Polyamine-modulated factor 1 binds to the human homologue of the 7a subunit of the *Arabidopsis* COP9 signalosome: implications in gene expression

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Polyamines have been identified to play a role in the transcription of various growth-related genes. The recently discovered polyamine responsive element and the associated trans-acting proteins involved in polyamine-regulated transcription have provided a model system for the study of the role of polyamines in transcription. Polyamine-modulated factor 1 (PMF-1) was identified as one of the transacting factors that binds to NF-E2 related factor-2 (Nrf-2) to regulate the transcription of spermidine/spermine N^1-acetyltransferase (SSAT). The possibility that PMF-1 also binds to other proteins involved in transcriptional regulation cannot be ruled out. Using a yeast two-hybrid strategy, it was found that PMF-1 binds to a human homologue of the *Arabidopsis* COP9 signalosome subunit 7a (CSN 7) protein. In the present study, we describe human CSN 7, a 275-amino-acid-containing protein that may have a direct role in regulating gene expression. CSN 7 and PMF-1 bind to each other, as well as compete with each other for binding to Nrf-2. This competition for Nrf-2 binding and interaction with each other is implicated in the regulation of SSAT transcription. CSN 7 possesses a C-terminal coiled-coil domain similar to the domain that mediates the interaction between PMF-1 and Nrf-2, suggesting that coiled-coil domains also mediate the interaction between CSN 7 and PMF-1. Since CSN 7 does not contain a DNA-binding domain, its effects on transcription must occur in conjunction with binding to other proteins. The results presented here demonstrate that PMF-1 and Nrf-2 can act as protein partners of CSN 7.

Key words: coiled-coil, leucine zipper, transcription.

INTRODUCTION

The identification of a polyamine responsive element (PRE) and transacting protein factors that respond to polyamines and polyamine analogues has opened up an exciting new area in the study of polyamine function [1]. It has been postulated for several years that the polyamine content of a cell can directly or indirectly affect the spectrum of genes expressed and now existing data demonstrate that the polyamine content of cells can have a direct impact on gene expression [1–6]. Using the spermidine/spermine N^1-acetyltransferase (SSAT) gene, the rate-limiting step in polyamine catabolism as a model, studies have demonstrated a direct role for polyamines in transcriptional regulation of SSAT. The identification of polyamine-modulated factor 1 (PMF-1), and its association with NF-E2 related factor 2 (Nrf-2), in the regulation of transcription has consolidated the role of polyamines in the complex regulatory pathway of SSAT expression. However, it is also possible that PMF-1 may be associated with the other transcription factors and thereby affect the regulation of additional genes. To identify other proteins that interact with PMF-1 and which have the potential to modify gene expression, the yeast two-hybrid system was used. In the present paper the identification of one such PMF-1-binding protein is reported. The human homologue of a member of the COP9 signalosome complex [7–10] was found to interact with both PMF-1 and Nrf-2, with the potential to affect gene expression.

The COP9 signalosome proteins were first identified in the multicellular plant *Arabidopsis* and have been demonstrated to form a regulatory complex involved in light-activated development. Interestingly, the COP9 complex has considerable similarity to the lid sub-complex of the 26 S proteasome, a structure necessary for the degradation of many proteins [11]. This family of proteins is highly conserved from *Arabidopsis* to humans, with homologues appearing in essentially all multicellular organisms [12]. In each of the organisms studied, the COP9 complex appears to play a role in intracellular signalling and transcriptional control. However, we report here that one of the subunits of this complex closely interacts with a human transcriptional co-factor important in polyamine-regulated transcription, suggesting the potential interplay between the polyamine status of a cell and COP9 signalosome-mediated regulation of gene expression.

MATERIALS AND METHODS

Chemicals and reagents

N^1,N^{11}-bis(ethyl)norspermine (BENSpm) was provided by Parke-Davis. 2-Difluoromethylornithine was obtained as a gift from the Marion–Merrell-Dow Research Institute (Cincinnati, OH, U.S.A.). Radionucleotides, [α-^{32}P]dCTP and [^{35}S]methionine were supplied by Amersham Pharmacia Biotech (Piscataway,

Abbreviations used: BENSpm, N^1,N^{11}-bis(ethyl)norspermine; CSN 7, COP9 signalsome subunit 7a; EMSA, electrophoretic mobility-shift assay; IPTG, isopropyl β-D-thiogalactoside; Nrf-2, NF-E2 related factor-2; NBE, Nrf-2 binding element; Ni-NTA, Ni^{2+}-nitrilotriacetate; ORF, open reading frame; PMF-1, polyamine-modulated factor 1; PRE, polyamine responsive element; SD-Trp, synthetic dextrose medium lacking tryptophan; SSAT, spermidine/spermine N^1-acetyltransferase.

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NJ, U.S.A.). The Matchmaker yeast two-hybrid system, yeast-culture media, human placenta Matchmaker cDNA library and a human multiple-tissue Northern-blot system were purchased from Clontech (Palo Alto, CA, U.S.A.). Lipofectin and TRIZOL total RNA reagents were purchased from Invitrogen (San Diego, CA, U.S.A.) The luciferase assay system and TnT-coupled transcription/translation reticulocyte lysate system were purchased from Promega (Madison, WI, U.S.A.). The Gal-XE chemiluminescent reporter gene assay system was purchased from ICN Pharmaceuticals (Cosa Mesa, CA, U.S.A.). Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose was purchased from Qiagen (Valencia, CA, U.S.A.). Restriction and DNA modifying enzymes were purchased from Life Technologies (Gaithersburg, MD, U.S.A.) New England Biolabs (Beverly, MA, U.S.A.) and Sigma Chemical Co. (St. Louis, MO, U.S.A.). Life Technologies synthesized all oligonucleotides used in the experiments. Other chemicals were purchased from Sigma, Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.) and J. T. Baker (Philipsburg, NJ, U.S.A.)

