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Identification of a Novel Tissue–Specific Transcriptional Activator FESTA as a Protein That Interacts with the Transcription Elongation Factor S-II

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Transcription elongation factor S-II was originally purified as a specific stimulator of transcription by RNA polymerase II. Recent studies suggest that S-II participates in gene-specific transcriptional activation *in vivo*, despite the fact that it directly binds RNA polymerase II and does not recognize specific DNA sequences. In this study, under the hypothesis that S-II requires co-factors to regulate the expression of specific-genes *in vivo*, we searched for factors that directly interact with S-II using a yeast two-hybrid system, and isolated a novel nuclear protein, FESTA. FESTA is expressed specifically in kidney and spleen, supporting our notion that S-II participates in gene-specific regulation. Two mRNA isoforms of FESTA encoding proteins with different sizes were identified and named FESTA-S and FESTA-L. FESTA contains a serine-rich region and a C-terminal tail that are highly similar to those of the ELL-associated factor EAF1. Reporter gene assays indicated that both GAL4-FESTA-S and GAL4-FESTA-L fusion proteins have trans-activating ability. Furthermore, deletion of the C-terminal tail of FESTA dramatically reduced its trans-activating ability and abolished its interaction with S-II. This study is the first report of a transcriptional activator that directly interacts with S-II and contains a transcriptional activation domain that cooperates with S-II via direct interaction.

Key words: S-II, tissue-specific factor, transcriptional activator, transcription elongation.

Abbreviations: Acc., accession number; 6-AU, 6-azauracil; FCS, fetal calf serum; GAL4 AD, GAL4 activation domain; GAL4 DBD, GAL4 DNA binding domain; GAL4 UAS, GAL4-responsive upstream-activating-sequences; GST, glutathione S-transferase; HTGS, high throughput genomic sequence; RT-PCR, reverse transcription–polymerase chain reaction; WGS, whole genome shotgun.

Transcriptional activation can be controlled by increased rate of initiation and/or enhanced efficiency of elongation. Several factors are known to affect transcription elongation by RNA polymerase II. The general transcription factor TFIIF supports both initiation and elongation. Other factors are specific regulators of transcription elongation (*1*). These cellular factors are categorized as follows: (i) P-TEFb, Elongin, MEN/ELL stimulate the rate of transcription elongation (*2–4*); (ii) S-II prevents transcription arrest (*5*, *6*); (iii) Elongator and FACT modify the chromatin structure and facilitate elongation through nucleosomes (*7*, *8*); (iv) DSIF and NELF induce negative regulation of elongation (*9*, *10*).

Among these, S-II is a unique cellular factor that exerts arrest-relief activity on RNA polymerase II. S-II stimulates nascent RNA chain elongation by exerting its arrest-relief activity and by preventing RNA polymerase II from stopping on the template DNA. Since S-II binds directly to RNA polymerase II (*11*) and does not recognize specific DNA sequences by itself, it is supposed to be a general transcription factor. However, recent studies indicate that S-II is involved in gene-specific regulation.

In yeast, disruption of *DST1*, which encodes S-II, does not affect viability under normal growth conditions but induces sensitivity to the nucleotide-depleting drug 6-AU (*12*). Recent studies indicate that S-II regulates the transcription elongation of *SSM1* and supports the induction of *PUR5* in response to 6-AU, suggesting that S-II is essential only under specific conditions. XSII-K1, a tissue-specific S-II subtype in *Xenopus laevis*, participates in the transcriptional regulation of mesoderm-specific genes such as *Xbra* and _-*actin*. How S-II regulates the transcriptional activation of specific genes, however, remains to be elucidated. We hypothesized that S-II is recruited to particular genes by co-factors that are capable of selecting target genes.

We searched for the factors that physically interact with S-II and isolated a novel transcriptional activator from mouse kidney, designated FESTA. The results indicate that FESTA is a tissue-specific transcriptional activator and suggest that FESTA cooperates with S-II in the transcriptional regulation of tissue-specific genes by directly interacting with S-II through its C-terminal activation domain.

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EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen—A PCR product encoding amino acid residues 1 through 182 of mouse S-II was amplified using pSII-3 (*13*) as a template and inserted in-frame into the *Sma*I site of pAS2-1 (Clontech, Palo Alto, CA). The resulting construct, pAS2-1/Ehr1-182, was used as bait in a two-hybrid screen and was co-transfected into *Saccharomyces cerevisiae* Y190 with a mouse kidney cDNA library based on the pGAD10 vector (Clontech). Two-hybrid screening and β-galactosidase assays were performed following the manufacturer's protocol. The plasmids harboring library cDNA were isolated from β-galactosidase-positive clones, then co-transfected individually into yeast strain Y190 with pAS2-1/Ehr1-182 or with the empty vector pAS2-1 as a negative control. The cDNA sequences of positive clones were analyzed using a 377XL DNA sequencer (PE Applied Biosystems, Foster City, CA). The nucleotide sequence of ELH5 was deposited in the DDBJ database under Acc. AB081298. FESTA-L cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) with a pair of specific primers, 5′-CATGAGTGGACCAGCGGGACTTGCATAC-3′ and 5′-TTCTCTGTAGCTTGATACTGC-3′.

Gene Identification—The contiguous sequence of genomic DNA containing exons 1 through 5 was obtained by assembling the Whole Genome Shotgun (WGS) trace data and mouse HTGS (Acc. AC116519) deposited in the MGSC Mouse Gene Server (http://www.ensembl.org/Mus-musculus/) using GENETYX-MAC/ATSQ (version 4.0.4.). A genomic DNA fragment containing exons 4 and 5 was amplified by PCR using 129/SvJ mouse genomic DNA as a template and sequenced. A genomic DNA fragment containing exons 6 and 7 was obtained through plaque hybridization from the 129/SvJ Mouse Genomic Library (Stratagene, La Jolla, CA).

