Transcriptional Activity of Serum Amyloid A-activating Factor-1 Is Regulated by Distinct Functional Modules*

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Serum amyloid A-activating transcription factor-1 (SAF-1) plays a major role in regulating transcription of several inflammation-responsive genes, including SAA and matrix metalloproteinase-1, that are implicated in the pathogenesis of reactive secondary amyloidosis, atherosclerosis, and arthritis. SAF-1 is a 477-amino acid protein with six zinc fingers. Its activation during inflammatory condition by a phosphorylation event that leads to an altered structure suggested possible structural modification of this protein as a leading cause of higher activity. However, no information is available regarding structural features that might regulate its activity. Here, we have characterized its functional domains, delineating activation and repression modules, DNA binding, and nuclear localization activities. Using GAL4AD chimeras and a DNA-binding assay with proteins prepared from various deletion constructs, the core DNA-binding domain of SAF-1 is mapped between amino acids 282 and 361, which contain second, third, and fourth zinc fingers. Results from several deletion and point mutants using green fluorescent protein reporter show that SAF-1 contains two independent nuclear localization signals; one is composed of a stretch of basic amino acids, and the other is a bipartite signal located within the core DNA-binding domain. SAF-1 contains several negative and positively functioning transactivation modules clustered at the two ends of this protein. Removal of any one of the terminal negative modules renders the SAF-1 protein functionally very active. These findings suggest that the terminal repression modules act in conjunction to regulate the functional activity of this protein.

Transcription factor SAF-1 has been shown to be activated in response to oxidatively modified low density lipoproteins and to increase SAA expression in the monocyte/macrophage cells (6). SAF-1 is also shown to induce matrix metalloproteinase-1 gene expression in the chondrocytes of osteoarthritic patients and to be involved in damaging overall structure of articular cartilage (3).

As a transcription factor, SAF-1 belongs to a family of proteins containing multiple Cys2-His2 type zinc fingers and its human and mouse homologs have been identified as MAZ (7) and Pur-1 (8), respectively. SAF-1/MAZ/Pur-1 regulates expression of a variety of genes including c-myc (7), insulin (8), serotonin 1A receptor (9), adenosivirus major late promoter (10), CD4 (11), γ-fibrinogen (12), PMNT (13), and CLC-K1 (14). SAF-1/MAZ/Pur-1 is also shown to interact with FACT (15), a novel zinc finger protein that is expressed at a higher level in the brain of patients suffering from Alzheimer’s disease and is implicated to play a role in the neurodegeneration associated with this pathology (16). SAF-1/MAZ/Pur-1 interacts with DCC (deleted in colorectal cancer) protein, a putative tumor suppressor during the neural differentiation of P19 EC cells (17).

Several reports have shown that SAF-1 is activated in response to several mediators of signal transduction (6, 18–21). These studies have suggested that structural modification, as a result of phosphorylation, leads to increased activity of SAF-1. Truncated SAF-1 protein has also been shown to be functionally more active compared with the native protein, indicating that structure plays a critical part in determining activity of this protein (20). In order to fully understand the role of SAF-1 in the induction of target gene expression under pathophysiological conditions, it is important to identify different structural features of this protein. To date, little is known as to how SAF-1 recognizes and binds to the promoter, its transactivation properties, or how it gains access to the nucleus to function as a transcription factor. Since regulation of many transcription factors is mediated by the availability of their functional modules, determining the functional modules of SAF-1 is a critical issue in understanding how SAF-1 functions as a transcriptional activator. In this study, we used a combination of in vivo and in vitro analyses to identify the domains involved in DNA binding, nuclear localization, and transcriptional activation. This report identifies a novel mechanism of regulation of SAF-1.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Rabbit synoviooctye (HIG82) cells, obtained from the American Type Culture Collection, were derived from the interarticular soft tissue of the knee joint of a normal female New Zealand White rabbit. These cells were cultured in Dulbecco's modified Eagle's medium containing high glucose (4.5 g/liter) supplemented with 7% fetal calf serum. Transient transfections of HIG82 cells were carried out using the calcium phosphate method (22). The amount of DNA in each transfection assay was kept the same by using carrier DNA. Reporter plasmids and the eucaryotic expression vectors containing...
various fragments of SAF-1 cDNA were transfected in amounts as indicated in the figure legends along with 1 µg of pSV-β-gal (Promega). The pSV-β-gal plasmid DNA was used as an internal control for measuring transfection efficiency. The cells were harvested 24 h post-transfection, and CAT activity in the cell extracts was determined as described previously (22). β-Galactosidase activity was assayed with the substrate o-nitrophenyl-β-D-galactopyranoside as described (22). Cell extracts containing equivalent amounts of β-galactosidase activity were used for a chlorophenol red acetyltransferase (CAT) assay. Prior to the CAT assay, each cell extract was heated at 60 °C for 10 min to inactivate endogenous acetylase. The cells used in transfection assays had no effect on β-galactosidase expression. All transfection experiments were performed at least three times.