Construction of plasmids

For construction of pAS2.1/PMF-1, the PMF-1 cDNA fragment excised from pCR2.1/PMF-1 with *Nco*I–*Xho*I was inserted into the pAS2.1 vector digested with *Nco*I–*Sal*I. To construct pcDNA3.1/COP9 signalosome subunit 7a (CSN 7), a 1350 bp CSN 7 cDNA (+141 to +1490 bp) was amplified by PCR with the primer pair P1 and P2 (Figure 1) using pACT2/CSN 7 as the template. The PCR product was digested with *Xba*I–*Eco*RI and then inserted into the pcDNA3.1(−) vector within the same restriction sites. For construction of pQE30/PMF-1, a 906 bp PMF-1 cDNA (+112 to +1017 bp) was amplified by PCR with the primer pair P3 and P4 (Figure 1). The PCR product derived from pCR2.1/PMF-1 was cut with *Sac*I–*Hin*dIII and then cloned into pQE30 vector digested with the same enzymes. To construct pQE30/CSN 7, an 885 bp CSN 7 cDNA (+142 to +1026 bp) amplified by PCR from pACT2/CSN 7 with the primer pair P5 and P6 was digested with *Kpn*I–*Bam*HI and then cloned into pQE30 vector in the same restriction sites. To construct pQE30/Nrf-2, a 2005 bp Nrf-2 cDNA (+39 to +2043 bp) was excised from pAS2.1/Nrf-2 with *Sma*I–*Sal*I and cloned into pQE30 vector digested with the same enzymes. The plasmids pcDNA3.1/PMF-1, pCR2.1/PMF-1, pBKH-93, pBKH-93/dPRE and pAS2.1/Nrf-2 were constructed as described previously [1]. Plasmid pACT2/CSN 7 was obtained from the yeast two-hybrid screening as described below.

Screening the cDNA library by yeast two-hybrid system

Yeast two-hybrid screening was performed with the Matchmaker two-hybrid system. *Saccharomyces cerevisiae* Y190 cells were first transformed with the bait plasmid pAS2.1/PMF-1 and selected on synthetic dextrose medium lacking tryptophan (SD-Trp). The transformants grown on the SD-Trp medium were subsequently transformed with a pACT2/placenta cDNA library and selected on medium lacking tryptophan and leucine (SD-Trp-Leu). The clones co-transformed with the bait and library were collected and re-plated on to the medium lacking tryptophan, leucine and histidine (SD-Trp-Leu-His) with 30 mM 3-amino-1,2,4-triazole to inhibit unselected growth of Y190 cells. The clones selected in this step were further assayed for their β-galactosidase activity. The pACT-2 library plasmids were purified from individual positive clones and amplified in *Escherichia coli*. Sequencing of the cDNA inserts in the positive clones was performed using a PerkinElmer ABI Automated DNA sequencer.

PCR primers for cloning pcDNA3.1/CSN 7
P1 5`-CGCGTTCTAGACATATGAGTGCGGAAGTGAAGGT
XbaI
P2 5`-AGGATGAACATGTCCAGTGACTCC

PCR primers for cloning pQE30/PMF-1
P3 5`-TCGAGAGCTCATGGTGGACACTTTTCTTCAG
SacI
P4 5`-GCATAAGCTTGTGGAGAGAACATGGTAAAGG
HindIII

PCR primers for cloning pQE30/CSN 7
P5 5`-CGAATTGGATCCATGAGTGCGGAAGTGAAGGT
BamHI
P6 5`-ATGGTAGGTACCTAAGAGGCAGGCAGGCAGCT
KpnI

PCR primers for amplifying cDNA inserts in pACT2 library
P7 5`-CTATTCGATGATGAAGATACCCCACCAAACCC
P8 5`-GTGAACTTGCGGGGTTTTTCAGTATCTACGAT

Oligonucleotides for generating NBE
P9 5`-GCACAGCAATGCTGAGTCATGATGAGTCATG
P10 5`-CAGCATGACTCATCATGACTCAGCATTGCTG

Oligonucleotides for generating PRE-containing DNA sequence
P11 5`-GACCGCTATGACTAAGCG
P12 5`-GGTCCGCTTAGTCATAGC
P13 5`-CGCTTAGTCATACGCTTAGTCATAGC

Figure 1 Primers and oligonucleotides used

In vitro transcription and translation

In vitro transcription and translation analyses were performed with the TnT-coupled transcription/translation system using [^{35}S]methionine according to the protocol of the manufacturer. Purified plasmid pcDNA3.1/CSN 7 or pcDNA3.1 vector was used as the template. The labelled translation products were separated by SDS/PAGE (12% gel) and exposed on Kodak X-Omat film.

RNA purification and Northern-blot analysis

Total cellular RNA from H157 and H82 cells was extracted using TRIZOL total RNA reagent according to the protocol from the manufacturer. Total RNA (10 μg) from H157 or H82 cells was separated on a denaturing 1.5% agarose gel containing 6% formaldehyde, transferred to GeneScreen membrane (NEN Life Science Products, Boston, MA, U.S.A.) and hybridized with a random primer-labelled CSN 7 cDNA as probe. The blot was washed and re-probed with an 18 S ribosomal cDNA probe to serve as a loading control. The results were quantified using Phosphor Image analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.). A human multiple-tissue Northern-blot analysis was used to examine the expression of CSN 7 in various human tissues according to the protocol of the manufacturer.

Expression and purification of CSN 7, Nrf-2 and PMF-1

The QIAexpress System (Qiagen, Chatsworth, CA, U.S.A.) was used in the expression of the target proteins. The cDNAs of CSN 7, Nrf-2 and PMF-1 were cloned in-frame into the pQE30 expression vector and transformed into the *E. coli* strain M15/pREP4 respectively. High-level expression of the target proteins was induced by exposure to 0.2 mM isopropyl β-D-thiogalactoside (IPTG) for 4 h. Ni-NTA resin was used to

purify the resultant His_6-tagged proteins according to the manufacturer-supplied protocol.