Northern Blot Analysis—Mouse Multiple Tissue Northern blot (Clontech) containing 2 μg of poly $(A)^+$ RNA was probed with a FESTA cDNA probe that encompasses the overlapping nucleotide sequence of ELH4 and ELH5, or with human β-actin cDNA probe. Probes were labeled with [α-^{32}P]dCTP using the random priming method. The blot was washed with 2× saline sodium citrate (SSC) and 0.1% SDS with agitation at room temperature, and with 0.1× SSC and 0.1% SDS, and then exposed to film (Kodak, Rochester, NY) for 4 days at –80°C.

Semi-Quantitative RT-PCR—Total cellular RNAs were extracted from kidneys and spleens of C57BL/6J mice using RNagent (Promega), then subjected to reverse transcription with Stratascript reverse transcriptase (Stratagene) using oligo(dT) primers. Polymerase chain reactions (PCR) were performed using gene-specific primers. Primers used for the amplification of FESTA-L specific region were 5′-CATGAGTGGACCAGCGGGACTTGCATAC-3′ and 5′-TCACAGTGATGTTGCTGCTG-3′. The primer set for FESTA-S amplification comprised 5′-CGGGATTGGCATGTTTGCAGTTCTGG-3′ and 5′-TTCTCTGTAGCTTGATACTGC-3′. The template cDNAs were appropriately diluted so that the signals of reference gene β-actin gave the same intensity. The PCR conditions were: (94°C 45 s, 68°C 1 min) × 5 cycles + (94°C 45 s, 56°C 45 s, 72°C 1 min) × 24 cycles. The intensity of each band was analyzed by Image Gauge software after staining with SYBR green (Takara Shuzo, Shiga).

Expression Vectors—Expression vectors of Xpress-tagged FESTA-L (residues 1–262), FESTA-S (residues 131–262), FESTAΔN (residues 163–262), and FESTAΔNC (residues 163–247) were constructed by inserting cDNA fragments into the *Eco*RV site of pcDNA3.1 HisA (Invitrogen, Carlsbad, CA). Expression vectors of GAL4 DBD-fused FESTA namely, pBIND/FESTA-L (residues 1–262), pBIND/FESTA-S (residues 131–262), pBIND/FESTAΔN (residues 163–262), and pBIND/FESTAΔNC (residues 163–247), were constructed by inserting the same cDNA fragments that were amplified by PCR into the *Eco*RV site of pBIND (Promega). pLuc, a control reporter plasmid that lacks GAL4 UAS, was constructed by deleting the *Nhe*I–*Kpn*I fragment from pG5Luc (Promega). A vector encoding GAL4-DBD–fused FESTA-L for the expression in yeast was constructed by inserting *Bam*HI–*Xho*I fragment excised from pcDNA 3.1 HisA/FESTA-L into the corresponding sites of pACT2 (Clontech). Vectors for FESTA-S (residues 131–262), FESTAΔN (residues 163–262), and FESTAΔNC (residues 163–247) were constructed by inserting cDNA fragments amplified by PCR into the *Sma*I site of pACT2.

Immunofluorescence Analysis—COS7 cells were transiently co-transfected with pFLAG-S-II and pcDNA3.1 HisA/FESTA-L or pcDNA3.1 HisA/FESTA-S using LipofectAmine 2000 (Invitrogen). After incubation for 24 h, the cells were washed with phosphate-buffered saline (PBS), fixed with 1.75% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.2% Triton X-100 for 5 min at room temperature, then blocked with 10% fetal calf serum (FCS). Cells were incubated with the primary antibody, 1 μg/ml anti-Xpress monoclonal antibody (Invitrogen) and 0.16 μg/ml anti-FLAG polyclonal antibody (SIGMA), or with mouse IgG_1 (Dako, Glostrup, Denmark) and rabbit immunoglobulin fraction (Dako). Then samples were incubated with secondary antibodies: Alexa 488–conjugated goat anti–mouse IgG (H+L) conjugate (Molecular Probes, Eugene, OR) to visualize FESTA, and Alexa 568–conjugated goat anti–rabbit IgG (H+L) conjugate to visualize S-II. Nuclei were stained with 10 μg/ml DAPI. Fluorescence images were obtained with an Olympus BH2-RFCA microscope (Olympus, Tokyo). Multiple images were collected and representative cells are shown. All images were digitally processed for presentation using Adobe Photoshop.

GST Pull-Down Assays—FESTA proteins were synthesized and labeled with [^{35}S]methionine *in vitro* using the expression vectors pcDNA3.1 HisA/FESTA-L, pcDNA3.1 HisA/FESTA-S, pcDNA3.1 HisA/FESTAΔN, and pcDNA3.1 HisA/FESTAΔNC. For production of GST-S-II fusion protein in *Escherichia coli*, pGEX2T/S-II was constructed by inserting an S-II cDNA fragment (residues 1–301) that was amplified by PCR into the SmaI site of pGEX-2T (Amersham Biosciences, Piscataway, NJ). GST-fusion proteins were expressed in *E. coli* BL21(DE3) and purified on Glutathione-Sepharose 4B beads according to the protocols provided by the supplier (Amersham Biosciences). Glutathione-Sepharose coupled to GST-S-II (10 μg) or GST alone (10 μg) was batch-absorbed to [^{35}S]methionine-labeled FESTA proteins. Unbound proteins were removed by washing four times

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FESTA-L     1   GGGCACCGAACTCAAGCTCGCAGCCAGTGCAGCTGTTCCAGAGGCTGTGGCAGGGGACGG   60

