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Aspirin promotes TFF2 gene activation in human gastric cancer cell lines

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Abstract Trefoil factor family (TFF) peptides promote cell migration, heal the mucosa and may suppress tumor growth. In reporter gene assays we show that aspirin (1–12 mM) evokes a six-fold up-regulation of *TFF2*, but not *TFF1* and *TFF3* transcription in human gastrointestinal cell lines. 6 h after application up-regulation of endogenous *TFF2* mRNA was observed. *TFF2* transcription was enhanced by indomethacin and arachidonic acid but repressed by staurosporine, suggesting mediation via protein kinase C. We mapped an aspirin responding element −546 to −758 bp upstream of *TFF2*. Up-regulation of *TFF2* by aspirin may partially explain the chemopreventive potential of low dose aspirin in gastrointestinal carcinogenesis.

Key words: Trefoil peptide; Spasmolytic polypeptide; Aspirin; Signal transduction; Transcriptional regulation; Carcinogenesis

1. Introduction

Peptides of the trefoil factor family (TFF) are characterized by a conserved motif known as the trefoil domain. This domain consists of some 40 amino acid residues in which six cysteines are disulfide-linked forming a clover leaf structure [1]. The conserved TFF domain is considered to interact with glycosylated (mucin) molecules at mucous surfaces in vertebrates. This idea is confirmed by recent results showing that TFF-like domains are ancient motifs already existing in invertebrates and that they are integrated in a variety of composed proteins expressed at mucous surfaces [2]. The three human *TFF* genes are clustered on chromosome 21q22.3 [3]. The highest expression can be found in specific epithelial cells of the gastrointestinal tract. In vivo TFF1 is mainly transcribed in the foveolar gastric cells [4], whereas TFF2 is found in the deeper glands of the stomach and duodenum [5]. Expression of TFF3 is fairly restricted to the goblet cells of the small and large intestine [6].

Although the molecular mechanism of TFF function is still unknown, results from several in vitro and in vivo studies revealed the ability of TFFs to protect epithelia against experimentally induced mucosal damage [7–9]. Endogenous expression of TFF as well as recombinant TFFs stimulate cellular motility, promote mucosal defense and wound healing and inhibit tumor cell proliferation [10–13]. *Tff1* knock-out mice show aberrant gastric mucosa and develop gastric carcinoma whereas $Tff3^{-/-}$ mice show impaired intestinal defence [7,8].

Supporting their cytoprotective function, TFFs are up-regulated in a variety of ulcerative, preneoplastic and neoplastic conditions, or when the integrity of the mucosa is threatened [3,11,14]. The regulation of these genes may be integrated into the acute phase response, inflammation and wound repair/restitution. In fact, we have recently demonstrated the activation of *TFF1* transcription by the transcription factor HNF-3, which also activates other endodermal derived and acute phase gene expression [15]. Moreover, the 5′-flanking region of *TFF1* contains several control elements responsive to estrogens, phorbol esters, c-Ha-ras, c-jun and other growth factors [16].

On the one hand, long lasting administration of acetyl salicylic acid (aspirin) is associated with gastric and intestinal cancer chemoprevention [17,18]. On the other hand, this widely used anti-inflammatory drug is known to cause gastric ulceration and damage. This drug inhibits the cyclooxygenase (COX), the key enzyme of the proinflammatory prostaglandin biosynthesis. Recently, activation of genes for TFF2 and COX-2 has been found during gastric adaptation to aspirin damage in rats [19]. This prompted us to investigate whether in human gastrointestinal cell lines in *TFF* gene regulation is affected by stimulation with aspirin. In this way we disclosed a considerable modulation of *TFF2* gene expression. Activation of *TFF2* transcription may be due to inhibition of the arachidonic acid metabolism. We suggest that aspirin may partially exert its tumor preventive effect by enhancement of the healing peptide TFF2 in vivo.

2. Materials and methods

2.1. Human cell lines and reporter plasmids

The poorly differentiated gastric adenocarcinoma cell lines MKN45 and KatoIII as well as the colon adenocarcinoma cell line LS174T were cultivated in RPMI1640 and DMEM supplemented with 20% FCS or 10% FCS, respectively [20]. Luciferase reporter plasmid pGL3 (Promega) was used to construct TFF recombinants using the 5′-flanking region of human TFF1 (position −1100 to +38), TFF2 (position −821 to +61), and TFF3 (position −867 to +63) [20,21]. TFF2 wild-type promoter (position −821 bp to +61 bp) inserted into plasmid pGL3 was used for deletion mutagenesis by using the GeneEditor™ in vitro site-directed mutagenesis system (Promega) as described [20,22]. All position numbers of the clones are displayed with respect to the TATA box. All constructs were verified by DNA sequencing of both strands.