**Reporter Plasmids**—The SAF-CAT reporter plasmid was constructed by ligating three tandem copies of the SAF-binding element of the rabbit SAA gene (2) into pBLCAT2 vector (23). The sequence of the SAF-binding element was 5′-CCCTTCCTCTCCACCCACCAAGCCCCCCCC/3′. For activation domain analysis, a reporter CAT gene in minimum basal activity was chosen (24). This plasmid, GAL4E1bCAT, containing five copies of the 17-bp GAL4 binding site (UASg) in front of the adenovirus minimal E1b promoter, was a gift from Erik Flemington. For the analysis of the repressor module, GAL4kCAT (25), which has much higher basal activity, was chosen. GAL4kCAT was prepared by using primers which amplify the GC-rich sequences present in the SAF-1 sequence, GC-methylated (BD Biosciences Clontech) was added to the PCR mixture. PCR products were subcloned in pTZ191U vector and sequenced for verification of the correct identity of each deletion construct. An internal deletion of SAF-1(D193–414) was created by PCR amplification of two segments, 1–192 and 415–477 amino acids of SAF-1, followed by ligation of these two segments in frame. Similarly, a deletion of SAF-1(477–1932) was created by PCR amplification of two segments, 142–192 and 214–477 amino acids of SAF-1 and ligation of the two fragments into pTZ191U. For transient transfection experiments in cultured cells, full-length and truncated SAF-1 sequences were subcloned in the pcDNA3 vector.

**Subcloning of SAF-1 Fragments**—Wild-type SAF-1 and its deletion mutants were constructed initially in plasmid vector pTZI91U (Invitrogen) and further subcloned into expression vectors pRSFET (Invitrogen) and pcdNA3 (Invitrogen). Deletion fragments of SAF-1 were generated by PCR using oligonucleotides containing sticky ends in the pBLCAT2 vector (23) that contains the minimal & promoter of the thymidine kinase gene of herpes simplex virus.

**Construction of GAL4DBDSAF1 Plasmids**—Plasmid RSV-GAL4DBD (26), encoding the DNA-binding domain (DBD) located within amino acids 1–147 of the yeast GAL4 gene, was used to prepare the GAL4DBDSAF-1 construct. Full-length SAF-1 cDNA and deleted SAF-1 fragments were fused in frame C-terminal to the GAL4 DNA-binding domain (GAL4DBD) at the unique BamHI site. This resulting clone was verified by DNA sequence analysis.

**Construction of GAL4ADSAF1 Plasmids**—A 514-bp HindIII-XhoI fragment, containing the GAL4 activation domain was isolated from pACT2 plasmid (BD Biosciences Clontech) and ligated to pcdNA3 vector at HindIII and XhoI sites. This fragment contains a nuclear localization signal, KKKKR, consisting of a stretch of basic amino acids. This plasmid, designated as pDGAL4AD, contains a cytochrome C oxidase immediate early enhancer/promoter that drives transcription of the GAL4AD gene. Full-length SAF-1 cDNA and deleted SAF-1 fragments were fused in frame at the C terminus of GAL4AD at the unique BamHI and XbaI sites. SAF-1 derivatives, ADSAF-1(282–361)ZFmut, with mutations located either within zinc finger 2, 3, or 4, designated as ZF2, ZF3, and ZF4, were generated by substituting two cysteine residues in each zinc finger with alanine residues. PCR amplification with primers containing mutated bases replacing the cysteine codon (TGC and TGT) with an alanine codon (GCC) was used to create the desired mutations. For ligation of N- and C-terminal regions of SAF-1 to the ZF2-ZF4 domain to construct ADSAF-1(282–361)N+C Tag plasmid, DNA sequences corresponding to amino acids 1–89 and 415–477 of SAF-1 were ligated in frame to that of amino acids 282–361. The resulting clones were verified by DNA sequencing.

**Construction of pHR-GFP Chimeras**—Full-length, truncated, and mutant sequences of SAF-1 were ligated to the pHR-GFP-C vector (Stratagene). It should be noted that pHR-GFP-C vector contains a built-in nuclear localization signal, for which GFP proteins stay in both nuclear and cytoplasmic fractions when cells are transfected with this vector. Truncated and mutated SAF-1 cDNAs were generated by PCR amplification using specific primers with the desired terminal sequences and mutated oligonucleotides to introduce base substitutions at the selected sites. The identity of each construct was verified by DNA sequence analysis.

**Fluorescence Microscopy for Nuclear Localization Analysis**—HIG82 cells, grown in a 24-well plate, were transfected with different SAF-1-GFP plasmid DNAs (0.5 µg of DNA for each plasmid) using the calcium phosphate method (22). The live cells in the 24-well plate were visualized 24 h after transfection using an inverted microscope equipped with UV light source and a filter that allows plasmid-derived green fluorescence protein to be visible. The fluorescing cells were photographed at a magnification of x400.

**Protein Preparation**—To prepare His-tagged proteins, wild-type and truncated SAF-1 cDNAs were subcloned into pPRSET vectors. Proteins expressed from these clones were purified by affinity chromatography using a nickel-Sepharose column (Invitrogen) following the manufacturer's protocol.