FESTA-L    61   CGAAGGCCAAAAGCGGGAGGCAGGGGACGAGGATGAGTGGACCAGCGGGACTTGCATACC  120

FESTA-L   121   TGGACCGTCGCGAGCGGGTTCTCAAGCTAGGCGAAAGTTTCGAGAAGCAGCCGCGCTGTG  180

FESTA-L   181   CCTTCCACACCGTGCGCTATGACTTCAAACCTGCTTCTATTGATACTTCTTGTGAAGGAA  240

FESTA-L   241   ATCTTGAGGTTGGCAAAGGTGAACAGGTGACAATAACTCTTCCAAATATAGAAGGTTCAA  300

FESTA-L   301   CTCCACCAGTCACAGTTTTCAAAGGTTCCAAGAGACCTTACTTAAAAGAATGCATTTTGA  360
FESTA-S     1                                                GGGATTGGCATGTT   13

FESTA-L   361   TTATTAACCATGATACTGGGGAATGTCGCCTAGAAAAGCTCAGCAGCAACATCACTGTGA  420
FESTA-S    14   TGCAGTTCTGGTCTGAAACATTAGCAAAGAAACTGGATAAAAAGAAACAATGTGGGAAAC   73

FESTA-L   421   AAAAAACAAGAGTGGAAGGGAGTAGCAGAATCCAGTACAGACTAGAACAACAGCAACAGC  480
FESTA-S    74   AAGAAAATGGAGTGGAAGGGAGTAGCAGAATCCAGTACAGACTAGAACAACAGCAACAGC  133

FESTA-L   481   AAATGTGGAATCTGCCTAGGACTTCCAATCTTGTACAGCATTCTCCATCAGAAGAGAAGA  540
FESTA-S   134   AAATGTGGAATCTGCCTAGGACTTCCAATCTTGTACAGCATTCTCCATCAGAAGAGAAGA  193

FESTA-L   541   TGTCTCCAACGTCTCTAATGGATGATATTGAAAGAGAACTGAAAGCAGAAGCTAGTCTTA  600
FESTA-S   194   TGTCTCCAACGTCTCTAATGGATGATATTGAAAGAGAACTGAAAGCAGAAGCTAGTCTTA  253

FESTA-L   601   TGGACCAGATGAGTAGTTGTGATAGTTCATCAGATTCCAAAAGTTCTTCATCTTCAAGTA  660
FESTA-S   254   TGGACCAGATGAGTAGTTGTGATAGTTCATCAGATTCCAAAAGTTCTTCATCTTCAAGTA  313

FESTA-L   661   GTGAGGATAGTTCTAGTGATTCTGAAGATGATGACCAATTCTCTCCTTTGGGTCCAAGGA  720
FESTA-S   314   GTGAGGATAGTTCTAGTGATTCTGAAGATGATGACCAATTCTCTCCTTTGGGTCCAAGGA  373

FESTA-L   721   AATACAGCTCGGAGCACCCTAGCATGTCTGCTGGGCCACAGTACAGGACTTCAGAGGCTG  780
FESTA-S   374   AATACAGCTCGGAGCACCCTAGCATGTCTGCTGGGCCACAGTACAGGACTTCAGAGGCTG  433

FESTA-L   781   ATGCTACTTGTCACCGACTTCAGGACCACAGTACCCTTCTGATGAGTACTTTACGAAGTG  840
FESTA-S   434   ATGCTACTTGTCACCGACTTCAGGACCACAGTACCCTTCTGATGAGTACTTTACGAAGTG  493

FESTA-L   841   ACTTGCAGCTGAGTGAGTCAGAAAGCGACAGTGAGGACTGAAGCAGTATCAAGCTACAGA  900
FESTA-S   494   ACTTGCAGCTGAGTGAGTCAGAAAGCGACAGTGAGGACTGAAGCAGTATCAAGCTACAGA  553

FESTA-L   901   GAAAACATTTGTGAGATGTGTAAGAATCTGTTTTGTATTGAGAATAAATATTCCTATGTT  960
FESTA-S   554   GAAAACATTTGTGAGATGTGTAAGAATCTGTTTTGTATTGAGAATAAATATTCCTATGTT  613

FESTA-L   961   TATGGAAATTGTGCAACTTTTGCTGAAAAATAAAGTTGGTTCAGAATTTTCAACT(polyA)
FESTA-S   614   TATGGAAATTGTGCAACTTTTGCTGAAAAATAAAGTTGGTTCAGAATTTTCAACC(polyA)
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B

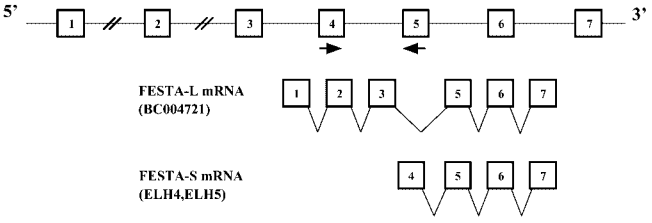

Fig. 1. **Structures of mouse FESTA.** (A) cDNA sequences of FESTA-L (GenBank Acc. BC004721) and FESTA-S (ELH5, Acc. AB081298). The numbers indicate the positions of nucleotide residues within each cDNA sequence. Blue and red letters denote FESTA-L and FESTA-S specific nucleotide sequences, respectively. The regions common to both types are in black. Putative translation initiator ATG codons are underlined. Translation stop codons are doubly underlined. The polyadenylation signals (AATAAA) are shaded. (B) Structure of FESTA gene. (Top) Schematic diagram showing the FESTA gene exons. Open boxes indicate exons. (Bottom) Diagrams showing the splicing of the transcripts for FESTA-L and FESTA-S. mRNA for FESTA-L is composed of exons 1, 2, 3, 5, 6, and 7. mRNA for FESTA-S is composed of exons 4, 5, 6, and 7. Arrows indicate the location of primers used in PCR (see text).

with 1 ml of binding buffer (20 mM HEPES, pH 7.3, 50 mM NaCl, 0.1% NP-40, 5 mM DTT, 10 mg/ml BSA) at 4°C. Bound proteins were harvested by boiling in SDS sample buffer and resolved by 14% SDS-PAGE, and images were obtained with BAS2000 (Fuji Film, Kanagawa).