2.2. Transient transfection assays

Cells grown to confluence were trypsinized and seeded to nearly 30% of confluence on 96-well plates the day before transfection. Transient transfection was performed by means of a liposome reagent

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Effectene (Qiagen, Hilden, Germany). 6 h after transfection, medium was exchanged and a stock solution of acetyl salicylic acid, indomethacin, arachidonic acid and staurosporine (pH 7.5, purchased from Sigma) was added to achieve the desired concentration. 40 h after stimulation cells were lysed and luciferase assays were performed in triplicates of quadruplicates. Renilla luciferase (pRL-CMV, Promega, WI, USA) and dual luciferase measurements (Promega, #E1910) were used to standardize transfection efficiency and calculate specific transcriptional activity [20]. Results were expressed as ratio of firefly luciferase to renilla luciferase and compared to the non-stimulated control (relative transcriptional activity). The standard deviation was less than 15%. Each experiment was independently performed three times.

2.3. Multiplex RT-PCR and Northern blotting

Total cellular RNA was prepared and reverse transcribed using oligo-dT primers. PCR primers were chosen from different exons to exclude contamination with genomic DNA [23]. To ensure that PCR was in the exponential phase, 22, 25 and 30 cycles of multiplex PCR with TFF1+GAPDH+TFF2 or TFF1+GAPDH+TFF3 primer pools were performed as described [22]. 15 μg of cellular RNA were subjected to Northern blotting. Blots were consecutively probed with TFF2, GAPDH and TFF1 cDNA generated by PCR and labeled by random priming using [^{32}P-α]dCTP. Three independent experiments revealed similar induction of TFF2 mRNA.

3. Results

3.1. Effect of aspirin on TFF reporter gene expression

To demonstrate the effect of aspirin on the gene expression of TFF peptides reporter gene analysis was performed in several gastrointestinal cell lines. *TFF2* transcription was upregulated in two gastric tumor cell lines, MKN45 and KatoIII 38 h after drug application (Fig. 1). At concentrations of 2.5 to 7 mM, a 6-fold transcriptional stimulation was observed in MKN45 (Fig. 1A). A similar dose response was also noticed in KatoIII (Fig. 1B). Higher concentrations of aspirin (15 and 25 mM) resulted in a growth reduction of both cell lines without any stimulation of *TFF2* expression. We observed a reduction of transcriptional activity after 2 days at concentrations above 15 mM ASA and a loss of cell adhesion after 3 days incubation above 25 mM ASA. Lower concentrations of aspirin (0.1 mM) showed no influence on the *TFF* transcriptional activity at all. To investigate whether stimulation by aspirin is a feature characteristic for all *TFFs*, reporter gene analysis was carried out using *TFF1*, *TFF2* and *TFF3* constructs in combination with the gastric

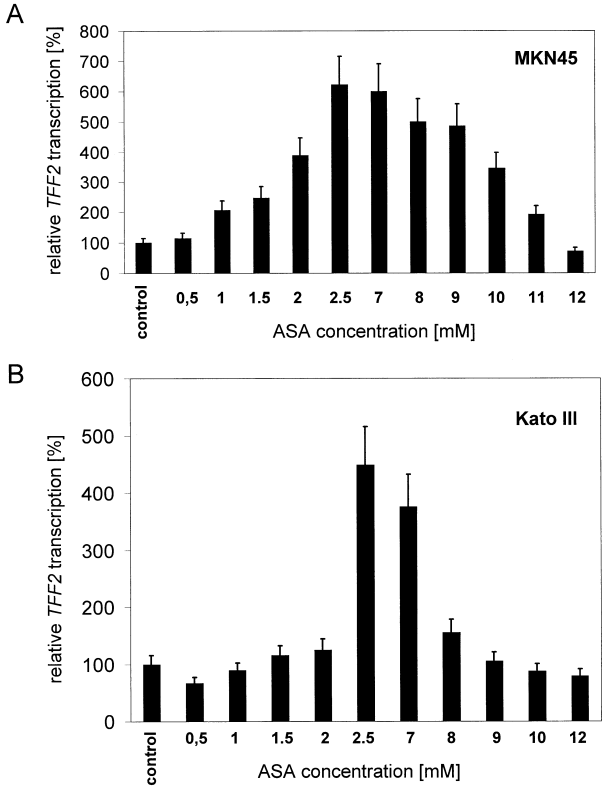

Fig. 1. Effect of acetylsalicylic acid (ASA) on the expression of TFF2 reporter gene constructs. Luciferase expression was determined 38 h after ASA stimulation in gastric cell lines MKN45 (A) and KatoIII (B) expressed in % of the non-stimulated control.

