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# Autoinduction of the trefoil factor 2 (TFF2) promoter requires an upstream *cis*-acting element

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**Abstract**

Trefoil factor 2 (TFF2)/spasmolytic polypeptide (SP) is a highly stable peptide which is abundantly expressed and secreted by mucous cells of the stomach and which functions in gastric cytoprotection. Previous studies from our group have shown that TFF2 is an immediate early gene capable of regulating its own expression through activation of the TFF2 promoter. We therefore aimed to investigate the *cis*-acting elements mediating this response in AGS cells transfected with TFF2 promoter–reporter gene constructs, using a TFF2-expression system resembling physiologic paracrine conditions. TFF2 peptide expression was achieved through stable transfection of AGS cells with a TFF2-expression construct. Stimulation of transiently transfected cells with this TFF2-containing conditioned media resulted in a significant increase in TFF2 promoter activity. Promoter stimulation was blocked by an anti-TFF2 antibody, indicating that it was mediated specifically by TFF2. Deletion analysis of the TFF2 promoter led to the identification of a specific response element located between −191 and −174 upstream of the transcriptional initiation site. This region of the promoter, which was designated SPRE (for spasmolytic polypeptide response element), was sufficient to confer responsiveness in a heterologous promoter system. Mutational analysis and electrophoretic mobility shift assays (EMSA) showed that a GAG motif was responsible for mediating promoter activation in response to TFF2 stimulation. Since auto- and cross-induction of TFF2 promoter is likely to be a means of rapid amplification of TFF2 expression in the critical first minutes following mucosal injury, these results should lead to insight into the molecular events initiating epithelial restitution and healing. 

*Keywords:* Trefoil peptides; Gene regulation; Restitution; SPRE

Trefoil factor 2/spasmolytic polypeptide (TFF2/SP) is a protein expressed and secreted predominantly by mucous neck cells in the gastric mucosa and Brunner's glands in the duodenum [1,2]. TFF2 is a member of a larger peptide family that includes at least three members (pS2/TFF1, SP/TFF2, and ITF/TFF3) that are expressed widely throughout the gastrointestinal tract [1,3,4]. The genes encoding these peptides have been mapped to the same 50 kb cluster region on chromosome 21q22.3 [5,6]. This family of peptides is characterized by one or more three-leaf clover or trefoil structural motifs and is highly protease resistant [1,3,4]. Both in vitro and in vivo studies have supported the notion that the trefoil peptides play a major role in protection, repair, and healing of the gastrointestinal mucosa [7–10].

TFF2 in particular is rapidly upregulated in response to mucosal injury [1,9]. Increased levels of TFF2 and other trefoil factors have been observed adjacent to ulcerations of the gastrointestinal tract [7]. Furthermore, upregulation in TFF2 expression has been demonstrated in both premalignant and malignant changes in the gastric mucosa, raising the possibility of a role in tumor suppression [11,12]. Recent studies from our group have shown that TFF2 transcription is regulated through growth factor pathways, and that upregulation of TFF2 gene expression requires both EGF receptor and MAP

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kinase activation [13]. In addition, studies from our laboratory suggest that TFF2 is capable of autoinduction; that is, TFF2 promoter activity increases in gastric epithelial cells in response to stimulation by exogenous recombinant TFF2 peptide (as well as by other trefoil peptides such as TFF3) [13]. Stimulation of AGS or KATO III cells with recombinant TFF2 results in increased steady-state TFF2 mRNA within 10 min with a maximum stimulation seen after 30 min. In addition, pretreatment of cells with cycloheximide enhanced responses to recombinant trefoils, suggesting TFF2 was an immediate-early response gene [13]. Similar responses to recombinant TFF2 stimulation were noted for the TFF1 gene. The increase in TFF2 mRNA following TFF2 peptide stimulation is most likely a transcriptional response, since the TFF2 promoter was also activated in response to trefoil peptide stimulation [13].

Previous studies of the human pS2 promoter had shown that a specific *cis*-acting region between −428 and −332 upstream of the translational start site was responsible for regulation by Ras, EGF, and phorbol esters [14]. To date, a similar *cis*-acting region has not been found in the TFF2 promoter. Several studies have investigated the human TFF2 promoter in various gastrointestinal cell lines and a number of potential transcription factor binding sites have been identified in the TFF2 promoter (including HNF3 and GATA6) [15–17]. However, the potential role of these *cis*-acting DNA elements in the growth factor-dependent regulation of TFF2 transcription has not been addressed.

In our earlier published study, we showed that the *cis*-acting response element mediating upregulation by recombinant TFF2 was contained within the basal 820 bp upstream of the translational start site of the human TFF2 promoter [13]. The aim of the current study was to identify and analyze the specific *cis*-acting elements in the human TFF2 promoter mediating this response. To create a more physiologic model for paracrine or autoinduction by TFF2, we generated a human cell-based expression system for TFF2, and used conditioned media from these overexpressing cells as a source of TFF2. This cell culture system more closely resembles the in vivo situation where TFF2 is locally produced and released after mucosal injury. Results from these studies confirm our previous reports with recombinant peptides, and further demonstrate that the *cis*-acting response element can be localized to a small upstream (−191 to −174) region of the TFF2 promoter.