Luciferase Assays—Reporter and effector constructs were prepared by standard alkaline lysis and two-rounds of cesium chloride gradient purification. NIH3T3 cells were transfected using LipofectAmine Plus (Invitrogen) with 100 ng of a pG5Luc or pLuc reporter plasmid, 100 ng of effector construct pBIND or pBIND derivatives, 100 ng of pCMV·SPORT-β-gal (Invitrogen), and 100 ng of pcDNA3.1. Transfected cells were grown for 48 h in Dulbecco's modified Eagle's medium (Sigma Chemical, St Louis, MO) supplemented with 10% FCS. Total cell lysates were prepared in Reporter Lysis Buffer (Promega), and firefly luciferase activity was assayed using Luciferase Assay System (Promega). β-Galactosidase activity was assayed using β-Gal Reporter Gene Assay, chemiluminescent (Roche Diagnostics, Mannheim, Germany). All luciferase activity values were normalized to β-galactosidase activity values. All transfections were performed in duplicate.

RESULTS

Identification of FESTA by Yeast Two-Hybrid Screening—To identify co-factors that function with S-II in transcriptional regulation, we searched for molecules that interact with S-II from a cDNA library of mouse kidney using a yeast two-hybrid system. Because full-length S-II (residues 1–301) fused to GAL4 DBD inhibited growth of yeast, the N-terminal portion of S-II (residues

Table 1. **Exon-intron boundaries and sizes of mouse FESTA.**

Exon number	Exon size (bp)	Splice acceptor	Splice donor	Intron size (kb)
1	198	GGGCACCGAA	ACCGTGCGCT/**gt**gagtgcag	n.d.
2	95	ctatttttt**ag**/ATGACTTCAA	AAATATAGAA/**gt**aagtagtt	n.d.
3	137	tcttaaac**ag**/GGTTCAACTC	AAAAAACAAG/**gt**aggtggtg	n.d.
4	84	GGGATTGGCA	AAGAAAATGG/**gt**aaaccaag	0.46
5	146	ttaattgc**ag**/AGTGGAAGGG	ATTGAAAGAG/**gt**aaaatcta	5.3
6	258	ctattttc**ag**/AACTGAAAGC	AGTACTTTAC/**gt**aagtatgc	6.5
7	182	tttattttt**ag**/GAAGTGACTT	TTTTCAACTA	–

Intronic sequences are shown in lower-case letters, and exon sequences are shown in upper-case letters. Sizes of exons 1 and 4 were calculated by defining the 5′ position of the EST clone (Acc. BC004721) and ELH5 as 1, respectively. Conserved splice donor and splice acceptor dinucleotides are in bold. n.d., not determined.

1–182) was fused to the GAL4 DBD and used as "bait." Among the 1.4 million clones screened, two positive clones were isolated and designated ELH4 and ELH5, respectively. Sequence analysis revealed that ELH4 and ELH5 encoded 164 and 175 overlapping amino acids, respectively. We named the novel protein encoded by these clones FESTA (A Factor interacting with Ehrlich S-II functions as a Transcriptional Activator), because subsequent studies revealed its property as a transcriptional activator.

Two FESTA mRNA Isoforms Are Expressed—BLAST searches of the GenBank database with the FESTA nucleotide sequences revealed an EST clone (Acc. BC004721) that contains a longer open reading frame than ELH5 (Fig. 1A). We named the 262 amino acid protein encoded by the EST clone FESTA-L. The C-terminal portion (residues 131–262) of the deduced amino acid sequence of FESTA-L was identical to those of ELH4 and ELH5, whereas the N-terminal portion (residues 1–130) was distinguishable. We hypothesized that the nucleotide sequences of the EST clone and ELH4/ELH5 consist of different sets of exons. To test this, we analyzed genomic DNA sequences that contain these cDNA sequences. We isolated a genomic DNA fragment by PCR using a pair of primers designed to obtain a genomic DNA fragment containing the nucleotide sequence specific to ELH4/ELH5 and the region common to these three cDNA clones (Fig. 1B, arrows). Nucleotide sequence analysis of the amplified 660-bp fragment revealed that the consensus splice donor/acceptor sequences, GT and AG, respectively, appear at the boundaries between the common region and the short-type cDNA (ELH4/ELH5) or the long-type cDNA (Table 1). These findings indicated that exons 4 and 5 are continuous on the mouse genome, and exon 4 is unique to the short-type cDNA (ELH4/ELH5). Moreover, the 5′-rapid amplification of cDNA ends (5′-RACE) method using a primer specific to exon 4 did not amplify cDNAs longer than ELH5 (data not shown). We also confirmed the structure of the *FESTA* gene by assembling genomic sequences retrieved from the MGSC Mouse Gene Server (Fig. 1B, and data not shown). Thus, we supposed that BC004721 and ELH4/ELH5 are composed of different sets of exons, and that ELH4/ELH5 are expressed from an alternative promoter. We named the putative 132 amino acids protein encoded by ELH4/ELH5 FESTA-S. Both FESTA-L and FESTA-S contained two characteristic regions rich in serine, glutamic acid, and aspartic acid (residues 187–209 and 249–262 in FESTA-L).

FESTA Is a Tissue-Specific Factor—To examine the tissue distribution of FESTA, poly (A)$^+$ RNAs from various mouse tissues were hybridized with FESTA cDNA probe. The results indicated that FESTA is expressed specifically in kidney and spleen (Fig. 2A). A single 1-kb transcript was detected specifically in kidney, whereas a larger 1.4-kb transcript was detected specifically in spleen. As we have identified two variants of FESTA, it is likely that these mRNAs correspond to FESTA-S and FESTA-L.