**Electrophoretic Mobility Shift Assay**—Electrophoretic mobility shift assay was performed with equal protein amounts of bacterially expressed proteins according to the methods described previously (2). Protein concentrations were measured by Bradford's method (27). Radiolabeled probe containing the SAF-binding element of the SAA promoter was prepared by using [α-32P]dCTP as the substrate to label the double-stranded oligonucleotide probe (2). Two complementary oligonucleotides, 5′-GGCTTCTTCTTCACCCACACCCGGGGG-3′ and 5′-CGAGG-CCGAGGGGACACACGGCGGGGGGG-3′, were annealed to prepare the double-stranded SAF-binding element.

**Western Immunoblot**—50 µg of protein from transfected HIG82 cell extracts were fractionated by electrophoresis in an SDS-5%/12% polyacrylamide gel. Proteins were electrophoretically transferred onto a nitrocellulose membrane and probed with various antibodies as described in the figure legends. Membrane was further incubated with horseradish peroxidase-conjugated secondary antibody and chemiluminescence reagent (Amersham Biosciences). Proteins were detected by autoradiography.

**RESULTS**

Inflammation-responsive transcription factor, SAF-1, and its human and mouse homolog, MAZ and Pur-1, regulate a number of genes with diverse cellular functions. Many of these SAF-1/MAZ/Pur-1-regulated genes are activated in different physiological conditions and in different tissues. Inducible nature of SAF-1 and its ability to interact with a well-defined promoter element as well as its ability to associate with several other regulatory proteins (15, 17, 28) suggest that different structural components might be involved in performing these diverse functions. To understand the mechanism of SAF-1 action, we sought to identify the functional motifs. The knowledge gained from the analyses of structural motifs of many transcription factors enabled us to predict some of the structural domains of SAF-1 and their functions (Fig. 1). Of the amino-terminal 150 amino acids of SAF-1, 31 residues are proline, including a stretch of 9 prolines located within residues 130–139. In addition, SAF-1 contains a total of three polyalanine tracts. Two of these are located at the N-terminal region between amino acids 89 and 108 and between amino acids 142 and 164. The third polyalanine tract is located at the carboxy-terminal region between amino acids 436 and 450. SAF-1 also contains a glycine-rich region containing 10 glycine residues within amino acids 244–257 and six potential zinc finger motifs that are of the Cys2-His2 type.

**In Vivo Mapping of the DNA-Binding Region of SAF-1**—For gross analysis, we used an in vivo mapping technique in which progressively deleted fragments of SAF-1 were fused to a heterologous activation domain (amino acids 768–881) of yeast GAL4 transcription factor to generate GAL4ADSAF-1 fusion proteins. A nuclear localization signal peptide sequence from SV40 T antigen was placed in-frame upstream of the GAL4AD activation domain for proper nuclear localization of the fusion protein in case the endogenous signal is deleted from the fragment of interest. Expression of the GAL4ADSAF-1 fusion proteins was directed by cytochrome C oxidase immediate early enhancer/promoter. This fusion approach allows assessment of the DNA-binding ability of the modular domain of a protein with...
out requiring the presence of an activation domain in the same protein. GAL4AD-SAF-1 chimeras were examined for analysis of promoter binding activity by transfecting HIG82 synovial cells and expressing CAT reporter gene (Fig. 2). The CAT reporter, SAF-CAT, contains the SAF binding element placed in front of the minimal tk promoter of pBLCAT2 vector (Fig. 2A), and thus expression of the CAT gene is dependent upon the binding of fusion proteins to this element. To assess the synthesis level of the fusion proteins in the transfected cells, Western immunoblot analysis was performed, which showed a comparable level of synthesis from each plasmid (Fig. 2F). Further analysis by immunofluorescence using anti-GAL4AD antibody showed that these GAL4AD-SAF proteins are localized into nucleus of the transfected cells (data not shown). As shown in Fig. 2B, ADSAF-1-(1–477), containing full-length SAF-1 protein, effectively activated transcription of SAF-CAT reporter. However, ADSAF-1-(1–192), containing 192 amino acids from the amino-terminal region of SAF-1, did not exhibit any DNA binding activity. Also, ADSAF-1(Δ193–414), with internal deletion of amino acid residues from 193 to 414, did not activate the reporter. In contrast, the ADSAF-1(193–414) construct activated SAF-CAT reporter very efficiently. These results indicated that DNA binding activity of SAF-1 resides within amino acids 193–414, containing all six zinc fingers of SAF-1.

To determine whether all six zinc fingers are required for optimal DNA-binding and to map the boundaries of functional minimum DNA-binding domain, we prepared several other constructs where individual or combinations of multiple zinc fingers were deleted. Data obtained from these AD-SAF-1 hybrids, shown in Fig. 2B, indicated that regions encompassing amino acids 282–414 and 193–361, represented by ADSAF-1-(282–414) and ADSAF-1(193–361), with deletion of either the first zinc finger or fifth plus sixth zinc fingers, respectively, were also able to bind quite significantly. Considerable DNA binding activity was also retained by the protein encompassing amino acids 282–361 that contained the second, third, and fourth zinc fingers. In contrast, ADSAF-1-(362–477), containing the fifth and sixth zinc fingers, exhibited almost no DNA binding activity. Thus, minimum functional DNA-binding domain was identified between amino acids 282 and 361 containing the second, third, and fourth zinc fingers. These results are specific, because the cells transfected with GAL4AD vector showed no activity, indicating that the increase in the reporter gene expression is solely due to the DNA-binding ability of attached SAF-1 fragments.