## Materials and methods

*Primer extension analysis and anchored polymerase chain reaction*

The transcriptional initiation site was mapped using a [$\gamma$-$^{32}$P]dCTP-end-labeled ($1 \times 10^7$ cpm/pmol) 24-base oligonucleotide probe (5′-GGT TTC TCA CTC CCC GCC AGG GCA-3′) that was designed from Exon 1 downstream of the translational start site of human TFF2 cDNA. The primer was annealed to poly(A)$^+$ RNA (5 μg) isolated from KATO III cells known to express TFF2 (data not shown) for 12 h at 30 °C in 1 M NaCl, 167 mM HEPES (pH 7.5), and 0.33 mM EDTA (pH 8.0). Transcripts were extended for 2 h at 37 °C using avian myeloblastosis virus reverse transcriptase (Perkin–Elmer Applied Biosystems, Foster City, CA). The extended product was subjected to electrophoresis on a denaturing urea 6% polyacrylamide gel (Novex, Carlsbad, CA), and the sizes of the extended products were determined by comparison with the [$\gamma$-$^{32}$P]dCTP-end-labeled ($1 \times 10^7$ cpm/pmol) pGEM Marker (Promega, Madison, WI).

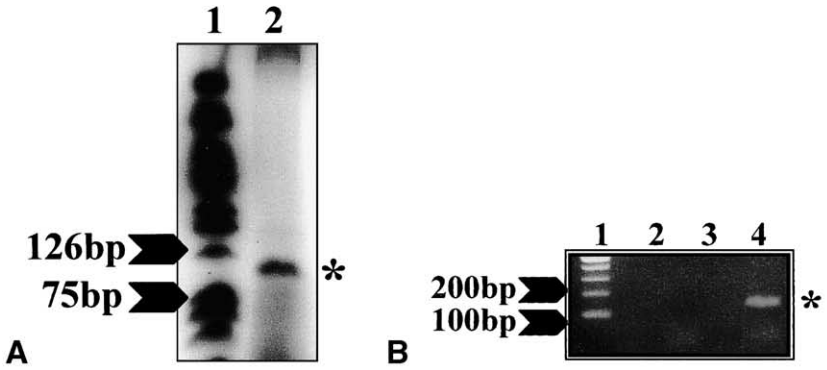
To confirm the results obtained by primer extension analysis, anchored polymerase chain reaction was carried out as described previously [18]. Briefly, first-strand cDNA was synthesized from 1 μg of mRNA from KATO III cells using the RT-PCR kit from Perkin–Elmer containing the above specific primer (5′-GGT TTC TCA CTC CCC GCC AGG GCA-3′). After removing the excess primer with a Centricon 100 spin filter (Amicon), the 3′-end of the first-strand cDNA was tailed in a 20 μl reaction mixture containing 1× tailing buffer, 1 mM dATP, and 15 units of terminal deoxynucleotidyltransferase (Life Technologies) for 10 min at 37 °C and then heated for 15 min at 65 °C. One microliter of the reaction mixture was used to synthesize the second-strand cDNA with 10 pmol of anchored oligo(dT)16 using 2.5 units of Taq DNA polymerase (Perkin–Elmer) at 72 °C for 7 min and then subjected to PCR with a nested primer (5′-CTC CCC GCC AGG GCA CAT AGC C-3′). PCR was performed in a thermal cycler (Perkin–Elmer) as described previously [19]. The anchored PCR products were resolved by electrophoresis on a 2.5% agarose gel with 100 bp DNA size markers. The product was gel-extracted using a commercial kit (Qiagen, Germany), cloned into PCR 2.1 vector with the TA cloning kit (Invitrogen, Carlsbad, CA), and sequence analysis performed.

*Cloning and construction of reporter constructs*

A series of human TFF2 promoter 5′-deletion constructs (hTFF2-luciferase) containing nucleotides upstream of the start site were made by PCR amplification of segments from the human TFF2 promoter utilizing the previously described [13] human TFF2 promoter fragment, containing 823 bp of 5′-flanking DNA upstream of the ATG as template. Given the results regarding the identity of the transcriptional start site this construct was renumbered and renamed 795TFF-Luc. The 5′ primers for the different constructs containing a *Kpn*I site were for the 479TFF2-Luc 5′-ATA CGC GTC ACA GCC CTG GGA GCA GTC TCT-3′, for the 209TFF2-Luc 5′-ATA CGC GTC AGG GCA GGA AGA GGT ATC ACC-3′, for the 191TFF2-Luc 5′-ATA CGC GTA GCA GGG AGA GAG TCA CCC-3′, for the 174TFF2-Luc 5′-ATA CGC GTC CCT GGC CCG GAA-3′, and for the 139TFF2-Luc 5′-ATA CGC GTT GCC TCT TGC CTC CT-3′. The 3′ primer containing a *Bgl*II site was constantly 5′-ATA GAT CTG TCT AGC TCA GCT GCA CCC CAG-3′. The PCR products were cloned into PCR 2.1 vector with TA cloning kit (Invitrogen, Carlsbad, CA). Sequence of cloned PCR products was confirmed by sequence analysis. Subsequently PCR products were subcloned into *Kpn*I/*Bgl*II sites of pGL2 basic vector (Promega, Madison, WI).