In order to test this idea, we performed semi-quantitative RT-PCR analysis using two pairs of primers that specifically amplify FESTA-S or FESTA-L (Fig. 2B). The

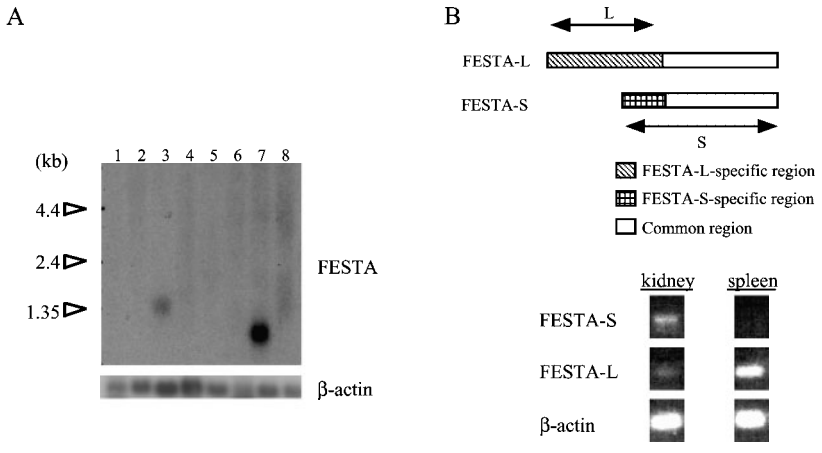

Fig. 2. **FESTA is a tissue-specific factor.** (A) Poly(A)$^+$ RNAs from mouse tissues were probed with a FESTA cDNA probe (Top). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. As a loading control, the same blot was probed with human β-actin cDNA (Bottom). Open arrows at the left of the autoradiogram indicate size markers. (B) Expression of FESTA cDNA isoforms were analyzed by semi-quantitative RT-PCR. (Top) Schematic illustrations of two isoforms of FESTA cDNA. Double-headed arrows indicate the regions amplified by PCR. (Bottom) Expression analysis of FESTA-S and FESTA-L in kidney and spleen.

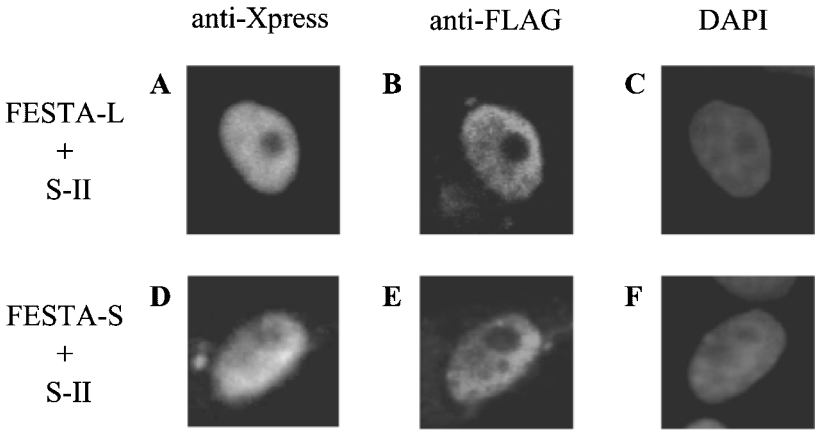

Fig. 3. **FESTA-L and FESTA-S co-localize with S-II in nucleoplasm.** COS-7 cells were transiently co-transfected with the Xpress-tagged FESTA-L or FESTA-S expression vector and FLAG-tagged S-II expression vector, then processed for immunofluorescence analysis using anti-Xpress antibody and Alexa 488–conjugated anti–mouse immunoglobulin antibody for FESTA-L (A) or FESTA-S (D) and anti-FLAG antibody and Alexa 568–conjugated anti–rabbit immunoglobulin antibody for S-II (B, E). DAPI was used to stain nuclei (C, F).

results indicated that FESTA-S is expressed specifically in kidney. FESTA-L was detected in both kidney and spleen, but quantitative analysis revealed that expression in kidney is much lower than that in spleen.

By searching the expressed sequence tags (EST) cluster database, Unigene (http://www.ncbi.nlm.nih.gov/entrez), we found that FESTA-L cDNA clones have been identified also from thymus, dendritic cells, germinal B-cells, and testis besides kidney and spleen. But we could not find FESTA-S cDNA other than from kidney cDNA library, indicating that FESTA-S is exclusively expressed in kidney.

FESTA Is a Nuclear Protein and Co-Localizes with S-II—We supposed that FESTA would localize to the nucleoplasm, since S-II has been shown to localize there (*14*). To examine the subcellular localization of FESTA-L and FESTA-S, indirect immunofluorescence analysis was performed with COS7 cells in which Xpress-tagged FESTA and FLAG-tagged S-II were co-expressed. Nucleoplasms were stained with anti-Xpress antibody in the cells expressing FESTA-L or FESTA-S, whereas nucleoli were not (Fig. 3, A and D). Nucleoplasms of the cells were stained with anti-FLAG antibody, indicating that S-II co-localized with FESTA (Fig. 3, B and E). When IgG from non-immunized mouse or rabbit was used as a primary antibody, nuclei were not stained (data not shown). These results demonstrated that FESTA is a nuclear factor, and that it co-localizes with S-II.

