 477 and 493 amino acids, respectively (Fig. 4A). The coding sequences in the SAF-1 mRNA-derived protein has several potential characteristic structural domains, which include three polyalanine tracts, one proline tract, and six zinc finger domains. Due to the insertion of the additional exon, designated as V’, the open reading frame of SAF-2 gained two additional zinc finger domains and created an in-frame premature translation termination codon, TGA, that deleted a C-terminal polyalanine tract (Fig. 4A). We synthesized both proteins by cloning the respective cDNAs in bacterial expression vectors. As seen in Fig. 4B, SAF-2 cDNA produced a protein of expected molecular mass of 51,118 daltons (lane 3), which was slightly larger than SAF-1 protein with an expected molecular mass of 48,685 daltons (lane 2). Mass spectroscopic analysis and microsequencing of peptides derived from these two proteins, purified by affinity chromatography and fractionation in polyacrylamide gel, also revealed the presence of altered amino acid sequence in SAF-2 (data not shown).

SAF-2 Is Widely Expressed in Adult Tissues—To examine the expression of SAF-2, a commercially available RNA blot was hybridized with a probe containing 223-bp unique sequences of SAF-2 mRNA. We detected SAF-2 mRNA at almost equal levels in several tissues (Fig. 5A). To detect SAF-2 protein without the interference from SAF-1, an antibody was prepared against the unique epitope of SAF-2 (amino acid residues 438–454 of SAF-2 protein). As seen in Fig. 5B, this rabbit polyclonal antibody cross-reacted with bacterially expressed SAF-2 (lane 2) but not with SAF-1 protein (lane 1). Western blot analysis was performed on total protein extracts from a variety of adult rabbit tissues using the antipeptide SAF-2 antibody. Consistent with the RNA expression pattern, the SAF-2 protein was detected at almost similar levels in all of the tissues that were examined (Fig. 5C).

SAF-2 mRNA and Protein Levels Are Reduced during Inflammation—The fact that some SAF-1-regulated genes are induced during inflammation prompted us to investigate whether the expression of SAF-2 was modulated during inflammation. To examine, we used human monocyte/macrophage cells, THP-1, that are highly responsive to several inflammatory agents, including IL-1, IL-6, PMA, and LPS and are known to overexpress serum amyloid A, one of the SAF-1-regulated genes (1, 3). The mRNA level of SAF-2 in IL-1- and IL-6-, PMA-, or LPS-treated THP-1 cells was noticeably less as compared with the untreated cells (Fig. 6A). The SAF-1 mRNA level, in contrast, was not affected by any of these inflammatory agents.
A lower level of SAF-2 mRNA was also seen in the liver tissues of rabbits that were challenged with several different inflammatory agents (Fig. 6B). These results indicated that the level of SAF-2 transcript is reduced during inflammation.

To determine whether the protein level of SAF-2 correlates with its mRNA level, we used Western immunoblot analysis of SAF-2 protein in liver tissues of untreated, turpentine-, LPS-, casein-, and silver nitrate-injected rabbits (Fig. 6C, lanes 1–5). The relative level of SAF-2 protein was considerably low in all inflamed rabbit liver tissues than the untreated tissue (Fig. 6C). Same samples were probed with an anti-SAF1 antibody directed against amino acids 456–472 of SAF-1 protein, a domain not present in the SAF-2 protein. The level of SAF-1 protein remained almost unchanged under these inflammatory conditions (Fig. 6D, lanes 1–5). Together, the data suggest that the level of SAF-2 mRNA and consequently the corresponding protein decline during many inflammatory conditions.

SAF-2 Binds to the SAF DNA-binding Element More Efficiently than SAF-1—To test whether changes in the structure of SAF-2 have altered its DNA binding ability and, if so, at what level compared with that of SAF-1, we performed DNA binding assay using purified bacterially expressed SAF-1 and SAF-2 proteins. Because phosphorylation has been shown to facilitate the DNA binding ability of SAF-1 (30), both proteins were phosphorylated with MAP kinase prior to the DNA binding assay. As shown in Fig. 7A, more DNA probe was bound by SAF-2 compared with an equivalent amount of SAF-1 (compare between lanes 2–5 and 6–8). By Western immunoblot analysis, we verified that a higher level of DNA binding ability of SAF-2 was not due to any differences in the input protein level (Fig. 7B) or due to any differences in phosphorylation level (Fig. 7C). Incidentally, both SAF-1 and SAF-2 proteins contain the same number of potential phosphorylation sites, which are located in the common region of these two proteins (3, 30).