For confirmation of relevance of the identified *cis*-acting region, sense and antisense oligonucleotides were designed with wild type and mutated sequences containing *Bam*HI and a mutated *Xho*I restriction sites. Sequences were wild type sense 5′-GAT CGA GCA GGG AGA GAG TCA CCC C-3′, wild type antisense 5′-TCG AGG GGT GAC TCT CTC CCT GCT C-3′, IK2 mutant sense 5′-GAT CGA GCA **TGT ACA** GAG TCA CCC C-3′, IK2 mutant antisense 5′-TCG AGG GGT GAC TCT **GTA CAT** GCT C-3′, AP1 mutant sense 5′-GAT CGA GCA GGG AGC **AGG TTA** CCC C-3′, AP1 mutant antisense 5′-TCG AGG GGT **AAC CTG** CTC CCT GCT-3′, serial mutations M1 sense 5′-GAT C**GG TAG T**GG AGA GAG TCA CCC C-3′, M1 antisense 5′-TCG AGG GGT GAC TCT CTC C**AC TAC** C-3′, M2

sense 5′-GAT CGA GCA **TTT GT**A GAG TCA CCC C-3′, M2 antisense 5′-TCG AGG GGT GAC TCT **ACA AA**T GCT C-3′, M3 sense 5′-GAT CGA GCA GGG A**TG TGT** CCA CCC C-3′, M3 antisense 5′-TCG AGG GGT G**GA CAC A**TC CCT GCT C-3′, M4 sense 5′-GAT CGA GCA GGG AGA GAG **GAG A**CC C-3′, M4 antisense 5′-TCG AGG G**TC TC**C TCT CTC CCT GCT C-3′. Single-stranded oligonucleotides for site-specific cloning were annealed and ligated upstream of the enhancerless thymidine kinase promoter of herpes simplex virus1 in the construct pT81. All constructs were confirmed by sequence analysis.




-829 tggggaactt cgggtcccct ctgtccttcc caccacactt ttcctctttt ctttccgggt

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-649 cccatggaac aaaaataacc catttcaggg gttggctgaa aatgaactta ttaaaacctg

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-529 tgctgaagat gggacaaagg gcatcgtgct gcggttgggg aggctctaac cacagccctg

-469 ggagcagtct cttacctcct ctgagatgct tcccttcctc agggaggggа cttttccatg

-409 ctatctgctg gcctgtacat tttccccagt aaacttggcc ctaatatttt ctaaattcct

-349 gtggtccctg cccactctat caatagaaat gcatagctta tcccttcctg ggtgtgaccc

-289 tgtgtgtgcc cagccccaga cctgcacgtg gccggttttc cacgctggca gcctggcatg

-229 acccaactct ctgtccaggg caggaagagg tatcac**cGAG CAGGGAGAGA GTCA**ccctgg

-169 cccggaagcc tcgcctgcac agggcacagc tgcctcttgc ctcctcttcg cctccacggt

-109 ggaagggctg gggccacggg gcagagaaga aaggttatct ctgcttgttg gacaaacaga

-49 ggggagat***ta taa***acatac ccggcagtgg acaccatgc

+1 **a ttctgcaagc caccctgggg tgcagctgag ctagac**

+38 ATGG GACGGCGAGA CGCCCAGCTC CTGGCAGCGC TCCTCGTCCT

C

Fig. 1. Mapping of the transcriptional start site. (A) Primer extension analysis. The labeled 24 mer oligonucleotide primer was annealed to poly(A)$^+$ RNA from KATO III cells, and extended products run on a 7 M urea, 6% polyacrylamide gel. Lane 1: [$\gamma$-$^{32}$P]dCTP-labeled pGEM DNA markers; Lane 2: Labeled products of primer extension analysis [size: about 110 bp (star)]. (B) Anchored PCR. Lane 1: 100 bp DNA ladder marker; Lane 2: Negative RT-PCR controls without addition of reverse transcriptase; Lane 3: Negative RT-PCR controls without addition of RNA; Lane 4: Specific RT-PCR [size: about 110 bp (star)]. (C) Labeling of the 5′-flanking region of the human TFF2 gene according to transcriptional initiation site. Primer sequences for generation of serial deletion reporter constructs are underlined; the identified SPRE *cis*-acting region is presented in bold capital letters; the TATA-box is shown in bold underlined italic; the 5′ untranslated region is presented in bold (with the start site been designated +1.); and the first part of the translational coding region is shown in capital letters.

*Tissue culture and transfections*

*Materials*. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin–streptomycin, and EDTA–trypsin were obtained from Biowhittaker, Walkerville, MA. AGS-cells (American Type Culture Collection) were maintained in DMEM containing 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin, KATO III cells (American Type Culture Collection) were maintained in DMEM containig 20% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin, Phorbol ester PMA was obtained from Sigma (St. Louis, MO), and anti-TFF2 antibody is described below.

*Tissue culture and transfections*. For transient transfections AGS cells were plated in six-well plates (Costar) at a density of $1 \times 10^6$ cells/35-mm well and transfected the following day. Transient transfections of cultured cells were carried out using the calcium phosphate precipitation technique (DNA transfection kit, Eppendorf) as described previously [19] using 1 μg of reporter DNA per well. After transfection, cells were serum starved for 12 h in serum free medium (Ultraculture, Biowhittaker). Conditioned media containing TFF2, anti-TFF2 antibody, and/or PMA at maximal stimulatory concentration ($10^{-7}$ M) were added 12 h after transfection. Cells were harvested and luciferase assays performed at 36 h post-transfection. Luciferase assays were carried out using luciferin, ATP, coenzyme A (Promega system) with a monolight luminometer (Analytical Luminescence Laboratory). Incubations were performed in triplicate or quadruplicate, and results calculated as the mean ± SE. Values for luciferase activity were expressed as a fold-increase in luciferase activity compared over untreated controls. Activities in all transfection experiments represent the mean ± SE of at least three independent transfections. Activities varied <15% among transfection experiments.

Stably transfected AGS cells overexpressing human TFF2 were generated by transfection of an expression construct containing the human TFF2 cDNA cloned downstream of the CMV promoter in the *Eco*RI site of pcDNA 3.1 vector. The vector also contained the neomycin resistance gene. Stable clones were selected with G418 and expression verified by Northern and Western blot analyses.