Transient transfection assay

For transient transfection, 4×10^5 H157 cells were seeded in each 35 mm-diameter culture dish and cultured in RPMI 1640 medium containing 5 mM 2-difluoromethylornithine for 48 h to reduce endogenous polyamines and background transcription as described previously [13]. Lipofectin-mediated transfection was performed with constructs as indicated in the Results section. After a 5 h incubation, the DNA–Lipofectin complex-containing mixture was replaced by RPMI 1640 medium containing 5 mM 2-difluoromethylornithine. Forty-eight hours after transfection, the cells were exposed to 5 μM BENSpm for 2 h. The cells were harvested, quick-frozen and subsequently prepared for luciferase activity measurements as per the instructions of the manufacturer. To account for variations in transfection efficiency, the luciferase activity was normalized to β-galactosidase activity.

Electrophoretic mobility-shift assay (EMSA)

EMSA was performed as described previously [13]. Briefly, DNA binding reactions were performed in a buffer (25 μl final volume) containing 1–3 μg of purified protein, 14 mM Hepes (pH 7.9), 84 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.3 mM $MgCl_2$, 0.4 mM PMSF, 1 μg of poly(dI/dC) and 10 % glycerol. The reaction mixture was incubated at 0 °C for 15 min, after which time the radiolabelled probes (10000–20000 c.p.m.) were added and the incubation was continued at room temperature for a further 30 min. In competition assays, the unlabelled DNA fragments were added to the binding reaction before the addition of radiolabelled probe. DNA–protein complexes were resolved on a 5 % polyacrylamide gel (37.5 : 1 acrylamide/bisacrylamide) with 0.5 × TBE buffer using 150 V at 4 °C. To generate a 34 bp Nrf-2 binding element (NBE; 5′-GCACAGCAATGCTGAG-TCATGATGAGTCATGCTG-3′) and a 30 bp PRE-containing element (5′-GACCGCTATGACTAAGCGTATGACTAAGC-G-3′, bases underlined represent the PRE element) for use as probes or competitors, two complementary and synthetic oligonucleotides (P9 and P10 for NBE; P11 and P13 for PRE-containing element) were annealed and filled in by DNA polymerase I Klenow fragment. A concatenated PRE-containing DNA with a mean length of 200 bp, which was also used for the competitor, was generated from two complementary and synthetic oligonucleotides (P11 and P12) as described previously [13].

Computer analysis

The sequences in the database with homology with the human CSN 7 cDNA were identified using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST). DNA and protein alignments were performed by BCM Pairwise Sequence Alignment system (http://searchlauncher.bcm.tmc.edu:9331/multi-align/multi-align.html). The coiled-coil content in the human CSN 7 protein was predicted using the methods of Lupas [14–16]. A program using this method can be found at the following URL: http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html.

RESULTS

Identification of CSN 7 as a PMF-1 binding protein

PMF-1 has been found to be involved in the regulation of SSAT transcription by interacting with the Nrf-2 transcription factor as a result of exposure to exogenous polyamines or polyamine analogues. To identify other factors that may regulate gene expression by binding to PMF-1, the yeast two-hybrid system was used to screen for PMF-1 binding proteins. To construct the bait plasmid pAS2.1/PMF-1, the PMF-1 cDNA containing the entire PMF-1 open reading frame (ORF) was fused to the Gal4 DNA-binding domain. Preliminary testing demonstrated that this fusion plasmid is not self-activating with respect to the reporter genes in the host Y190 cells in the absence of the Gal4 DNA activation domain. A placenta cDNA library constructed in the pACT2 vector that expresses proteins fused to the Gal4 transcriptional activation domain was then screened by the HIS3 jump-start procedure. Twenty-seven clones from approx. 10^6 yeast transformants were identified to be positive in activating the Lac Z and HIS 3 reporter genes. The cDNA inserts in these positive clones were amplified by PCR with Matchmaker 5′ (P7) and 3′ Amplimer (P8) and digested with the restriction enzyme *Alu*I. Gel electrophoresis/ethidium staining results demonstrated three different cDNA inserts. The longest and most prevalent cDNA was determined to be approx. 1.9 kb and was ultimately designated as CSN 7. Only clones co-transformed with pAS2.1/PMF-1 and pACT2/CSN 7 were capable of growth on SD-Trp-Leu-His selection medium and demonstrated a positive reaction in β-galactosidase analysis, verifying transcriptional activation of the reporter genes.

Sequencing the insert in pACT2/CSN 7 revealed a cDNA of 1868 bp that contains an ORF of 275 amino acids with a calculated molecular mass of 30 kDa (Figure 2). A translation initiation codon (shown underlined) is found at nucleotide 142 (GCA-GTGATGA) with six out of ten matches for the Kozak consensus sequence (5′-GCCA/GCCATGG). The polyadenylation signal sequence (AATAAA) is found at nucleotide 1767, 20 bp upstream of the polyadenylated [poly(A)] tail. A search of the GenBank® database revealed a highly homologous sequence: the mouse COP9 complex subunit 7a. The human CSN 7 (GenBank® accession no. AF210052) described here has 76.9 % identity with the full-length mouse cDNA (GenBank® accession no. AF071316), 91.3 % identity within the ORF and 98.9 % identity at the amino acid level. It should be noted that using the new nomenclature the human homologue is referred to as CSN 7 [10]. Out of 275 amino acids, only three are different between the two species (human Glu^{90}–mouse Asp^{90}; human Arg^{172}–mouse Gln^{172}; human Pro^{147}–mouse Ser^{147}).

Human CSN 7 contains a predicted C-terminal coiled-coil domain

Using the methods of Lupas et al. [14–16], human CSN 7 was found to possess a coiled-coil domain in the C-terminal region spanning approximately from amino acid 180 to 240. No leucine zipper region was found in the entire 275 amino acid sequence, suggesting that the protein–protein interactions between CSN 7 and other proteins are likely to occur in the coiled-coil region [17].

In vitro transcription and translation of human CSN 7 cDNA

In vitro transcription and translation of full-length CSN 7 cDNA produced a major band with an apparent molecular mass of approx. 31 kDa, which is in agreement with the predicted ORF (Figure 3).