Since the minimum DNA-binding domain spanning amino acids 282–361 contains three zinc fingers, the relative significance of each was assessed (Fig. 2C). Mutation of any one of these three zinc fingers reduced the DNA binding activity, suggesting that each zinc finger contributes toward the DNA-binding function of the minimum DNA-binding domain.

It was interesting to note that the GAL4AD construct, ADSAF-1-(1–477), containing full-length SAF-1 protein was a relatively inferior transactivator compared with some of its truncated derivatives (Fig. 2B). These results suggested that terminal regions of SAF-1 may have an adverse effect on the minimum DNA-binding domain of SAF-1. To test this possibility, we prepared several constructs in which the surrounding sequence of the minimum DNA-binding domain of SAF-1 was progressively deleted either from the N-terminal or C-terminal region (Fig. 2D). The removal of terminal amino acid sequences 1–89 and 415–477 from N and C termini, respectively, increased the DNA binding activity quite significantly. To test whether the terminal sequences have any effect on the minimum DNA-binding domain of amino acids 282–361 containing zinc fingers 2–4, the N and C termini were ligated in frame with this region. Compared with ADSAF-1-(282–361), the ADSAF-1-(282–361)N-C tag construct showed much lower activity (Fig. 2E). This finding suggested that terminal sequences of SAF-1 pose a negative effect that could be due to interference with the binding of the protein to the promoter or due to an inhibitory activity of the terminal regions. Further studies, described in the legends to Figs. 4 and 5, revealed an inhibitory activity of the terminal regions.

**DNA-binding Domain of SAF-1**—To test whether the region of SAF-1, identified in the above studies, directly and independently interacts with the SAF promoter, in vitro DNA-binding studies were performed using affinity-purified subunits of SAF-1 protein. A DNA-binding assay was performed using equal amounts of these proteins and a radioactively labeled SAF-binding element as the probe (Fig. 3A). The quality of individual subunit proteins was verified by SDS-PAGE analysis (Fig. 3B). Proteins expressed from the N-terminal fragment SAF-1-(1–192) and centrally deleted SAF-1-(193–414) showed no DNA binding activity (Fig. 3A, lanes 6 and 10). A high level of DNA binding activity was seen with protein expressed from the 193–414 fragment (Fig. 3A, lane 12), but other proteins expressed from the fragments including 282–414 and 193–361 were also quite active (Fig. 3A, lanes 13 and 14). High level of DNA binding activity was also exhibited by the protein expressed from the SAF-1-(282–361) fragment (Fig. 3A, lane 11). However, carboxyl-terminal protein containing amino acids from 362 to 477 that spans the fifth and sixth zinc fingers of SAF-1 showed no DNA binding activity (Fig. 3A, lane 5). These results suggested that a domain of SAF-1 that spans the second, third, and fourth zinc fingers is necessary for direct interaction with DNA. Noticeably, full-length SAF-1 binds poorly (Fig. 3A, lane 1), but terminal truncation either from the amino-terminal end (Fig. 3A, lane 2) or carboxyl-terminal end (Fig. 3A, lane 7) recovers the DNA binding activity. This finding is consistent with earlier observations described in Fig. 2.

**Mapping of the Transactivation Domains of SAF-1**—To determine whether SAF-1 contains any autonomous transactivation domain, we prepared constructs capable of expressing chimeric proteins containing various segments of SAF-1 fused to the GAL4DBD of yeast GAL4 transcription factor placed under the control of the RSV promoter. GAL4DBDSAF-1 chi-
meras were tested for their ability to transactivate a CAT reporter gene (Fig. 4). The reporter plasmid (GAL4E1bCAT), shown in Fig. 4A, contains a GAL4 DNA-binding element placed in front of the E1b minimal promoter; therefore, expression of the CAT gene is dependent on the ability of the fusion protein to bind to the SAF binding element. B–E, the indicated regions of SAF-1, shown in parenthesis, were fused in frame to the GAL4 activation domain (AD). GAL4AD-SAF-1 fusion constructs or GAL4AD (0.5 μg) was cotransfected with the SAF-CAT reporter (1.0 μg) into HIG82 cells. CAT activity results shown are averages of three independent experiments. F, Western immunoblot analysis of GAL4AD-SAF-1 fusion proteins, identified as a–i, a’–d’, a”–h”, and a”–b”, which were expressed in HIG82 cells. Fusion proteins in the transfected cell extracts were detected using anti-GAL4AD antibody.