C-Terminal Tail of FESTA Is Essential for the Interaction with S-II—Next, we investigated whether S-II physically interacts with FESTA *in vitro*. We assessed the binding of FESTA with S-II using a GST pull-down assay. GST-S-II fusion protein expressed in *E. coli* was purified and coupled to Glutathione-Sepharose beads. GST alone was coupled to the beads and used as a negative control. [^{35}S]methionine-labeled FESTA proteins were synthesized *in vitro* (Fig. 4A). Both FESTA-L and FESTA-S bound to GST-S-II but not to GST alone (Fig. 4B, lanes 1 and 2). These results suggest that both FESTA isoforms function with S-II. Next, we analyzed the region in FESTA that is required for the interaction. We constructed deletion mutants of FESTA, FESTAΔN (residues 163–262), which contains both the serine-rich region and the C-terminal tail, and FESTAΔNC (residues

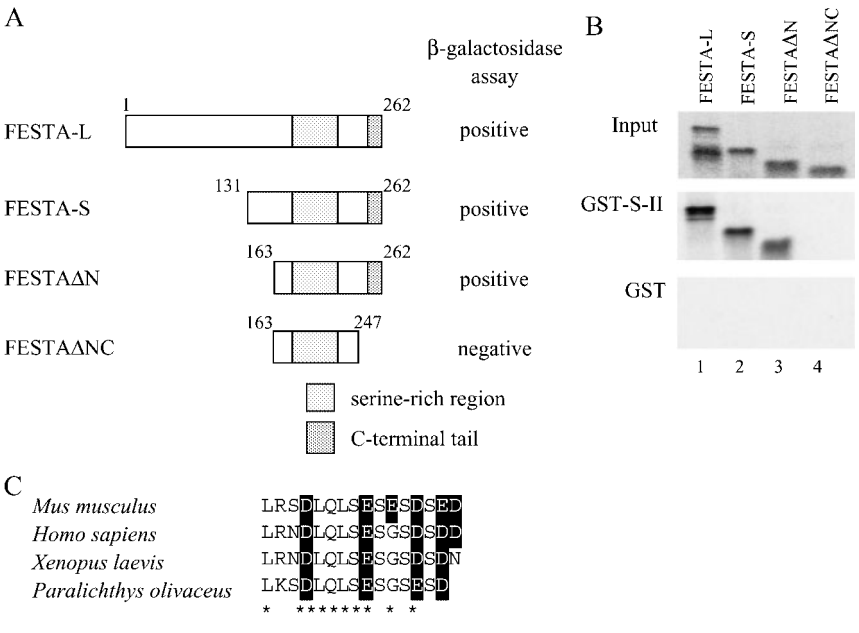

Fig. 4. **FESTA interacts with S-II *via* C-terminal tail.** (A) Schematic representations of FESTA constructs used in the experiment. Interaction with S-II was scored using the yeast two-hybrid system. The results of β-galactosidase assays in yeast two-hybrid assays are indicated by "positive" or "negative." (B) GST pull-down assays were performed using *in vitro* translated [^{35}S]methionine-labeled FESTA proteins and bacterially expressed GST-S-II fusion protein. Glutathione-Sepharose beads coupled to GST-S-II (middle) or GST alone (bottom) were batch-absorbed to various radio-labeled FESTA proteins. Five percent of the input FESTA proteins are shown (top). (C) C-terminal tails of FESTA are conserved among many vertebrate species. Alignment of the deduced amino acid sequences of vertebrate homologues, *Mus musculus* (Acc. BC004721), *Homo sapiens* (Acc. BC014209), *Xenopus laevis* (Acc. AW640366), *Paralichthys olivaceust* (Acc. AU050237). Acidic residues are shaded. Asterisks indicate amino acid residues conserved among all proteins.

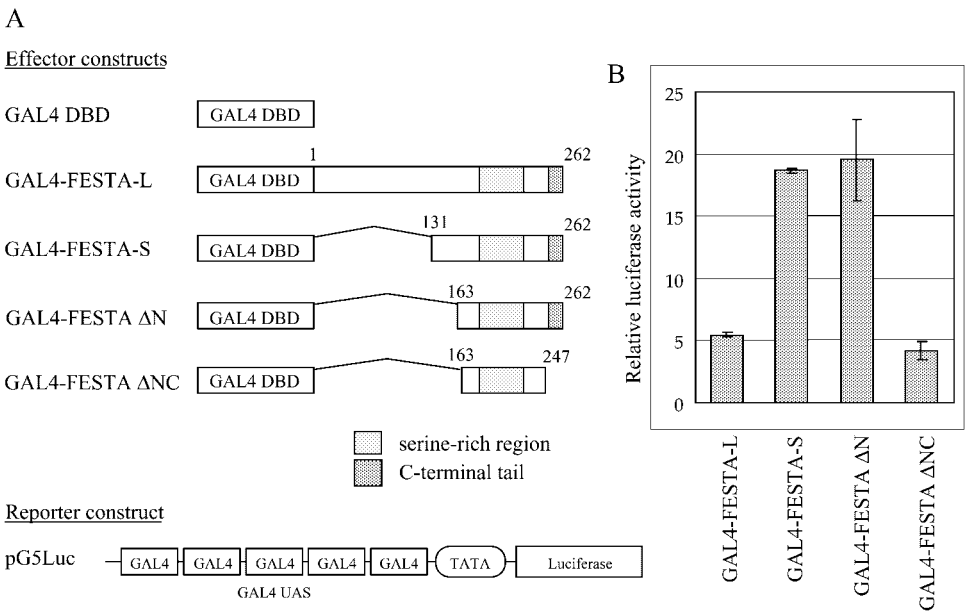

Fig. 5. **FESTA contains transcriptional activation domains.** (A) Schematic representations of effector and reporter constructs used in the experiment. pBIND is an expression vector of GAL4 DBD. GAL4 DBD alone or GAL4 DBD fusion proteins were expressed from pBIND or its derivatives. pG5Luc is a reporter construct containing five copies of the GAL4 UAS upstream of the adenovirus major late promoter. (B) NIH3T3 cells were co-transfected with reporter construct pG5Luc, pBIND derivatives expressing various GAL4 DBD-FESTA fusion proteins, and the internal standard plasmid pCMV·SPORT-β-gal. Relative luciferase activity induced by each FESTA construct was calculated by defining the luciferase activity in the presence of pBIND as 1. Error bars represent the standard deviations of two independent experiments.