Expression of CSN 7 in human tissues

The expression of CSN 7 mRNA in a variety of normal human tissues was evaluated by Northern-blot analysis using random-primer labelled CSN 7 cDNA as a probe. An mRNA transcript

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1     AGCGACTCTTCAGGGAGGTGGCAGGAAAGGCTTGGAACAGCTGCCGGAGG
51    TGACGGAGCGGCGGCCCCGCCCGGTGCGCTGGAGGTCGAAGCTTCCAGCT
101   CTGGACATCCTGAGCCCAAGTCCCCCACACTCAGTGCAGTGATGAGTGCG
                                          M  S  A
151   GAAGTGAAGGTGACAGGGCAGAACCAGGAGCAATTTCTGCTCCTAGCCAA
       E  V  K  V  T  G  Q  N  Q  E  Q  F  L  L  L  A  K
201   GTCGGCCAAGGGGGCAGCGCTGGCCACACTCATCCATCAGGTGCTGGAGG
         S  A  K  G  A  A  L  A  T  L  I  H  Q  V  L  E
251   CCCCTGGTGTCTACGTGTTTGGAGAACTGCTGGACATGCCCAATGTTAGA
      A  P  G  V  Y  V  F  G  E  L  L  D  M  P  N  V  R
301   GAGCTGGCTGAGAGTGACTTTGCCTCTACCTTCCGGCTGCTCACAGTGTT
         E  L  A  E  S  D  F  A  S  T  F  R  L  L  T  V  F
351   TGCTTATGGGACATACGCTGACTACTTAGCTGAAGCCCGGAATCTTCCTC
         A  Y  G  T  Y  A  D  Y  L  A  E  A  R  N  L  P
401   CACTAACAGAGGCTCAGAAGAATAAGCTTCGACACCTCTCAGTTGTCACC
      P  L  T  E  A  Q  K  N  K  L  R  H  L  S  V  V  T
451   CTGGCTGCTAAAGTAAAGTGTATCCCATATGCAGTGTTGCTGGAGGCTCT
       L  A  A  K  V  K  C  I  P  Y  A  V  L  L  E  A  L
501   TGCCCTGCGTAATGTGCGGCAGCTGGAAGACCTTGTGATTGAGGCTGTGT
         A  L  R  N  V  R  Q  L  E  D  L  V  I  E  A  V
551   ATGCTGACGTGCTTCGTGGCTCCCTGGACCAGCGCAACCAGCGGCTCGAG
      Y  A  D  V  L  R  G  S  L  D  Q  R  N  Q  R  L  E
601   GTTGACTACAGCATCGGGCGGGACATCCAGCGCCAGGACCTCAGTGCCAT
       V  D  Y  S  I  G  R  D  I  Q  R  Q  D  L  S  A  I
651   TGCCCGAACCCTGCAGGAATGGTGTGTGGGCTGTGAGGTCGTGCTGTCAG
         A  R  T  L  Q  E  W  C  V  G  C  E  V  V  L  S
701   GCATTGAGGAGCAGGTGAGCCGTGCCAACCAACACAAGGAGCAGCAGCTG
      G  I  E  E  Q  V  S  R  A  N  Q  H  K  E  Q  Q  L
751   GGCCTGAAGCAGCAGATTGAGAGTGAGGTTGCCAACCTTAAAAAAACCAT
       G  L  K  Q  Q  I  E  S  E  V  A  N  L  K  K  T  I
801   TAAAGTTACGACGGCAGCAGCAGCCGCAGCCACATCTCAGGACCCTGAGC
         K  V  T  T  A  A  A  A  A  A  T  S  Q  D  P  E
851   AACACCTGACTGAGCTGAGGGAACCAGCTCCTGGCACCAACCAGCGCCAG
      Q  H  L  T  E  L  R  E  P  A  P  G  T  N  Q  R  Q
901   CCCAGCAAGAAAGCCTCAAAGGGCAAGGGGCTCCGAGGGAGCGCCAAGAT
       P  S  K  K  A  S  K  G  K  G  L  R  G  S  A  K  I
951   TTGGTCCAAGTCGAATTGAAAGAACTGTCGTTTCCTCCCTGGGGATGTGG
         W  S  K  S  N
1001  GGTCCCAGCTGCCTGCCTCTTAGGAGTCCTCAGAGAGCCTTCTGTG
1051  CCCCTGGCCAGCTGATAATCCTAGGTTCATGACCCTTCACCTCCCCTAAC
1101  CCCAAACATAGATCACACCTTCTCTAGGGAGGAGTCAAATGTAGGTCATG
1151  TTTTTGTTGGTACTTTCTGTTTTTTGTGACTTCATGTGTTCCATTGCTCC
1201  CCGCTGCCATGCTCTCTCCCTTGTTTCCTTAAGAGCTCAGCATCTGTCCC
1251  TGTTCATTACATGTCATTGAGTAGGTGGGTAGCCCTGATGGGGGTCGCTC
1301  TGTCTGGAGCATAACCCACAGGCGTTTTTTCTGCCACCCCATCCCTGCAT
1351  GCCTGATCCCCAGTTCCTATACCCTACCCCTGACCTATTGAGCAGCCTCT
1401  GAAGAGCCATAGGGCCCCCACCTTTACTCACACCCTGAGAATTCTGGGAG
1451  CCAGTCTGCCATGCCAGGAGTCACTGGACATGTTCATCCTAGAATCCTGT
1501  CACACTACAGTCATTTCTTTTCCTCTCTCTGGCCCTTGGGTCCTGGGAAT
1551  GCTGCTGCTTCAACCCCAGAGCCTAAGAATGGCAGCCGTTTCTTAACATG
1601  TTGAGAGATGATTCTTTCTTGGCCCTGGCCATCTCGGGAAGCTTGATGGC
1651  AATCCTGGAAGGGTTTAATCTCCTTTTGTGAGTTTGGTGGGGAAGGGAAG
1701  GGTATATAGATTGTATTAAAAAAAAAAAGGTATATATGCATATATCTATA
1751  TATAATATGACGCAGAAATAAATCTATGAGAAATCTATCTACAAAAAAAA
1801  AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
1851  AAAAAAAAAAAAAAAAA
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Figure 2 Nucleotide and predicted amino acid sequences of the human CSN 7 cDNA

The ATG initiation codon and the TGA stop codon are in boldface and the AATAAA polyadenylation signal is underlined.

of approx. 2 kb was observed and appears to be expressed in multiple tissues. High steady-state levels of CSN 7 mRNA were observed in the brain, heart and skeletal muscle (Figure 4).