![Fig. 4. Cloned cDNAs for SAF-1 and SAF-2 produce distinct proteins.](image)

**A**  
Both SAF-1 and SAF-2 proteins initiate at the same ATG codon in exon 1 but terminate at TGA codons located in exon V and V giving rise to proteins that contain 477 and 493 amino acids, respectively. Different structural domains and their locations with starting and ending amino acids are marked. **B**  
Bacterially expressed SAF-1 and SAF-2 proteins were fractionated in an SDS-polyacrylamide gel. Migration positions of these proteins in lanes 2 and 3 are identified. Lane 1 contains bacterial cell extract from vector-transfected cells. Numbers at left show molecular masses in kilodaltons.

![Fig. 5. SAF-2 is widely expressed in adult tissues.](image)

**A**  
Northern analysis of a RNA blot (purchased from Clontech) containing 2 μg of mouse poly(A)+ RNA per lane from heart (lane 1), brain (lane 2), spleen (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and testis (lane 8) was performed using a 32P-labeled SAF-2-specific cDNA probe. The same RNA blot was washed in boiling water to remove the radiolabeled probe and re-probed with 32P-labeled actin cDNA probe. **B**  
Bacterially expressed SAF-1 (lane 1) and SAF-2 (lane 2) proteins (5 μg each) were fractionated in an 11% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-SAF-2 peptide (amino acids 438–454) antibody. Lanes 3 and 4 show Coomassie Blue staining of the respective proteins, as indicated in the figure. **C**  
Cell extracts (50 μg of protein in each lane) from liver (lane 1), lung (lane 2), kidney (lane 3), brain (lane 4), skeletal muscle (lane 5), and heart (lane 6) tissues of an adult rabbit were fractionated in an 11% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and immunoblotted with anti-SAF-2 peptide (amino acids 438–454) antibody.
the DNA-protein complexes was done by scintillation counting of the radioactive band as shown in Fig. 7A and plotting the data (Fig. 7D). These data suggested that increased radioactivity in SAF-2-specific complexes, as seen in Fig. 7A, lanes 2–5, is mostly due to a higher affinity of SAF-2 for the probe.

SAF-2 Acts as a Transcriptional Repressor of SAF-1—As it was evident that SAF-2 interacts more efficiently with SAF DNA-binding promoter elements than SAF-1, we were interested in comparing the transcriptional activation properties of these two proteins. HeLa cells were transiently transfected with a CAT reporter gene containing multiple copies of SAF DNA-binding elements and FLAG-tagged expression plasmid containing SAF-1 or SAF-2 cDNA. Co-transfection of the reporter with pCMVSAF-1 plasmid showed a high level of CAT activity in a dose-dependent manner (Fig. 8A). In contrast, transfection of the cells with the same amount of pCMVSAF-2 DNA displayed much lower levels of CAT gene expression. This result suggested that pCMVSAF-2 may have lower transactivation potential than pCMVSAF-1. To verify that lower transactivation potential of SAF-2 did not result from low ectopic expression of SAF-2 protein in the transfected cells, we performed Western blot assay using anti-FLAG antibody. As seen in Fig. 8D, expressions of SAF-1 and SAF-2 proteins were similar. As these transfection assays were conducted using a short SAF DNA-binding element-driven reporter gene, it became important to examine the transactivating potential of SAF-2, in the context of a natural promoter. HeLa cells were transfected with 0.6 SAA-CAT reporter plasmid, containing the 600-bp upstream promoter region of the SAA gene and increasing concentrations of either pCMVSAF-1 or pCMVSAF-2 expression plasmids (Fig. 8C). SAF-2 displayed lower transactivation ability than that of SAF-1. In view of poor transactivation property of SAF-2, it became important to know the effect of SAF-2 on the transactivation property of SAF-1, when both proteins were simultaneously expressed in the cell. To monitor ectopic expression of these proteins without any interference from the other, SAF-1 and SAF-2 cDNAs were cloned in two different tagged expression vectors. HeLa cells were transfected with 0.6 SAA-CAT reporter plasmid, a constant amount of pCMVFLAG-SAF-1 plasmid, and an increasing concentration of pCMVHis-SAF-2 expression plasmid DNA (Fig. 8D). The total amount of DNA in all transfection mixtures was kept same using empty vector DNA. Transactivation of 0.6 SAA-CAT reporter by pCMVFLAG-SAF-1 was reduced in a dose-dependent manner by pCMVHis-SAF-2. It is noteworthy to mention that reporter gene expression was not completely abolished even at the highest concentration of SAF-2. An explanation for this could be that SAF-2 is a poor transactivator of SAA promoter rather than an inhibitor of transcription that is transcriptionally inactive. Western blot assay using anti-FLAG and anti-His tag antibodies showed that ectopic expression of SAF-2 at higher concentrations did not prevent ectopic expression of SAF-1 (Fig. 8E). To ensure that different tags used for monitoring expression of the transfected plasmids had no undue influence on the results, we performed a reciprocal assay in which HeLa cells were transfected in a similar fashion with pCMVHis-SAF-1 and pCMVFLAG-SAF-2 expression plasmids. The result of this experiment (data not shown) was no different from what is shown in Fig. 8D. Together, these results showed that co-expression of SAF-2 can considerably reduce the transactivation potential of SAF-1.