*RNA isolations and Northern blot analysis*

Total RNA from AGS-TFF2 stable transfected cells and KATO III cells was prepared using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. mRNA was isolated from total RNA samples using the Oligotex mRNA isolation kit (Qiagen, Germany).

Twenty micrograms of total RNA was separated on 2.2 M formaldehyde, 1.0% agarose gels. Probes for TFF2 and GAPDH cDNA were a [α-$^{32}$P]dCTP labeled by random priming. Membranes were prehybridized for 2 h and hybridized overnight using ULTRAhyb buffer (Ambion, Austin, TX) at 65 °C. Following hybridization the membrane was washed twice for 20 min each in 2× SSC, 0.1% SDS at 25 °C, then once in 0.2× SSC, 0.1% SDS at 55 °C for 20 min. Membranes were exposed to X-ray film for 16–36 h at −80 °C.

*Western blotting*

For Western blotting a polyclonal anti-TFF2 antibody was raised against full length recombinant human TFF2/SP (gift of L.Thim [20]), generated, and obtained from Covance, Denver, PA. This antibody recognizes rat and mouse TFF2 (unpublished observations). Equal amounts of protein (whole cell lysates or immunoprecipitates) were separated on 10–20% Tris–tricine polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, MA) using transfer buffer consisting of 192 mM glycine and 25 mM Tris. Nonspecific binding was blocked with Tris-buffered saline plus Tween20 (TBST) (10 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween20) containing dried milk (5%) overnight at 4 °C. Membranes were then incubated with primary antibody in TBST dried milk (2%) overnight at 4 °C. The membrane was washed three times with TBST and then incubated with sheep anti-rabbit IgG–horseradish peroxidase conjugate (Amersham) (1:10,000 dilution) in TBST dried milk (2%) for 2 h at room temperature. After three washes with TBST, bands were visualized by enhanced chemiluminescence system (NEN Life Science, Boston, MA).

*EMSA–gel shift experiments*

Nuclear extracts from AGS cells were prepared by NP-40 detergent lysis and 0.5 M NaCl extraction as described previously [20]. Protein concentrations were determined by the Bio-Rad protein assay. EMSAs were performed by incubating the extracts with 4 fmol of double-stranded oligonucleotide probe (40,000 cpm) end-labeled with [α-$^{32}$P]dCTP (Amersham) by Klenow DNA polymerase (New England Biolabs, Beverley, MA) in a 20 μl binding reaction mixture containing 25 mM HEPES (pH 7.9), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1 μg of poly(dI-dC), and 10 μg of nuclear extracts. The wild type, double-stranded synthetic probe used in this study was: sense 5′-TCG ACT GAC TCT CTC CCT GCT CGG TGA TAC CTC TTC CTG G-3′, antisense 5′-CAG GAA GAG GTA TCA CCG AGC AGG GAG AGA GTC AGT CGA-3′. The sequences of the mutant probes (M1–M4) were the same as described above for the heterologous pT81 promoter constructs (see Cloning and construction of reporter constructs section). After incubation at room temperature for 10 min, samples were loaded onto a 6% nondenaturing polyacrylamide gel and electrophoresed in 0.5× Tris borate/EDTA at 10 V/cm. The gels were dried and exposed to Kodak X-AR film for 2–12 h at room temperature.

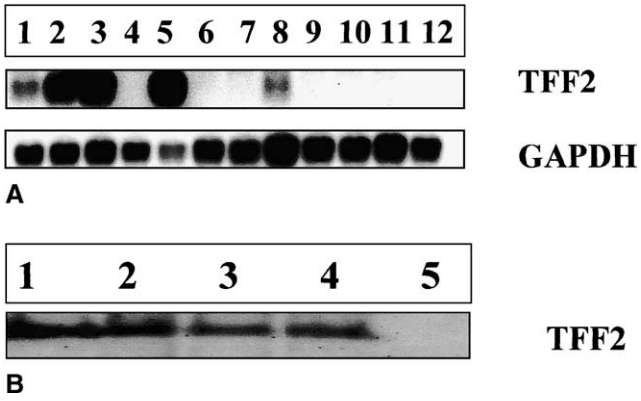


Fig. 2. Generation of stably transfected AGS cells overexpressing TFF2. (A) Northern blot for TFF2 mRNA. Total RNA was isolated from clones of stably transfected AGS cells and probed with a random primed human TFF2 cDNA probe. Each lane contains 10 μg of total RNA. Lanes 1–5: AGS cell clones expressing the TFF2 sense expression construct; lanes 6–10: AGS cell clones expressing the TFF2 antisense expression construct; lanes 11 and 12: untransfected AGS cells. The blot was also hybridized with a random primed GAPDH cDNA probe (shown on the bottom) to control for equal loading. (B) Western blot for TFF2 peptide. Conditioned media from the sense TFF2-expressing AGS cell lines (lanes 2, 3, and 5 above) and one antisense TFF2-AGS cell line (lane 6 above) were run on protein gels and probed with an anti-TFF2 antibody (see Materials and methods). Lane 1: Positive control containing 100 ng of recombinant TFF2 peptide; Lane 2, Conditioned media from the AGS/sense TFF2 cell line #2; Lane 3, Conditioned media from AGS/sense TFF2 cell line #3; Lane 4, Conditioned media from AGS/sense TFF2 cell line #5; Lane 5, Conditioned medium from AGS/antisense TFF2 cell line #6.