Expression of CSN 7 in human lung cancer cells

To determine the effects on CSN 7 expression after exposure to a polyamine analogue, the non-small cell lung cancer line NCI H157 and the small cell lung cancer line NCI H82 were exposed to 10 μM BENSpm for 24 h. These cell lines were chosen because they have been found to differentially express SSAT and the transcriptional regulators of SSAT, PMF-1 and Nrf-2 [1,13].

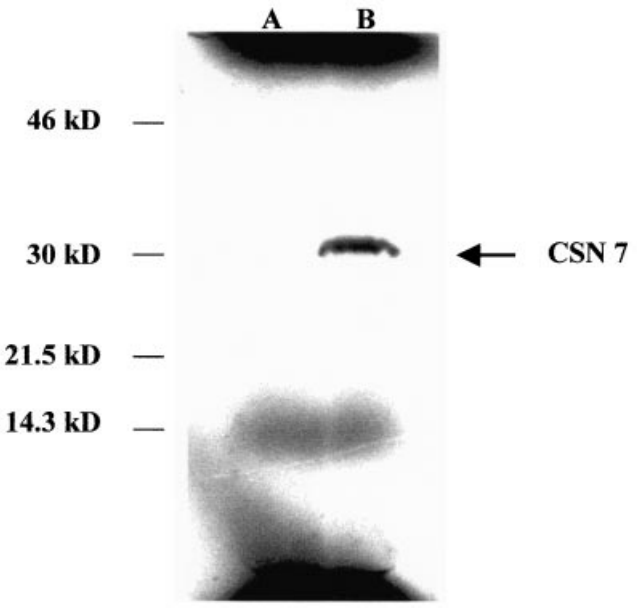


Figure 3 *In vitro* transcription and translation of CSN 7

The assay was performed in the presence of [^{35}S]methionine with 2 μg of plasmid as template in a 50:1-TnT reaction. The labelled translation products were separated by SDS/PAGE (12% gel). The templates used in the assays are pcDNA3.1 vector (lane A) and pcDNA3.1/CSN 7 (lane B). The arrow indicates the position of CSN 7 protein.

Therefore it was of interest to determine whether CSN 7 is also differentially expressed in these lines. After exposure, the total cellular RNA was extracted from treated and control cells of each line and examined by Northern-blot analysis using labelled CSN 7 cDNA as a probe. Both cell types exhibited significant basal levels of CSN 7 expression. However, H157 cells expressed approx. 70% greater amounts of CSN 7 in the basal state than NCI H82 cells (Figure 5). Both cell types responded to BENSpm exposure with a modest (approx. 50%) increase in CSN 7 expression. It is not clear whether these changes in CSN 7 in response to analogue exposure or differences between cell types are significant.

CSN 7 is an Nrf-2 partner

For Nrf-2 to activate transcription it apparently requires heterodimerization with another protein. In many cases it first heterodimerizes with a member of the small Maf family of proteins and then binds to its cognate sequence, the NBE [18]. However, in the case of binding to the PRE and activating SSAT transcription, it appears that Nrf-2 remains constitutively bound to the PRE, but transcription is only activated after PMF-1 binds to the DNA-bound Nrf-2 in the presence of excess polyamines or polyamine analogues [1]. To evaluate the protein–protein interaction between the three proteins CSN 7, Nrf-2 and PMF-1, cDNAs were cloned in-frame into the prokaryotic expression vector pQE30. High-level expression of the target proteins in *E. coli* strain M15[pREP4] was induced by 0.2 mM IPTG. The expressed proteins contain a His_6 tag in their N-terminus and were purified using the Ni-NTA system under native conditions (Nrf-2) or denaturing conditions (PMF-1 and CSN 7) according to the method of the manufacturer (Figure 6). The purified

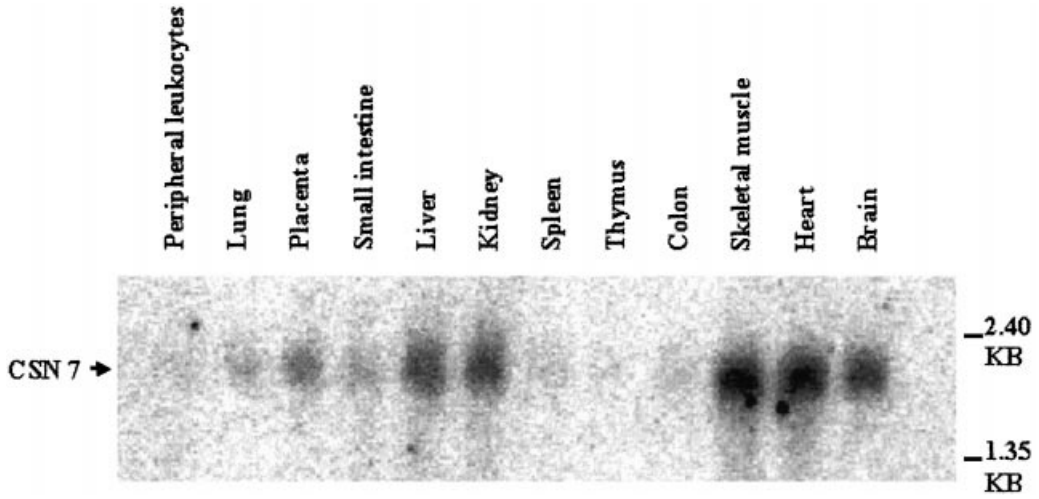


Figure 4 Expression of CSN 7 in human normal tissues

The expression level of CSN 7 mRNA was evaluated by Northern-blot analysis using mRNA from 12 normal human tissues. Random primer-labelled human CSN 7 cDNA was used as a hybridization probe. The arrow indicates the position of the CSN 7 mRNA.