163–247), which lacks the C-terminal tail (Fig. 4A). FESTAΔN bound to GST-S-II but not to GST alone, indicating that it retained the S-II binding capacity (Fig. 4B, lane 3). On the other hand, FESTAΔNC did not bind to either GST-S-II or GST alone (Fig. 4B, lane 4). These findings indicate that the C-terminal tail of FESTA is essential for the interaction with S-II, and the serine-rich region alone is not sufficient for the interaction. Consistent results were obtained from the yeast two-hybrid assay, suggesting that FESTA interacts with S-II through its C-terminal tail *in vivo* (Fig. 4A).

The C-terminal tail is conserved among vertebrate homologues (Fig. 4C), and thus the ability of FESTA to interact with S-II might also be conserved.

FESTA Contains a Transcriptional Activation Domain that Overlaps the S-II Binding Region—As FESTA contains several acidic regions and a serine-rich region that are characteristic of various transcriptional activators, we next examined whether FESTA functions as a transcriptional activator. We co-transfected NIH3T3 cells with pBIND/FESTA-S or with pBIND/FESTA-L, which express FESTA-S or FESTA-L fused to GAL4 DBD, and pG5Luc luciferase reporter driven by tandem GAL4-responsive upstream-activating-sequences (GAL4 UAS) (Fig. 5A). Compared with GAL4 DBD alone, GAL4-FESTA-L and GAL4-FESTA-S activated luciferase expression 5.5-fold and 18.7-fold, respectively (Fig. 5B). Activation by FESTA was not observed when the reporter construct pG5Luc was replaced with a control reporter construct pLuc, which lacks GAL4 UAS (data not shown). Although the capacities to bind to S-II were indistinguishable, GAL4-FESTA-L had lower transcriptional activity than GAL4-FESTA-S in the reporter gene assay. Possible explanations for this paradox are that cellular inhibitory factors compete with S-II in binding with FESTA-L, or some post-translational modifications alter the trans-activating competency of FESTA-L.

To investigate whether the interaction with S-II is important for the transcriptional activity of FESTA, we measured the trans-activating abilities of FESTA deletion mutants: FESTAΔN, which binds to S-II; and FESTAΔNC, which does not. GAL4-FESTAΔN activated reporter gene expression as well as GAL4-FESTA-S, while the trans-activating ability of GAL4-FESTAΔNC was decreased to 22% of that of GAL4-FESTA-S (Fig. 5B). These results indicate that loss of the C-terminal 15 amino acids residues of FESTA reduces its trans-activating ability, suggesting the involvement of the C-terminal tail of FESTA in efficient transcriptional activation, which is induced *via* the interaction with S-II. GAL4-FESTAΔNC exhibited a lower residual transcriptional activating ability than did GAL4-FESTAΔN, indicating that the regions remaining in FESTAΔNC, possibly the serine-rich region, were still functional in an activation mechanism that occurs independently of the interaction with S-II. Thus, we concluded that FESTA contains at least two activation domains, which are S-II-dependent and S-II-independent.

DISCUSSION

In this study, we isolated a novel nuclear factor FESTA as an S-II interacting factor. Tissue distribution analysis and database searches revealed that there are two tissue-specific FESTA variants, FESTA-S and FESTA-L, each showing a distinct expression pattern. GST pull-down and two-hybrid assay indicated that FESTA directly interacts with S-II *in vitro* and *in vivo*, and the C-terminal tail of FESTA is essential for the interaction. The investigation of transcriptional activity of FESTA using a luciferase assay system indicated that FESTA contains at least two transcriptional activation domains, one in the C-terminal tail and the other in residues 163–247, possibly in the serine-rich region. The analysis of deletion mutants suggested that the C-terminal tail of

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mouseFESTA-L      1 MSGPAGLAYLDRRERVLKLGESFEKQPRCAFHTVRYDFKPASIDTSCEGNLEVGKGEQVT  60
humanFESTA-L      1 MNSAAGFSHLDRRERVLKLGESFEKQPRCAFHTVRYDFKPASIDTSSEGYLEVGEGEQVT  60
mouseEAF1         1 MNGTAN-PLLDREEHCLRLGESFEKRPRASFHTIRYDFKPASIDTSCEGELQVGKGDEVT  59
humanEAF1         1 MNGTAN-PLLDREEHCLRLGESFEKRPRASFHTIRYDFKPASIDTSCEGELQVGKGDEVT  59
                    *   *    *** *  * ******* **  *** ************ ** * ** *  **

mouseFESTA-L     61 ITLPNIEGSTPPVTVFKGSKRPYLKECILIINHDTGECRLEKLSSNITVKKTRVEGSSRI 120
humanFESTA-L     61 ITLPNIEGSTPPVTVFKGSKKPYLKECILIINHDTGECRLEKLSSNITVKKTRVEGSSKI 120
mouseEAF1        60 ITLPHIPGSTPPMTVFKGNKRPYQKDCVLIINHDTGEYVLEKLSSSIQVKKTRAEGSSKI 119
humanEAF1        60 ITLPHIPGSTPPMTVFKGNKRPYQKDCVLIINHDTGEYVLEKLSSSIQVKKTRAEGSSKI 119
                    **** * ***** ***** * ** * * *********  ****** * ***** **** *

mouseFESTA-L    121 QYRLEQQQQQ---------------MWNLPRTSNLVQHSPSEEKMSPTSLMDDIERELKA 165
humanFESTA-L    121 QYRKEQQQQQ---------------MWNSARTPNLVKHSPSEDKMSPASPIDDIERELKA 165
mouseEAF1       120 QARMEQQPARPPQPSQPPPPPPPPMPFRAPTKPPAGPKTSPLKDNPSPEPQLDDIKRELRA 179
humanEAF1       120 QARMEQQPTRPPQTSQPPPPPPPPMPFRAPTKPPVGPKTSPLKDNPSPEPQLDDIKRELRA 179
                    * * ***                                 **     **    *** *** *