## Results

*Transcription of the human TFF2 gene starts 38 bp upstream of the translation inititation site (ATG)*

The transcriptional start site of the human TFF2 promoter was analyzed from poly(A)$^+$ RNA prepared from KATO III cells known to express human TFF2 [13,15]. Primer extension was performed using a 24 mer reverse oligonucleotide complementary to the human cDNA sequence of TFF2 starting from 77 bp downstream of the ATG. The primer extension analysis revealed a product of about 110 bp in size (Fig. 1A). Anchored PCR was then performed (see Materials and methods) resulting in a similar sized product shown in Fig. 1B. The PCR product was cloned into PCR 2.1 vector and sequence analysis was performed. Sequence analysis of four different clones showed identical 5′ sequences downstream of the introduced poly A-tail starting with 5′-ATT CTG CAA GCC ACC CTG-3′. Based on these results the transcriptional start site could be mapped to 26 bp downstream of the TATA-box and 38 bp upstream of the translational start site of human TFF2. As a result of these findings, the numbering and labeling of the human TFF2 promoter luciferase reporter constructs were revised (Fig. 1C).

*Production of TFF2 containing conditioned media from AGS cells overexpressing TFF2 after stable transfection*

In order to identify the *cis*-acting elements mediating activation of the TFF2-promoter in response to TFF2