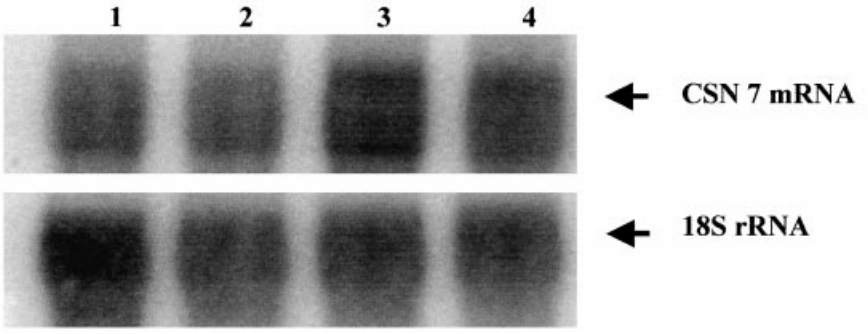


Figure 5 Expression of CSN 7 mRNA in the human lung cancer cells

Total cellular RNA (10 μg) was used in each lane for the Northern-blot analysis with labelled human CSN 7 cDNA as probe. Lane 1, H82 cells treated with 10 μM BENSpm for 24 h; lane 2, H82 untreated cells; lane 3, H157 cells treated with 10 μM BENSpm for 24 h; lane 4, H157 untreated cells. The arrows indicate the positions of CSN 7 mRNA and 18 S rRNA.

proteins were then used in EMSA with labelled NBE (closely related to the PRE). It should be noted that NBE was used as the probe rather than the PRE because the results from EMSA using the PRE would be difficult to interpret, since Nrf-2 binds to the PRE regardless of protein partnering [13]. The results of these experiments indicate that the Nrf-2/CSN 7 complex binds to the NBE with a greater affinity than the Nrf-2/PMF-1 complex. None of the three proteins (Nrf-2, PMF-1 or CSN 7) was capable of binding and shifting the NBE oligomer alone. Interestingly, PMF-1 addition to the Nrf-2/CSN 7 complex resulted in a decrease in the binding affinity of Nrf-2/CSN 7 for the NBE (Figure 7). It should be noted that CSN 7 was identified originally as a PMF-1 binding protein in this experiment. Therefore it is likely that the formation of PMF-1/CSN 7 complex resulted in a decrease in CSN 7 availability as a partner for Nrf-2.

Competition assays indicated that the labelled oligomer shifted by Nrf-2/PMF-1 or by Nrf-2/CSN 7 could be efficiently competed with unlabelled NBE-containing oligomer. It should also be noted that an unlabelled PRE-containing oligomer and a concatenated PRE DNA efficiently compete for the bands shifted by both complexes (Figure 7). These results indicate that the Nrf-2/CSN 7 complex can also efficiently bind to the PRE.

Functional analysis of CSN 7 by transient transfection

To determine whether CSN 7 could functionally influence PRE-mediated transcription of the SSAT gene, two reporter plasmids were constructed. One plasmid, pBKH-93, contains the minimal promoter region of the SSAT gene (-93 to 1 bp) upstream of the luciferase gene. The second plasmid, pBKH-93/dPRE, contains the minimal promoter region and a 30 bp oligonucleotide containing two PRE consensus sequences (TATGACTAA) cloned into a site upstream of the minimal SSAT promoter. For expression of PMF-1 and CSN 7, cDNAs for each were cloned into the pcDNA3.1 expression vector. A PRE-mediated induction of luciferase expression in BENSpm-treated H157 cells was demonstrated in the transient transfection assay. Co-transfecting PMF-1 or CSN 7 along with reporter plasmid could

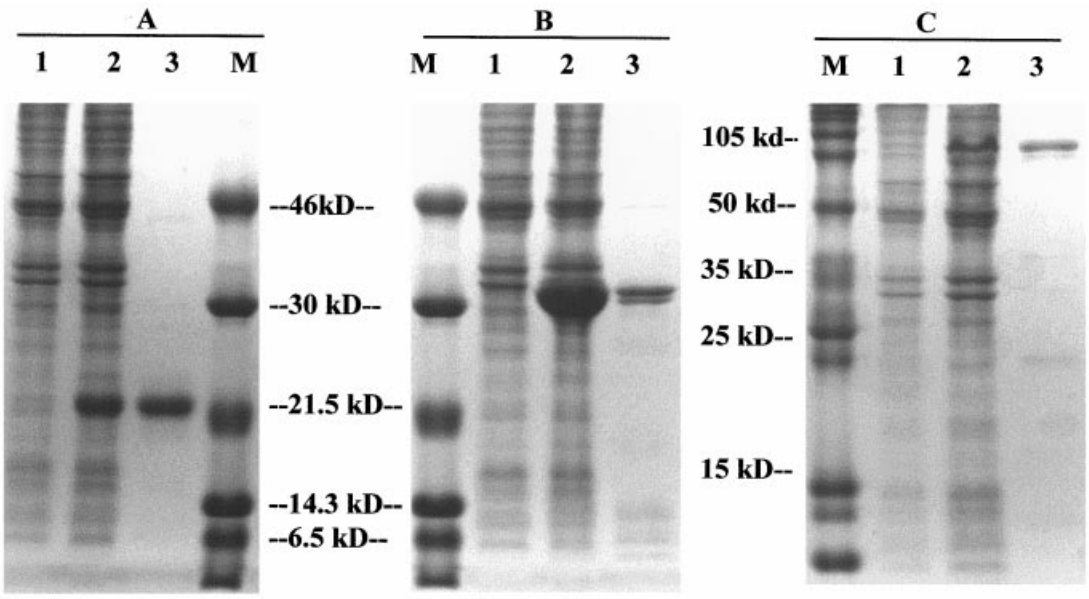


Figure 6 Expression and purification of PMF-1 (A), CSN 7 (B) and Nrf-2 (C) proteins

cDNAs of PMF-1, CSN 7 and Nrf-2 were cloned into pQE30 vector and expressed in *E. coli* strain M15[pREP4]. Target proteins with N-terminal His_6 tags were purified using the Ni-NTA system. Proteins were resolved on an SDS/12%/polyacrylamide gel and stained with Brilliant Blue R250. Lane 1, cell lysate before IPTG induction; lane 2, cell lysate induced by 0.2 mM IPTG for 4 h; lane 3, purified protein; M, protein markers.