mouseFESTA-L    166 EASLMDQMSSCDSSSDSKSSSSSSSEDSSSDSEDDDQFSPLGPR-KYSSEHPSMSAGPQY 224
humanFESTA-L    166 EASLMDQMSSCDSSSDSKSSSSSSSEDSSSDSEDEDCKSSTSDTGNCVSGHPTMT---QY 222
mouseEAF1       180 EVDIIEQMSSSSGSSSSDSESSSGSDDDSSSSAGED-NGPASPP-QPSHQQPYNS----- 232
humanEAF1       180 EVDIIEQMSSSSGSSSSDSESSSGSDDDSSSSGGED-NGPASPP-QPSHQQPYNS----- 232
                    *     ****   ** * * *** * * ** *   *                *

mouseFESTA-L    225 RTSEADATCHRLQDHSTLLMSTLRSDLQLSESESDSED                      262
humanFESTA-L    223 RIPDIDASHNRFRDNSGLLMNTLRNDLQLSESGSDSDD                      260
mouseEAF1       233 RPAVANGTSR--PQGSSQLMNTLRNDLQLSESGSDSDD                      268
humanEAF1       233 RPAVANGTSR--PQGSNQLMNTLRNDLQLSESGSDSDD                      268
                    *             *  ** *** ******* *** *
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Fig. 6. **FESTA-L exhibits strong similarity to EAF1.** Deduced amino acid sequences of mouse FESTA-L (Acc. BC004721), human FESTA-L (Acc. BC014209), mouse EAF1 (Acc. AK016628), and human EAF1 (Acc. AF272973) were aligned using the CLUSTALW algorithm (version 1.81). The numbers indicate the positions of amino acid residues within each protein. Acidic residues are marked in red. Asterisks indicate amino acid residues conserved among all proteins.

FESTA is important for both the interaction with S-II and the transcriptional activation. Our study presents the first evidence that S-II directly interacts with a tissue-specific transcriptional activator, and suggests a molecular mechanism in which S-II participates in gene-specific transcriptional regulation through interaction with an activation domain of tissue-specific transcriptional activators.

Tissue-specific expression of FESTA suggests that FESTA participates in the regulation of tissue-specific gene expression, especially in kidney and spleen. It is possible that downstream genes possess transcriptional pausing sites, and that S-II stimulates elongation through these transcriptional block sites. At present, it is not clear whether FESTA recognizes specific DNA sequences. Well-characterized trans-activators such as GAL4 and VP16 are composed of distinct DNA binding domains and transcriptional activation domains (*15*, *16*). Although FESTA contains transcriptional activation domains, it does not contain DNA-binding motifs that are apparent from its primary structure. This is also the case with AF4, which was found to be fused to MLL DNA-binding domain in acute leukemias (*17*). Whether FESTA recognizes specific DNA sequences directly or indirectly through interactions with other DNA-binding factors has to be elucidated to reveal the events that occur downstream of the interaction between S-II and FESTA.

Blau *et al.* reported that activation domains of some trans-activators, such as VP16, p53, and E2F1, stimulate not only the rate of initiation but also the efficiency of transcription elongation by RNA polymerase II (*18*), but the molecular mechanism is not yet known. Among the above trans-activators, VP16 possesses acidic regions that play important roles in transcriptional activation (*19*). The deduced amino acid sequence of FESTA-L indicates that it is a highly acidic protein (calculated pI 4.8), particularly in its C-terminal region (Fig. 6). It is possible that S-II, which has a basic character, is a target of these acidic transcriptional activators and stimulates transcription elongation of the genes selected by these activators.

In yeast, S-II was shown to participate in gene-specific regulation particularly in the induction of *SSM1* and *PUR5* genes in response to 6-AU (*20*, *21*). It is likely that interaction with S-II and other gene-specific regulators might function in the induction of these genes in response to 6-AU, though we could not find any open reading frames in the yeast genome that encode a protein with significant similarity to FESTA. The strategy to isolate S-II-interacting factors in yeast might identify such regulators with equivalent function to mouse FESTA.

It is also of great interest to determine how the interaction between S-II and FESTA contributes to transcriptional activation. We analyzed whether FESTA alters the activity of S-II *in vitro*, but we could not observe a significant effect of FESTA on the RNA polymerase II-stimulating activity of S-II (data not shown). It is possible that FESTA requires other factors besides S-II and RNA

polymerase II to activate transcription *in vivo*, and that FESTA and these factors constitute a macromolecular transcription complex. Identification of such a macromolecular complex may greatly contribute to solving the question.

During the course of our study, we found that FESTA-L exhibits strong similarity to ELL-associated factor 1 (EAF1) (*22*). EAF1 is a ubiquitously expressed factor isolated from bone marrow as an ELL-associated factor by yeast two-hybrid screening. The amino acid sequence alignment revealed strong similarity between FESTA-L and EAF1 spanning the N-terminal portion (residues 1–127 in mouse FESTA-L), the serine-rich region, and the C-terminal tail (Fig. 6). There are also serine-rich regions in the transcriptional activation domains of several translocation partner proteins of MLL in acute leukemia, including AF4, LAF4 and AF5q31(*23–25*) Thus, it is conceivable that the trans-activation induced by FESTAΔNC is due to the function of the serine-rich region. As the C-terminal tail is highly conserved between FESTA and EAF1 (12 of 15 residues), it is likely that EAF1 also binds to S-II. The structural similarity between FESTA and EAF1 also suggests that S-II and ELL have cooperative or competitive functions that are mediated by the C-terminal tails of FESTA/EAF1. ELL negatively regulates S-II-induced nascent transcript cleavage *in vitro* (*26*). Whether the inhibitory effect of ELL on S-II is modified in the presence of FESTA/EAF1 *in vivo* remains to be elucidated.

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