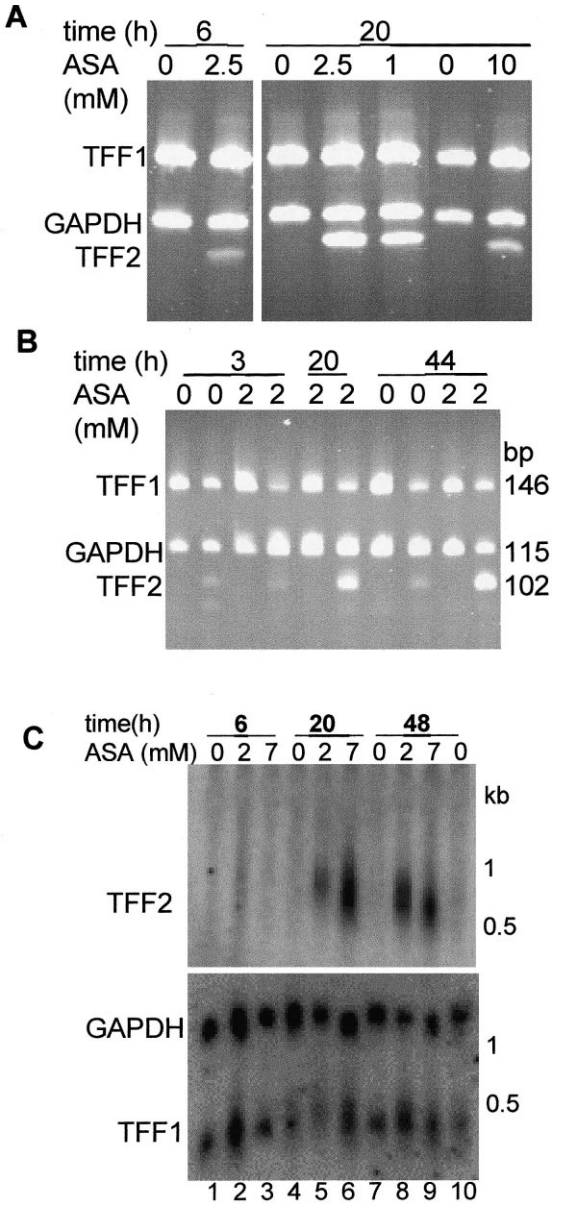

Fig. 2. Effect of ASA on the endogenous TFF mRNA expression in MKN45. A: Multiplex RT-PCR assays using TFF1, TFF2 and GAPDH oligos, following stimulation with 1, 2.5, and 10 mM ASA or without ASA. B: Stimulation with 2 mM ASA for 3, 20 and 44 h. Even lanes display TFF1, TFF2 and GAPDH multiplex assays, odd lanes display TFF1, TFF3 and GAPDH assays. C: Northern blot from cells incubated with 2 and 7 mM ASA or without ASA (0), for 6, 20 and 48 h, respectively.

cell lines MKN45, KatoIII and the colon cell line LS174T (Table 1). No considerable activation of TFF1 and TFF3 reporter gene expression was observed in all of the three cell lines.

3.2. Aspirin stimulates endogenous TFF2 gene expression

As shown in previous studies, TFF reporter gene expression resembles endogenous TFF gene expression in a variety of cell lines [20]. To analyze whether aspirin also causes up-regulation of endogenous *TFF2* transcription, cells were cultivated and stimulated under identical conditions as for reporter gene analysis. Specific mRNA levels of TFF1, TFF2, TFF3 and GAPDH were determined after semi-quantitative multiplex RT-PCR (Fig. 2A). In MKN45, application of aspirin preceded an increase of TFF2 mRNA, whereas neither TFF1 nor TFF3 mRNA were influenced (Fig. 2B). Up-regulation of TFF2 mRNA was first detected 6 h after stimulation by aspirin and reached its highest point after 20 h. Similar transcriptional up-regulation of *TFF2* was observed in multiplex RT-PCR using β-actin as an internal standard (data not shown). Northern blots revealed considerable induction of TFF2 mRNA in cells 20 h and 48 h after stimulation by aspirin confirming the results generated by RT-PCR (Fig. 2C, lanes 5, 6 and 8, 9).