**Fig. 2.** Activity of GAL4AD-SAF1 fusion constructs in vivo. A, physical map of SAF-CAT reporter plasmid, where CAT expression is dependent on the ability of the fusion protein to bind to the SAF binding element. B–E, the indicated regions of SAF-1, shown in parenthesis, were fused in frame to the GAL4 activation domain (AD). GAL4AD-SAF-1 fusion constructs or GAL4AD (0.5 μg) was cotransfected with the SAF-CAT reporter (1.0 μg) into HIG82 cells. CAT activity results shown are averages of three independent experiments. F, Western immunoblot analysis of GAL4AD-SAF-1 fusion proteins, identified as a–i, a’–d’, a”–h”, and a”–b”, which were expressed in HIG82 cells. Fusion proteins in the transfected cell extracts were detected using anti-GAL4AD antibody.

meras were tested for their ability to transactivate a CAT reporter gene (Fig. 4). The reporter plasmid (GAL4E1bCAT), shown in Fig. 4A, contains a GAL4 DNA-binding element placed in front of the E1b minimal promoter; therefore, expression of the reporter CAT gene is dependent on the binding of fusion proteins with the help of GAL4DBD and subsequent action of the specific activation domain of the test fusion protein. Amino acids 1–147 of GAL4 protein are sufficient to confer dimerization, binding to GAL4 promoter, and nuclear localization. However, this protein by itself does not activate transcription from the GAL4E1b-CAT minimally active reporter. Transcriptional activation occurs only when an activation domain of a transcription factor is attached to the GAL4DBD. Results obtained from progressively deleted C-terminal GAL4DBDSAF1 chimeras indicated that SAF-1 contains several positively acting as well as negatively acting transactiva-
tion domains (Fig. 4B). While full-length GAL4DBDSAF-1-(1–477) activated GAL4E1bCAT expression by about 35-fold, removal of C-terminal amino acids from 415 to 477 considerably reduced CAT gene activity, suggesting the presence of a positively acting transactivation domain in this region. Further removal of C-terminal amino acids up to residue 390, DBDSAF-1-(1–389), significantly reduced the CAT expression level. Together, these results indicated that a transactivation domain is present within amino acids 390–477 of SAF-1. Since the C-terminal region was further deleted up to amino acid 362, DBDSAF-1-(1–361), there was a noticeable increase in the reporter gene activity, which declined when amino acids up to residue 331 were deleted. Together, these results indicated that a negatively acting regulatory domain is present between amino acids 362 and 389, and a positively acting regulatory domain is present between amino acids 331 and 361.

Analysis of activity of constructs GAL4DBDSAF-1-(1–281), GAL4DBDSAF-1-(1–213), and GAL4DBDSAF-1-(1–192) further indicated that a negatively functioning domain exists between 214 and 281 and that a positively functioning domain exists between amino acids 1 and 213.

To analyze N-terminally located transactivation domain, we prepared a series of progressively deleted GAL4DBDSAF-1 constructs (Fig. 4C). Results obtained from this group of chimeras confirmed the presence of a transactivating domain at the N-terminal region spanning amino acids 1–213 and a negatively acting repression module between amino acids 214 and 281. In all cases, activation or repression of the GAL4E1bCAT reporter was dependent on the presence of specific regions of SAF-1 protein, since no detectable level of CAT activity was
Together, the above results indicate the presence of three transactivation domains: 90–213, 331–361, and 390–450. To assess the relative activity of these three domains, we compared their transactivation potentials. Results shown in Fig. 4F revealed that whereas domains spanning amino acids 90–213 and 390–450 possess similar activity, the domain spanning amino acids 331–361 holds a relatively weaker activity.

Interestingly, deletion of the C-terminal 27 amino acids, from 451 to 477, increased the CAT activity as revealed by the comparison of transactivation activity between DBDSAF-1-(415–477) and DBDSAF-1-(415–450) (Fig. 4E). These data suggested that the C-terminal 27 amino acids may contain a repressor module. Similarly, an increase in transactivation potential due to removal of the first 59 amino acids in DBDSAF-1-(60–213), when compared with DBDSAF-1-(1–213) (Fig. 2D), suggested that the region spanning amino acids 1–59 may also contain a repressor activity.

Analysis of the Terminal Repressor Modules—To test whether the regions 1–59 and 451–477 of SAF-1 contain repressor modules, we used a CAT reporter plasmid that generates a high basal activity, suitable for examining transcriptional repression. The minimal tk promoter sequence of the thymidine kinase gene of the herpes simplex virus promotes high level transcription of the CAT reporter gene. The presence of a GAL4-binding element sequence in the promoter region of this reporter plasmid allows recruitment of a protein at the promoter region with the help of GAL4DBD that acts as a bait. If the test protein has a potential transcription repressor activity, interaction of GAL4DBD-repressor fusion protein to the GAL4-binding element of the GAL4tkCAT vector would recruit the repressor domain at the promoter to suppress CAT expression. Transfection of cells with the GAL4tkCAT plasmid resulted in high level of CAT activity (Fig. 5A). Co-transfection of GAL4DBD plasmid had no adverse effect on CAT expression, indicating no inhibitory activity of the GAL4DBD itself. However, when the cells were co-transfected with DBDSAF-1-(1–59) and DBDSAF-1-(450–477), there was inhibition of CAT expression. Further deletion of the amino-terminal repressor domain showed that a protein containing only first 27 amino acids of SAF-1, GAL4DBDSAF-1-(1–27), is able to repress...
transcription. Transfection with GAL4E1bCAT (Fig. 5B) showed relatively very low activity, which explained why these terminal repression modules adjacent to transactivation domains remained undetected in the previous assay (Fig. 4). pBLCAT2, which is similar to GAL4tkCAT but lacks the GAL4-binding element, showed no response (Fig. 5C), suggesting that repression of CAT activity requires recruitment of the repressor modules to the promoter region of the reporter plasmid.