The possibility that SAF-2, due to its higher DNA binding activity (Fig. 7), might influence the DNA binding ability of SAF-1 by competing for the same element was tested by using a constant amount of FLAG-tagged SAF-1 protein and increasing levels of His-tagged SAF-2 protein in a DNA binding assay (Fig. 9). Both proteins were phosphorylated with MAP kinase prior to the DNA binding assay. A single band was seen when both proteins were used alone (lanes 1 and 5). However, when these proteins were combined, a new band, designated as the complex A, was seen (lanes 2–4). Complex A appears to be the heterodimer of SAF-1 and SAF-2 because addition of anti-FLAG or anti-His tag antibody inhibited this complex (lanes 6 and 7). Interestingly, as the concentration of SAF-2 protein was increased, although it favored the formation of complex A, it did not prevent the formation of the SAF-2-specific complex (lanes 2–4). Together these data suggested that the reduced level of SAF-1-mediated transactivation could be, at least in part, due to the formation of SAF-1/SAF-2 heterodimer, which may have lower levels of transactivation potential than the SAF-1 homodimer.

**DISCUSSION**

This study provides first evidence of the existence of an alternatively spliced member, SAF-2, of the SAF family of transcription factors. We show that the splicing pattern of
SAF-2 varies during inflammation, and SAF-2 protein negatively regulates another member, designated as SAF-1, of the family. These data shed new light on the mechanistic details of SAF-regulated genes.

The SAF-1 transcription factor, containing six Cys2His2-type zinc fingers, regulates a variety of genes, and some of them, such as SAA and $\gamma$-fibrinogen, are induced during inflammation. Here we show that the gene coding for SAF-1 generates another functionally distinct isoform, SAF-2, by utilizing the alternate splicing mechanism. Due to alternate splicing of the pre-mRNA, a small exon containing 223 nucleotides is inserted at the C-terminal end of SAF-2 mRNA. Consequently, SAF-1 and SAF-2 proteins are identical at the N-terminal end up to 426 amino acids. Due to the insertion of 223 nucleotides in the mRNA, SAF-2 protein gained two additional zinc fingers, but this event also generated a premature termination codon resulting in deletion of 51 amino acids at the C-terminal end of SAF-2 protein. These differences in the structures suggested that SAF-1 and SAF-2 might have similar but distinct functions. Indeed, we show that SAF-2 with two additional zinc fingers exhibits much higher levels of DNA binding ability than SAF-1 (Fig. 7). However, increased DNA binding ability did not get translated to higher transactivating ability, instead SAF-2 displayed lower transactivation potential. We speculate that one of the reasons may be deletion of 51 amino acids at the C-terminal end that includes a 17-amino acid-long polyalanine tract that is present in SAF-1 (Fig. 1). The polyalanine tract and adjacent sequences were shown to function at low concentration as a transcriptional activator in Drosophila cells (32).