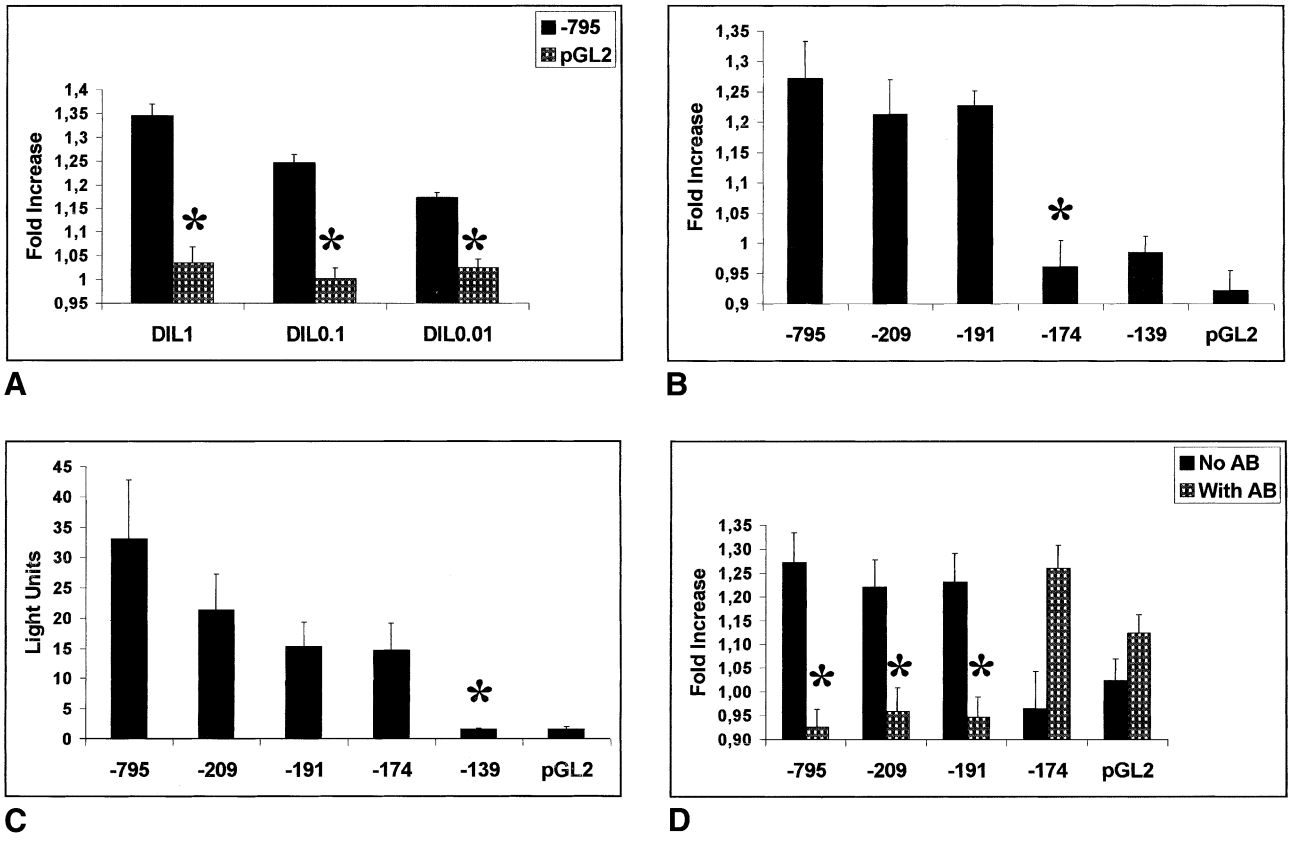


Fig. 3. Regulation of the human TFF2 promoter by TFF2 peptide. AGS cells were transiently transfected with reporter (luciferase) gene constructs containing the full length human TFF2 promoter (−795 bp) or a deleted promoter as indicated. Cells were harvested 36 h after transfection and luciferase activity measured. Results shown represent the mean ± SE calculated from at least three independent transfections. (A) Dose–response to TFF2. Pure conditioned media (DIL-1), a 1:10 dilution (DIL0.1), and a 1:100 dilution (DIL0.01) were used to stimulate AGS cells transfected with the −795 bp hTFF2-luciferase construct (solid black bars) or the empty vector pGL2 Basic (hatched bars). Promoter activity is shown as -fold response over controls stimulated with control media. (B) Localization of the SP/TFF2 response element. AGS cells were transiently transfected with various deletions of the human TFF2 promoter, and then stimulated with TFF2-containing conditioned media. The -fold increase over control media is shown. The responsive region appears to lie between −191 and −174 ($^*p < 0.01$). (C) Basal activity of human TFF2 promoter deletion constructs. The human TFF2 promoter deletion constructs were transfected into AGS cells, and absolute promoter activity assessed. Promoter activity is shown as light units relative to the activity of a RSV-promoter driven luciferase reporter-construct, RSV-Luc. Significant ($^*p < 0.01$) basal enhancing activity appears to be localized between −174 and −139. (D) Immunoneutralization of TFF2 blocks promoter activation. AGS cells were transfected with human TFF2 promoter-luciferase constructs or empty vector (pGL2 basic) and then stimulated with conditioned media, with or without the addition of an immunoneutralizing anti-TFF2 antibody. Addition of the anti-TFF2 antibody results in significant inhibition of stimulation for the −795, −209, and −191 bp promoter constructs ($^*p < 0.01$).

peptide stimulation we decided to establish a system resembling physiologic paracrine conditions in the stomach. We therefore cloned the TFF2 cDNA in a CMV-promoter driven expression construct (pcDNA 3.1) in both sense and antisense orientations and established several stable AGS cell lines overexpressing either sense or antisense cDNA. Northern blot experiments confirmed the expression of TFF2 mRNA (Fig. 2A) in the individual stable cell lines, and identified three cell lines highly overexpressing TFF2 mRNA (lanes 2, 3, and 5, Fig. 2A). Western blot analysis revealed that the conditioned medium from these cell lines (lanes 2, 3, and 4, Fig. 2B) indeed contained high levels of TFF2 peptide, thus demonstrating production and secretion of TFF2 (Fig. 2B). For the majority of experiments, TFF2 promoter stimulation was reproduced using conditioned media from stable AGS cell lines 1, 3, and 4 corresponding to lanes 2, 3, and 5 in Fig. 2A.

*Activation of the TFF2 promoter by conditioned medium containing TFF2 occurs through a specific cis-acting region*

In order to examine the effect of endogenously produced TFF2 peptide on TFF2 promoter activity, we added conditioned media from TFF2 overexpressing AGS cells to AGS cells transiently transfected with TFF2-luciferase reporter constructs. We initially conducted experiments to assess the dose responsiveness of this effect. Conditioned medium was collected and a volume of 500 ml was lyophilized after dialysis. The total protein content of the resuspended conditioned media was measured and three different dilutions were used for stimulation. We observed a dose response on promoter activity with increasing concentrations of conditioned media from 1:100 (DIL 0.01) up to 1:1 (DIL 1) (Fig. 3A). We were then able to localize a *cis*-acting element mediating this response to a region of the promoter ranging from −191 to −174 bp (Fig. 3B). When examining the basal activity of the different reporter constructs, there was no difference between the −191 and the −174 bp containing reporter constructs suggesting that there is no basal enhancing element located in this region. Moreover, we observed about a 10-fold decrease in basal activity between −174 and −139 suggesting that there might be an additional basal enhancing element located in this region (Fig. 3C). Finally in order to address the specificity of the response, we examined the effect of an immunoneutralizing anti-TFF2 antibody to the conditioned medium containing TFF2. The addition of anti-TFF2 antibody to the conditioned medium completely abrogated the promoter stimulation (Fig. 3D). Taken together, these data demonstrate the activation of the TFF2 promoter through TFF2 peptide is mediated by a specific *cis*-acting region (−191 to −174). Analysis of the identified region for known transcription

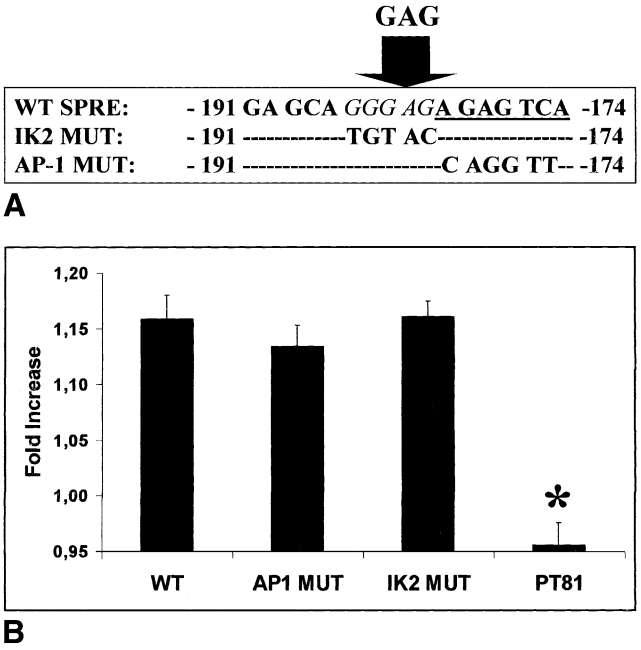


Fig. 4. Analysis of the putative IK2 and AP1 sites. (A) Schematic representation of the SP response element (SPRE), the sequence of the IK2 (italic), and AP1 (underlined) mutations, and the location of the GAG-motif (arrow). (B) Functional analysis of wild type and mutant (SPRE) *cis*-acting elements. The oligonucleotide sequences shown above were cloned upstream of the heterologous thymidine kinase promoter in the construct pT81, and responses to TFF2 assessed after transfection into AGS cells. Promoter activity is shown as -fold increase over stimulation with control media. Results shown represent the mean ± SE calculated from at least three independent transfections.

factor binding sites using commercially available software programs (DNA-Star, Lasergene, Madison, WI, Transfac Software [33]) revealed three homologies to previously described elements, which included AP1 and IK2 sites, as well as a GAG motif (Fig. 4A).