Functional Domains of SAF-1—Having identified the functional DNA binding, activation, and repression domains of SAF-1, we have developed a map of SAF-1 protein that locates different motifs distributed across the molecule (Fig. 6). One obvious question is: What is the optimum functional unit of SAF-1 that can accomplish its role as a transcription factor? To address this issue, we generated a series of SAF-1 constructs with progressive deletion from both the N and C termini (Fig. 7). Proteins expressed from these plasmids were found to promote transcription at varying rates. Terminal truncation from both ends increased the activity of the protein, and the maximum activity was found within a derivative that contains amino acids 193–450 (Fig. 7C). Interestingly, comparison of activity between pcDSAF-1-(193–477) and pcDSAF-1-(214–477) constructs revealed that deletion of region spanning amino acids 193–213 significantly reduces the functional activity (Fig. 7C). To test whether this region is crucial for the functional activity, it was deleted from full-length SAF-1, and the deleted construct was assessed for functional activity (Fig. 7D). Loss of activity of the deletion mutant pcDSAF-1-(142–477)(Δ193–213) suggested the functional importance of this region. However, this region spanning amino acids 193–213 independently does not have any activity (Fig. 7D). The data suggest that the functional entity located within amino acids 193–213 acts in conjunction with the surrounding domains of SAF-1. In this context, we should mention that full-length SAF-1 protein is functionally weak, and earlier studies (20) have indicated that unfavorable folding of the protein might be responsible for reduced activity. Could terminal truncation be involved in generating active SAF-1? Indeed, proteolytic processing of SAF-1 may be involved in removing terminal amino acids and in this manner could create derivatives that are functionally superior, as seen in this study. Thus, the finding of the functional motifs and the analysis of activation mechanism of SAF-1 by terminal truncation, observed in the current study, provides an important regulatory mechanism for SAF-1 activation.

SAF-1 Contains Two Independent Nuclear Localization Signals—In previous studies, we detected SAF-1 activity mostly in the nucleus, suggesting the presence of a mechanism of nuclear transport of this protein (1, 2). Conventionally, nuclear localization of a protein is achieved by the presence of a nuclear localization signal (NLS) sequence. Analysis of the amino acid sequence of SAF-1 identified a region rich in basic amino acids, KKRRSK, resembling consensus NLS, between amino acids

![Fig. 6. Schematic illustrating activation domains, negative regulatory regions, and core DNA-binding unit in SAF-1. The numbers in parenthesis represent amino acid positions of SAF-1.](http://www.jbc.org/)

![Fig. 7. Transcriptional activities of SAF-1 mutants. A. physical map of the SAF-CAT reporter plasmid. The ability of SAF-1 deletion mutants to activate transcription was determined by co-transfection of HIG82 cells with 0.5 μg of the specified SAF-1 expression plasmid and 1.0 μg of SAF-CAT reporter. B. progressive C-terminal deleted fragments of SAF-1 were cloned in expression plasmid pcDNA3 that contains cytomegalovirus early promoter/enhancer. CAT activity results shown are averages of three separate experiments. C, terminally deleted fragments of SAF-1 were cloned in pcDNA3, and resulting constructs were assayed for transcriptional activation function as described in B. D, an internally deleted SAF-1 mutant lacking amino acids 193–213 (pcDSAF-1-(142–477)(Δ193–213)) and a derivative containing amino acids 193–213 (pcDSAF-1-(142–477)(Δ193–213)) were assayed for functional activity as described in B.](http://www.jbc.org/)
183 and 188 of SAF-1. A second site rich in similar basic amino acid residues was located between amino acids 304 and 322 of SAF-1 (Fig. 8A). To determine whether these regions contain functional NLS motifs, we fused amino acid residues 1–477, 1–192, and 193–477 of SAF-1 to the open reading frame of GFP and transfected HIG82 cells with these constructs along with control empty vector, phrGFP plasmid. The phrGFP plasmid itself contains a nuclear localization signal, and for that reason GFP is present both in the nucleus and cytoplasm of vector transfected cells (Fig. 8B, a). However, increased nuclear localization of GFP was observed with SAF-1-(1–477)-GFP, SAF-1-(1–192)-GFP, and SAF-1-(193–477)-GFP constructs as compared with the empty vector (Fig. 8B, b–d). This result suggested that two NLS sites are present in SAF-1 protein; one is located within amino acids 1–192, and the other is located within amino acids 193–477. To further verify the basic amino acid cluster located between 183 and 188 of SAF-1 as a functional NLS motif, we prepared two constructs in GFP vector, one containing wild-type sequences and the other containing mutated sequences of this region. The KKRKSK sequence provided efficient nuclear localization of the GFP (Fig. 8C, a) whereas the mutated sequence showed almost no additional nuclear localization (Fig. 8C, b). A similar result of a lower level of nuclear localization was obtained when the same mutation was introduced in the full-length SAF-1 (Fig. 8C, c). Two additional plasmids containing either wild-type or mutated sequences of amino acids from 304 to 322 were also tested. Transfection of cells with SAF-1-(304–322)-GFP plasmid showed increased levels of nuclear localization of GFP (Fig. 8C, d) relative to the mutant plasmid (Fig. 8C, e). When the mutated amino acids 304–322 were introduced into the full-length SAF-1, a similar result of lower level of nuclear localization was obtained (Fig. 8C, f). Together, the data suggest that both of these two distinct NLS motifs have similar activity. These data indicated that mutation of either site lowers the nuclear localization of SAF-1, and apparently one site cannot compensate for the loss of the other.