Many genes are known to produce alternatively spliced mRNAs, each encoding a different protein to provide economic and efficient regulation of gene expression. It is estimated that more than one-third of human genes are alternatively spliced (33). This versatile regulatory mechanism of gene expression is utilized during development (34), apoptosis (35), sex determination (20), and hormonal regulation (36) of genes. It is noteworthy that inflammation-regulated splicing of SAF pre-mRNA will be, to our knowledge, the first report of this type. Changes in the relative abundance of SAF-2 in response to different inflammatory conditions were demonstrated by both RNA and protein analysis (Fig. 6). At present, the physiologic or pathophysiologic role of SAF-2 is not fully known, but the shift in relative abundance of SAF-2 mRNA and protein during inflammation is highly suggestive of its biological importance. SAF-1, the major isoform of this family, exhibits low but sustained transcriptional activity under normal conditions. The activity of SAF-1 is highly induced in response to inflammatory agents via phosphorylation by a variety of protein kinases including protein kinase C (5), MAP kinase (30), and protein kinase A (37). We speculate that under normal conditions, SAF-1 is additionally regulated by the SAF-2 isoform, which has higher DNA binding ability but very poor transactivation property. By competing for the same promoter elements, under normal conditions, SAF-2 further regulates expression of SAF-1-regulated genes. The presence of SAF-2 at a low level in almost all tissues that have been examined (Fig. 5).
supports this hypothesis. During inflammation, when the activity of SAF-1 needs to be at its maximum to support inflammation-induced gene expression, SAF-2-driven regulation is lifted by reducing SAF-2 mRNA-specific splicing. Thus the two splice variants could be involved in maintaining a balance for the regulatory function provided by SAF family of proteins. Similar to our observations, cAMP-responsive element-binding protein modulator and STAT3/H9252, splice variants of cAMP-response element-binding protein and STAT3, act as dominant negative regulators of cAMP-response element-binding protein and STAT3 actions (22, 23).

The precise removal of introns from pre-mRNAs is highly complex and requires accurate recognition and pairing of the correct 5′ and 3′ splice sites. Because sequence similarity is always not sufficient to guarantee correct selection of the proper 5′ splice sites, splice site recognition is regulated by additional elements termed as exonic splicing enhancer (ESE) elements (38). Although the majority of ESEs has been reported to be purine-rich, examples of non-purine-rich sequences are recently becoming known. ESEs are interacted with various splicing factors, such as SR proteins, heterogeneous nuclear ribonucleoproteins, small nuclear ribonucleoproteins, and some other novel alternative splicing factors (reviewed in Ref. 39). These factors participate in both constitutive and alternate splicing of pre-mRNAs. Furthermore, identification of polypyrimidine track binding proteins that can repress (40) or positively regulate (41) selection of an exon complicates the whole matter. The presence of SAF-2 mRNA at a much lower level than SAF-1 suggests that this splice site is recognized less favorably. One explanation could be the absence of purine-rich elements in SAF-2-specific exon V′. Instead, a CA-rich motif is present at this region. SR proteins that interact with purine-rich sequences are primarily involved in constitutive splicing, whereas the CA-rich element is shown to interact by alternative splicing factors (42). It is

**FIG. 8.** Transactivation potential of SAF-2. A, HeLa cells were transfected with SAF-CAT2 reporter plasmid (2.0 µg of DNA). Where indicated, increasing amounts (0.5, 1.0, 1.5, and 2.0 µg) of pCMV-FLAG-SAF-1 or pCMV-FLAG-SAF-2 expression plasmid DNAs were added to the transfection mixture. The cells were transfected by incubating 16 h in the presence of DNA in calcium phosphate. Following glycerol shock, the cells were incubated for additional 24 h and then harvested. CAT activity was determined as described under “Experimental Procedures.” The results shown are averages of three separate experiments. B, Western immunoblot analysis of transfected cell extracts with anti-FLAG antibody was performed as described under “Experimental Procedures.” Arrows indicate positions of SAF-1 and SAF-2 proteins expressed in transfected cells. C, HeLa cells were transfected with 2.0 µg of 0.6 SAA-CAT3 reporter plasmid together with (0.5, 1.0, 1.5, and 2.0 µg) of pCMV-FLAG-SAF-1 or pCMV-FLAG-SAF-2 expression plasmid DNA, as described above in A. D, HeLa cells were transfected with 2.0 µg of 0.6 SAA-CAT3 reporter plasmid DNA. In addition, some transfection mixtures contained 2.0 µg of pCMV-FLAG-SAF-1 DNA and increasing amounts (0.5, 1.0, 1.5, and 2.0 µg) of pCMV-His-SAF-2 DNA. E, Western immunoblot analysis for SAF-1 and SAF-2 expression in the transfected cells. Anti-FLAG and anti-His tag antibodies were used to detect respective levels of SAF-1 and SAF-2 proteins in transfected cells.