*Analysis of the putative AP1 and IK2 sites in the specific cis-acting region mediating response to TFF2*

In order to further investigate the −191 to −174 element, we cloned this element upstream of the TK-promoter into the heterologous promoter construct pT81. We created wild type (WT), AP1 mutated (AP1MUT) and IK2 mutated (IK2MUT) constructs and compared the activation of these constructs by stimulating with TFF2-containing conditioned medium. We mutated the putative AP1 site (−181 AGAGTCA-173) to −180 CAGGTTA-173 and the putative IK2 site (−186 GGGAG-180) to −186 TGTAC-180 (Fig. 4A). These constructs were transfected individually into AGS cells and the response to TFF2 stimulation was examined. These studies revealed that the wild type sequence as well as the AP1- and IK2-mutated sequences confer TFF2 response to the heterologous promoter construct (Fig. 4B).

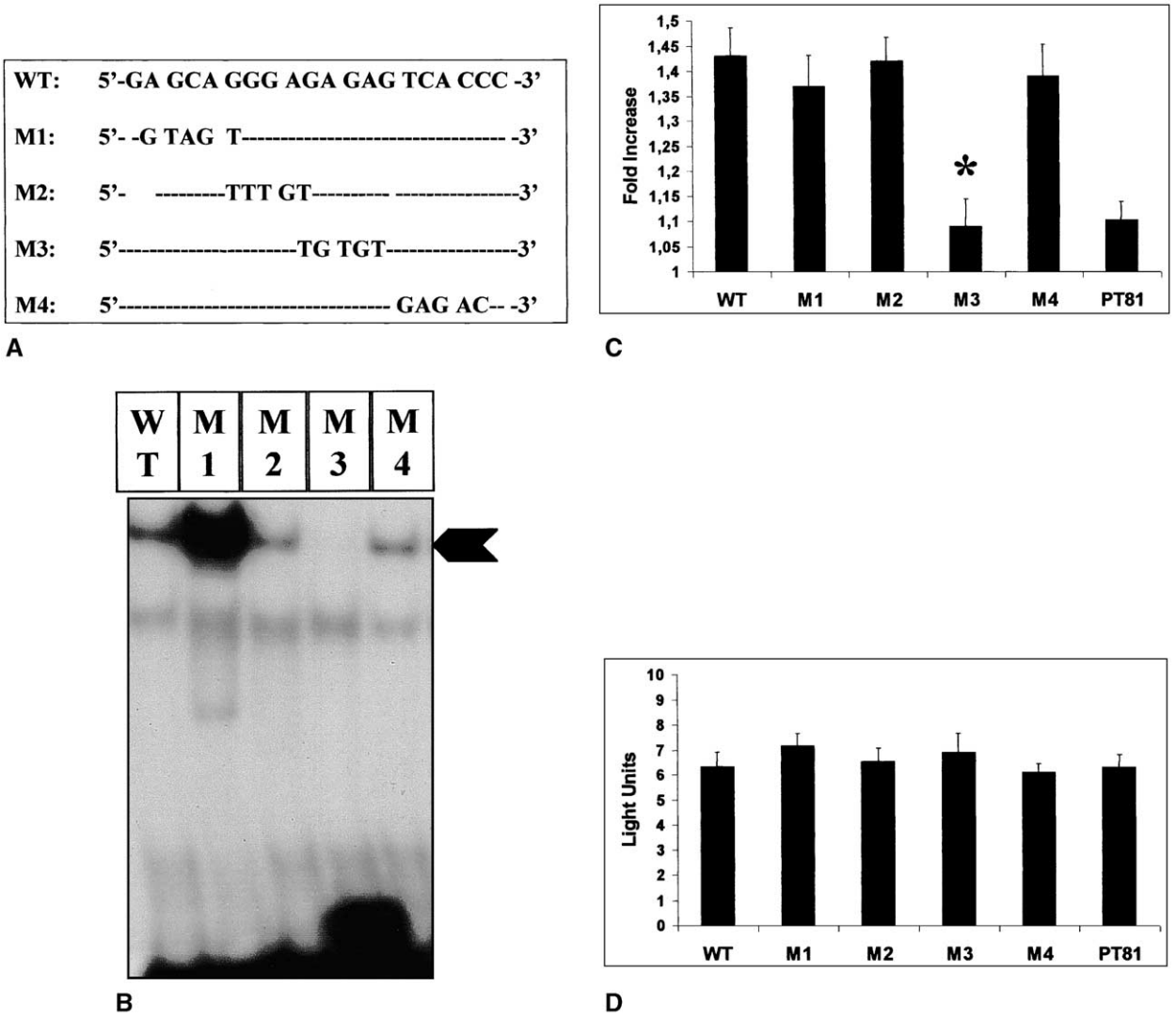


Fig. 5. Analysis of the spasmolytic polypeptide response element (SPRE) through scanning mutagenesis. (A) Schematic representation of the wild type SPRE and the scanning mutations (M1–M4) used in EMSA experiments and reporter gene (luciferase) assays. (B) EMSA analysis. Wild type or mutated [$\alpha$-$^{32}$P]dCTP-labeled probes (shown in Fig. 5A) were incubated with AGS cell nuclear extracts and analyzed by gel mobility shift assays. Arrow shows the specific delayed complex which is absent with the mutant M3 probe. (C) TFF2 responses by mutant reporter gene constructs (M1–M4). Heterologous (pT81) promoter reporter gene constructs containing wild type or mutant SPRE sequences (shown in Fig. 5A) as well as the parental vector pT81 were transfected into AGS cells and stimulated with TFF2-containing conditioned media. Promoter activity is shown as -fold increase over stimulation with control media. The M3 mutant showed significantly less response to TFF2 compared to the other constructs ($^{*}p < 0.01$). (D) Basal activity of the mutant reporter gene constructs (M1–M4) compared to wild type SPRE and the parental vector pT81. The light units (X1000) relative to the activity of a RSV-promoter driven luciferase reporter-construct are shown, and represent the mean $\pm$ SE calculated from at least three independent transfections.

*A GAG motif is sufficient to mediate the TFF2 induced TFF2 promoter activation*

Since the TFF2 promoter responsiveness was not abrogated by mutations in the putative AP1 and IK2 sites we generated an additional series of scanning mutations in the −191 to −174 element (Fig. 5A) which were then analyzed by EMSA (Fig. 5B) and reporter gene assays (Fig. 5C). Mutation of the region −182 GAGAGT −175 to −182 TGTGTC −175 (M3) did result in loss of DNA binding of the AGS-cell nuclear extract to the oligonucleotide as shown in Fig. 5B. Furthermore analysis of the corresponding TFF2-TK-promoter mutants with mutations in this core GAG element confirmed a loss of responsiveness to TFF2 peptide stimulation (Fig. 5C). The basal activity for all the TFF2 pT81 constructs (both wild type and mutants) was not increased over the parent pT81 vector control (Fig. 5D), again indicating that the identified element is not a significant basal enhancer.

## Discussion

In this study we report the identification of a novel TFF2/SP response element which we have designated SPRE (for SP response element). This element is contained within the 823 bp 5′-flanking region that we found in previous studies to be responsive to autostimulation by TFF2. We show here that the TFF2 responsive region can be further localized to an 18 bp element (−191 to −174), and through additional mutation analysis demonstrate that the key nucleotides constitute a GAG-motif. This element was capable of confering responsiveness to TFF2 on a heterologous promoter, and also of binding in a specific fashion nuclear factors present in AGS cell extracts. The element also appears to mediate response to phorbol ester (data not shown), consistent with our earlier study showing that TFF2 and PMA activate the human TFF2 promoter through a MAP kinase signaling pathway.