**DISCUSSION**

This study provides an understanding of the functional domains of SAF-1 transcription factor and identified a previously unknown mechanism by which activity of this transcription factor is regulated. Core DNA-binding domain is surrounded by multiple activation domains, some of which function as positive regulators of transcription, whereas several others function as transcription repressors. Together these modular domains give rise to the unique functional properties and complexity of SAF-1 that lies with these mul-

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**FIG. 8.** Domains involved in the nuclear localization of SAF-1. A, schematic of various fragments of SAF-1 cloned in frame N-terminal to the GFP protein using phrGFP (Stratagene) plasmid. B, HIG82 cells were transfected with phrGFP vector (a), SAF-1-(1–477)-GFP (b), SAF-1-(1–192)-GFP (c), and SAF-1-(193–477)-GFP (d) fusion constructs and visualized by fluorescence microscopy 24 h post-transfection. C, HIG82 cells were transfected with SAF-1-(183–188)-GFP (a), mutant SAF-1-(183–188)-GFP (b), full-length SAF-1 with mutant basic cluster NLS-(183–188) (c), SAF-1-(304–322)-GFP (d), mutant SAF-1-(304–322)-GFP (e), and full-length SAF-1 with mutant bipartite NLS-(304–322) (f) fusion constructs. Transfected cells were visualized by fluorescence microscopy 24 h post-transfection. The subcellular localization of GFP fusion protein of each construct in both B and C is summarized as follows: C = N, staining throughout the cell; N > C, predominant nuclear localization of the protein.
multiple negative and positive functioning transactivation modules clustered at both ends of this protein. The core DNA binding domain of SAF-1 encompasses zinc fingers 2–4 (amino acids 282–361). Nuclear transport of SAF-1 is accomplished by two independent nuclear localization signals, one of which is present within the core DNA-binding domain and the other of which located within a transactivation domain.

SAF-1 was originally identified as a regulator of SAA gene expression under inflammatory condition. Increased SAA synthesis during a variety of inflammatory episodes was found to be due to activation of DNA binding ability of SAF-1 via phosphorylation (1, 2). Several signal transducing protein kinases including mitogen-activated protein kinase, protein kinase C, and cyclic AMP-dependent protein kinase have been shown to phosphorylate this transcription factor (19–21). Results presented here suggest that, besides the phosphorylation site, other domains of SAF-1 are also involved in regulating its overall transcriptional activity. From the analyses of GAL4DBDSAF-1 chimeras, several negative and positively acting transactivation modules were identified (Fig. 4). A schematic diagram is presented to illustrate the location of these domains (Fig. 6). The negative and positive modules are clustered and positioned at both ends of SAF-1. Among these, two terminal negative modules appeared to be very important. When any one of these two repression modules is altered by deletion, the modified SAF-1 protein becomes highly efficient in terms of both DNA binding activity (Figs. 2 and 3) as well as transactivation potential (Figs. 4 and 7). These two repression modules, however, are independently capable of suppressing transcription from the heterologous GAL4 DNA-binding domain (Fig. 5). A simple interpretation of this finding is that these repressor modules assemble at the promoter region with the help of a specific DNA-binding protein such as GAL4DBD in Fig. 5 and subsequently mediate their action via their independent interaction with some component(s) of the basal transcription complex or other co-repressor molecules assembled at the promoter site. Interestingly, although both terminal repressor modules are capable of repressing transcription, when one is removed, transcriptional activity of SAF-1 is almost fully gained, and removal of the second terminal repression domain only slightly increased its transcriptional activity (Fig. 7C). One possible explanation for this finding is that removal of one of the two terminal repression domains, while facilitates its interaction with the promoter, brings about a conformational change of the protein, rendering the remaining terminal repressor module less effective as a repressor. Thus, both terminal repression domains are needed to regulate SAF-1 function, and activation would possibly involve terminal truncation. Existence of such a truncated form of SAF-1 molecules that could be generated by specific protease action in SAF-1-activated cells is yet to be reported. However, we cannot rule out such a possibility, because proteosome inhibitors have been found to suppress activation of SAF-1. Another possibility that also cannot be ruled out is that in vivo the terminal ends of SAF-1 are physically interacted with by a protein(s) that is a repressor(s) of SAF-1 function. There are several examples of transcription factors that contain the repression module acting as the site of physical interaction of another regulatory protein. One of the best characterized examples is Ets-1, a member of the Ets family. Ets-1 contains an autoinhibitory domain that functions intramolecularly to repress DNA binding and thus prevents transactivation (29). Binding of the core-binding factor o2 to Ets-1 counteracts the inhibition by increasing the DNA binding affinity of Ets-1 (30).