**FIG. 9.** Competition of DNA binding activity of SAF-1 by SAF-2. Purified FLAG-SAF-1 and His-tagged SAF-2 proteins were used in EMSA with 32P-labeled SAF-binding oligonucleotide probe. SAF-1 and SAF-2 proteins were added in different amounts (in µg) as indicated in the figure. Migration positions of SAF-1- and SAF-2-specific complexes are indicated. The heterodimer of SAF-1 and SAF-2 is designated as complex A and indicated by an arrow. Some binding assay mixture contained anti-FLAG or anti-His tag antibody (Ab) as indicated.
noteworthy that during inflammation, alternative splicing reaction that generates SAF-2 mRNA was reduced even further. We speculate that down-regulation of the splicing factor involved in the selection of exon V is probably one of the reasons of low SAF-2 mRNA level during inflammation. Although there are at present no reports of conditionally regulated splice factors, splicing factors that are developmentally regulated and play an important regulatory role in cell-specific alternative splicing during normal development and disease are already known (43, 44). Further studies are necessary for the identification and characterization of the exonic splicing enhancers and the trans-acting factors to gain insight into the inflammation-induced splicing mechanism that controls SAF-1 and SAF-2 mRNA synthesis.

Acknowledgments—We are grateful to Michael Blanar for providing HeLa cDNA library in pAR(31R159/60). We also thank David Pintel and Kleus Jensen for their help in the RNase protection assay.

REFERENCES
1. Sipe, J. D. (1994) Crit. Rev. Clin. Lab. Sci. 31, 225–354
2. Uhlar, C. M., and Whitehead, A. S. (1999) J. Biol. Chem. 274, 8698–8707
3. Her, S., Bell, R. A., Bloom, A. K., Siddall, B. J., and Wong, D. L. (1999) J. Biol. Chem. 274, 8698–8707
4. Uchida, S., Tanaka, Y., Ho, H., Suitoh-Obara, F., Inazawa, J., Yokoyama, K. K., Sasaki, S., and Marumo, F. (2000) Mol. Cell. Biol. 20, 7319–7331
5. Brivanlou, A. H., and Darnell, J. E., Jr. (2002) Science 296, 813–818
6. Hunter, T. (2000) Cell 100, 113–127
7. Uchida, S., Tanaka, Y., Ito, H., Saitoh-Ohara, F., Inazawa, J., Yokoyama, K. K., Sasaki, S., and Marumo, F. (2000) Mol. Cell. Biol. 20, 7319–7331
8. Adams, M. D., Rudner, D. Z., and Rao, D. C. (1996) Curr. Opin. Cell Biol. 8, 331–339
9. Wang, J., and Manly, J. L. (1997) Curr. Opin. Genet. & Dev. 7, 205–211
10. Lapp, A. Z. (1998) Annu. Rev. Genet. 32, 279–305
11. Adachi, K., and Rutter, W. J. (1992) Science 256, 1014–1018
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
14. Ray, B. K., and Ray, A. (1997) Biochemistry 36, 4662–4668
15. Ray, A., and Ray, B. K. (1994) Mol. Cell. Biol. 14, 4324–4332
16. Ray, A., Yu, G. Y., and Ray, B. K. (2002) Mol. Cell. Biol. 22, 1027–1035
17. Shepheard, J., Reic, M., Olson, S., and Gravel, B. R. (2002) Mol. Cell. Biol. 22, 211–210