TFF2 is expressed specifically in the mucous neck cells of the stomach, but it is highly upregulated in regenerating tissues and has been identified as an important promoter of cell migration in the initial phase of mucosal healing [21,22]. TFF2 immunoreactivity is significantly increased within hours after injury of the gastric mucosa by indomethacin [23] and TFF2 is protective against mucosal injury in the upper and lower gastrointestinal tracts in animal models [24,25]. Given that cytoprotective effects have been observed after both oral and parenteral administration of TFF2, there has been speculation about the existence of specific trefoil receptors [23]. Although it remains unclear whether TFF2 acts through a specific receptor, candidate molecules for a putative trefoil receptor have been recently described [26]. Taking these results into consideration, we used a model system where AGS cells overexpress TFF2 in order to mimic physiologic autocrine and paracrine conditions in the stomach and enable TFF2 mediated promoter upregulation after mucosal injury.

The initial 18 bp sequence of the identified *cis*-acting element was found by computer-based homology search [27] to contain two potential transcription factor binding sites. An IK-2 element could be identified (−186 GGGA −181) as well as an AP1-like sequence (−180 GAGTCA −173). AP-1 (Fos-/jun heterodimer) is a well-known transcription factor complex (consensus binding sequence 5′-TGACTCA-3′) [28], and mediates response to phorbol ester and related signaling pathways. Target genes are involved in the proliferative response during healing of gastric mucosa after stress induced ulceration [29]. Furthermore the AP-1 complex appears to be involved in cell proliferation and survival [28]. IK2 (consensus binding site 5′-GGGA-3′) and the other members of the Ikaros family of transcription factors have been detected in analysis for regulators of T-cell development, and have been shown to be important in the complex changes of gene expression regulation during lymphocyte differentiation and activation [30]. However these two binding sites were not involved in mediating TFF2-dependent promoter activation. Instead, mutation analysis revealed that a GAG element appears to be critical for promoter activation and nuclear factor binding. While the identity of the relevant nuclear factor is unclear, there have been previous reports of GAG-binding transcription factors in *Drosophila*, and several genes have been identified as targets for this transcription factor called GAF [31,32]. There have also been reports on GAGA-motifs and GAF-like DNA binding proteins in regulating gene expression regulation in mammalian cells [33–35]. Interestingly, binding sites for the GAF transcription factor are usually found close to or even overlapping other key regulatory elements [36]. This could explain the surrounding IK2 and AP1 sites in the SPRE and is an important question for further analysis.

Overall the GAG element appears to mediate transcriptional response to stimulation by TFF2, and possibly other trefoil factors. However the GAG element does not appear to be a basal enhancer, since it did not confer higher basal activity to a heterologous promoter reporter gene system, and deletion of the element from the endogenous promoter did not result in loss of basal activity. Thus these observations underscore the importance of the identified *cis*-acting element as a critical regulatory region mediating responsiveness to TFF2 peptide and possibly other growth factors.

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