Among the three positively functioning transactivation modules of SAF-1, activation domain I is quite broadly localized between amino acids 90 and 213, encompassing two polyalanine tracts, one polyproline tract, and the first zinc finger. By using progressively deleted SAF-1 constructs, we determined that within this broad transactivation domain, the region containing amino acids 193–213 is essential for maintaining high transcriptional activity of SAF-1 (Fig. 7). Comparison of transcriptional activity of pcDSAF-1-(193–477) and pcDSAF-1-(214–477) supports this conclusion. Furthermore, deletion of amino acids 193–213 also reduced the transcriptional activity, verifying the functional significance of this region. Interestingly, this region, which encompasses a zinc finger domain, alone does not possess any transcriptional activity (Figs. 4D and 7D). One possible explanation for this paradoxical result is that the region surrounding amino acids 193–213 may provide a structural support for the activation domain activity of this region of SAF-1. This possibility is based on the findings that the N-terminal region spanning amino acids 90–213 is active (Fig. 4D). Examination of the amino acid sequence within 193–213 revealed no known unique element with potential functional significance. Noticeably, two polyalanine tracts, one polyproline tract, and the basic NLS of SAF-1 are located just upstream of this region. Further studies are needed to identify critical structural motif within amino acids 193–213 and its potential cross-talk with the surrounding regions.

SAF-1 contains a total of six zinc finger domains. Three (fingers 1, 4, and 5) of the six zinc fingers of SAF-1 contain consensus (F/Y)XXCXX2–4CXXFXLX2HX3–4H sequence (31), whereas the remaining three (fingers 2, 3, and 6) contain a substitution in one of the conserved hydrophobic residues thought to stabilize the formation of the zinc finger. Zinc finger proteins bind to DNA through a generally conserved docking arrangement with each finger’s α-helix fitting into the major groove of the DNA double helix (32, 33). For most transcription factors, the DNA binding domain functions in a modular manner: the isolated binding domain and full-length protein bind to DNA identically (34, 35). This concept was not exactly followed in SAF-1 protein. The DNA binding affinity of the full-length bacterially expressed protein was much less than several shorter fragments containing a minimum of three zinc fingers, which were identified as the core DNA-binding unit (Fig. 3). The regions outside the zinc finger motifs were examined for DNA binding activity. We have no evidence for a second domain that may modulate the affinity of DNA binding, as is the case for Sp1 (36). In contrast, our data suggest that transcriptional activity of SAF-1 is regulated via terminal sequences, which have regulatory effect on the function of SAF-1 (Figs. 2 and 3).

In SAF-1, two nuclear localization signals are present. One is composed of a cluster of six basic amino acids, and the second one is a bipartite signal. The bipartite NLS is present within the core DNA binding domain of SAF-1. The location of the NLS in a DNA-binding domain is also described for some other proteins, including a number of transcription factors such as MyoD (37), NF-κB (38), or SOX-9 (39). Incorporation of NLS within the DNA-binding domain that frequently contains many basic amino acid residues is an economic design, which may be the result of an evolutionary process. The other interesting feature was the presence of two redundant nuclear localization signals. Why SAF-1 contains redundant NLS signals is presently not very clear. We speculate upon several possibilities. First, two NLS signals would reduce the susceptibility of a nuclear protein to total loss of nuclear translocation by NLS-inactivating point mutations. Second, interaction with some cellular proteins at times may mask one of the two NLSs of SAF-1. Third, two signals may simply function, either addi-

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2 A. Ray, D. Kumar, P. Ray, and B. K. Ray, unpublished observation.
respectively or cooperatively, to ensure maximum nuclear localization. Further studies will provide an explanation for the necessity of dual nuclear localization signals in SAF-1.

One notable feature of SAF-1 is that it belongs to a Cys2His2 type zinc finger family of proteins. Several members of this family of transcription factors are known to directly interact with each other. For example, Sp1 which contains three such zinc fingers at its C-terminal region (36) binds to GATA1 and family of transcription factors are known to directly interact with SAF-1. SAF-1 contains dual nuclear localization signals in SAF-1. These data should aid in future efforts to understand the regulation of SAF-1-targeted genes under different pathological conditions.

In summary, the data presented here delineate the structural domains of SAF-1. These data should aid in future efforts to understand the regulation of SAF-1-targeted genes under different pathological conditions.

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