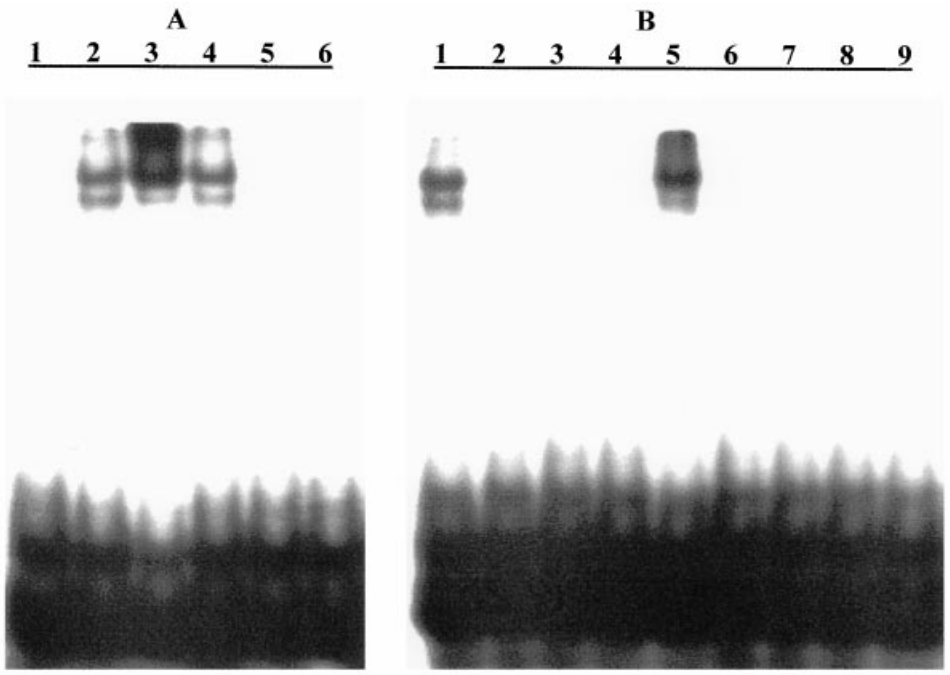
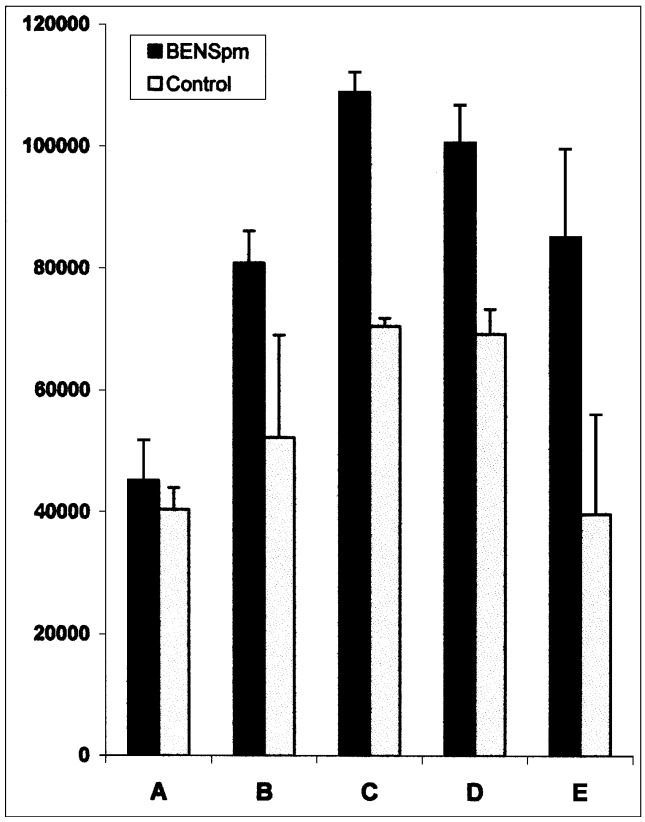


Figure 7 EMSA for the interaction between the human PMF-1 or CSN 7 and Nrf-2

(**A**) Nrf-2/PMF-1 and Nrf-2/CSN 7 complexes can shift up NBE probe. EMSA was performed with labelled NBE probe and purified proteins. Lane 1, 1 μg Nrf-2; lane 2, 1 μg Nrf-2 + 1 μg PMF-1; lane 3, 1 μg Nrf-2 + 1 μg CSN 7; lane 4, 1 μg Nrf-2 + 1 μg CSN 7 + 1 μg PMF-1; lane 5, 1 μg PMF-1; lane 6, 1 μg CSN 7. (**B**) Competition assay was performed with labelled NBE as the probe. The purified proteins used in the EMSA: lanes 1–4, 1 μg Nrf-2 + 1 μg PMF-1; lanes 5–8, 1 μg Nrf-2 + 1 μg CSN 7. The competitors used in the assay: lanes 2 and 6, PRE-containing element (30 bp); lanes 3 and 7, concatenated PRE-containing DNA; lanes 4 and 8, unlabelled NBE (34 bp). Lane 9, labelled probe only.

A



B

Constructs used in the transient transfection

Constructs	A	B	C	D	E
pBKH-93	0.5 μg				
pBKH-93/dPRE		0.5 μg	0.5 μg	0.5 μg	0.5 μg
pcDNA3.1/PMF-1			0.5 μg		0.5 μg
pcDNA3.1/CSN 7				0.5 μg	0.5 μg
pcDNA3.1 vector	1.5 μg	1.5 μg	1.0 μg	1.0 μg	0.5 μg
pSV–β-gal	0.4 μg	0.4 μg	0.4 μg	0.4 μg	0.4 μg

Figure 8 Involvement of CSN 7 in pre-mediated transcriptional activation

H157 cells were pretreated for 48 h with 5 mM 2-difluoromethylornithine and co-transfected with control plasmid pSV-β-galactosidase (pSV-β-gal) and indicated constructs. (**A**) Effects of CSN 7 on PRE-mediated luciferase expression. (**B**) Constructs used in the co-transfection.

increase the luciferase expression level further, but co-transfection with PMF-1 and CSN 7 resulted in inhibition of PRE-mediated transcriptional activation in the absence of BENSpm treatment. Exposure to 10 μM BENSpm relieved this inhibition (Figure 8). It should be noted that transfection with Nrf-2 expression constructs had no significant effect on reporter construct expression as demonstrated previously [1].

DISCUSSION

PMF-1 was previously identified as a transcriptional cofactor of Nrf-2 involved in facilitating the polyamine-inducible transcription of SSAT [1]. However, the possibility that PMF-1 could also interact with other regulatory proteins could not be ruled out. Therefore we used the yeast two-hybrid system in an attempt to find other proteins with which PMF-1 interacted. The most prevalent protein to be identified was the human homologue to subunit 7 of the *Arabidopsis* COP9 signalosome, CSN 7. It should be noted that, although an expressed sequence tag (GenBank® accession no. AA137103) of the human CSN 7 and a partial protein sequence [19] have been identified, no full-length human CSN 7 cDNA has been described previously. Further, no functional role has been described for the human CSN 7 homologue.

For PMF-1 to alter the rate of transcription of SSAT, it must first interact with the Nrf-2 transcription factor that appears to be constitutively bound to the PRE [13]. However, when Nrf-2 binds to the NBE it first heterodimerizes with another protein [18]. Interestingly, from the EMSA studies with the NBE and the functional studies with the PRE-containing constructs, it appears that the binding of PMF-1 and CSN 7 to Nrf-2 is mutually exclusive. PMF-1/Nrf-2 or CSN 7/Nrf-2 is capable of shifting the cognate sequence, NBE. However, when PMF-1 protein is added to the CSN 7/Nrf-2 complex, the amount of NBE shifted is greatly decreased. These results suggest that, under certain circumstances, PMF-1 and CSN 7 can act as competitors and may thus regulate transcription in opposing manners. These results also suggest that CSN 7 may have a role aside from its interaction with the larger complete signalosome, and may also influence the role of Nrf-2-mediated transcription of other genes [18,20].

It should be noted that the homologous plant COP9 complex contains kinase activity capable of phosphorylating c-*jun*, potentially altering the transcription of Ap1-regulated genes [19]. We and others have demonstrated previously that the depletion of polyamines from human cells can lead to a specific decrease in the expression of early-growth-related genes including c-*jun*, c-*myc* and c-*fos* [2–6]. Changes in intracellular polyamine concentrations are known to alter the levels of PMF-1. If PMF-1 interferes with CSN 7 function in the human signalosome complex, it is entirely possible that signal transduction mediated by this protein complex could be affected.

The results of this study are currently limited by the fact that only one gene, the human SSAT, has been identified to contain a PRE and an ability to respond to increases in polyamine or polyamine analogue concentration by an increase in transcription. However, the existence and potential function of PREs in other genes is currently under study. It should also be noted that polyamines have been implicated in altering the expression of certain genes by an alternative method. Hobbs and Gilmour [21] have demonstrated that an increase in intracellular polyamine concentrations associated with the overexpression of ODC leads to an enhancement of histone acetylation. The mechanism by which this occurs is not known.

As an additional example of the potential effects of PMF-1/CSN 7 competition that may occur in various cell systems, Yahalom et al. [22] have demonstrated in *Arabidopsis* that CSN 7 can be localized to the nucleus and interact with the eukaryotic initiation factor eIF3e. This interaction was demonstrated to be a direct association between eIF3e and CSN 7 alone, not in association with the rest of the signalosome proteins. Their results suggest that other nuclear proteins can interact with CSN 7 and regulate transcription and/or message processing and subsequent translation. That PMF-1 may also compete for CSN 7 binding in the nucleus underscores the potential effect on the expression of multiple genes.

It is also important to note that Nrf-2 has transcriptional regulatory roles other than those associated with SSAT. Most notably, the Nrf-2/Maf-family heterodimer plays a critical role in the expression of antioxidant response genes that code for various antioxidant phase-II enzymes [20]. It is possible that, in

addition to competing with PMF-1 for Nrf-2 binding, CSN 7 also competes with the Maf-family proteins and may alter or affect the expression of antioxidant response element containing genes. Further study will be necessary to determine the role of CSN 7 in the expression of other Nrf-2-associated genes.

The potential role for polyamine concentrations in affecting gene expression has been demonstrated previously. However, the molecular mechanisms underlying their effects are only now starting to be elucidated. The inhibition of polyamine metabolism and the subsequent decrease in polyamines leads to a decrease in the expression of c-*myc*, c-*fos* and c-*jun* at the transcriptional level [2–6,23]. Also, it has been demonstrated that in some cell types c-*myc* expression can be specifically down-regulated by polyamine depletion at the translational level [24]. Since the identification of the PRE in the SSAT gene and the associated transcriptional activators, it has now become possible to investigate directly the polyamine-influenced genes and the transcription factors involved. The association of PMF-1 and Nrf-2 resulting in the increased transcription of SSAT as mediated with the PRE is but one potential mechanism by which polyamine-associated transcription factors can influence gene expression. The results presented here are consistent with the idea that PMF-1 may associate with factors other than Nrf-2 and lead to the up- or down-regulation of specific gene expression.

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