3.3. Disturbance of the arachidonic acid metabolism stimulates TFF2 expression

Indomethacin, a drug known to inhibit COX-1 was examined under similar conditions as aspirin to analyze the effect on *TFF2* activation. In fact, doses known to inhibit COX-1 also promote a 2-fold up-regulation of *TFF2* transcription (Fig. 3A). To investigate whether an increased internal concentration of arachidonic acid, the physiological substrate of COX-1, is responsible for *TFF2* activation, arachidonic acid was added to the cultivation medium. A 6-fold up-regulation of *TFF2* transcription was observed (Fig. 3B). Co-stimulation of the MKN45 cells with arachidonic acid and ASA resulted in a further increase of *TFF2* transcription, but no additive effect was achieved. This indicates that arachidonic acid and aspirin may influence the same signaling transduction pathway. Since arachidonic acid is known to activate protein kinase C, we furthermore treated MKN45 cells with staurosporine, a protein kinase C inhibitor (Fig. 3C). This experiment led to a 10-fold reduction of TFF2 reporter gene expression. Simultaneous addition of ASA and staurosporine did not reverse the inhibitory effect of staurosporine, but attenuated the effect, also indicating that a PKC pathway is involved in up-regulation of *TFF2* (see Fig. 5).

3.4. Localization of a TFF2 enhancing 5′-flanking region

In order to map a single cis-acting sequence motif responsible for the aspirin effect, various regions of the 5′-flanking sequences of *TFF2* were deleted. Thus, the reporter gene constructs were exposed to various concentrations of aspirin in MKN45 cells (Fig. 4). In comparison to the TFF2 wild-type reporter construct, deletions S546B, DM2 and DMyc resulted in a 5–9-fold reduction of reporter gene expression, whereas deletions DG6 and D3 still respond to aspirin. This indicates an aspirin responding element located 546 to 758 bp upstream to the TATA box.

Table 1
Effect of aspirin on TFF reporter gene expression

Cell line	TFF1	TFF2	TFF3
MKN45	100	620	110
KatoIII	120	450	130
LS174T	160	270	180

Displayed in % of the control (not stimulated). S.E.M. was ≤15% in triplicate experiments.

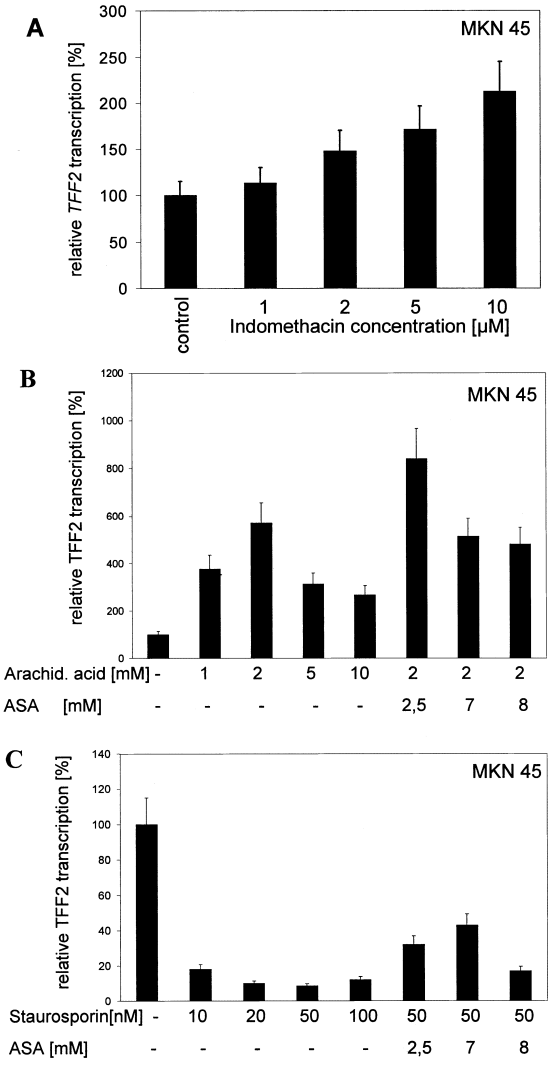

Fig. 3. Effect of indomethacin (A), arachidonic acid (B) and staurosporine (C) on the expression of *TFF2* reporter genes in MKN45.

4. Discussion

In our study we have shown that transcription of *TFF2* is up-regulated by administration of acetylsalicylic acid (ASA) especially at concentrations between 2.5 and 7 mM in gastrointestinal cell lines. This was monitored by reporter gene expression and endogenous gene expression using RT-PCR (Figs. 1 and 2). TFFs are gastrointestinal healing peptides that trigger cell migration and restitution in vitro as well as protecting and healing of experimentally induced ulceration in rodents [9]. In addition, TFF1 may act as tumor suppressor, since mice lacking *TFF1* develop adenocarcinoma, and expression of *TFF1* and *TFF3* is down-regulated in gastrointestinal

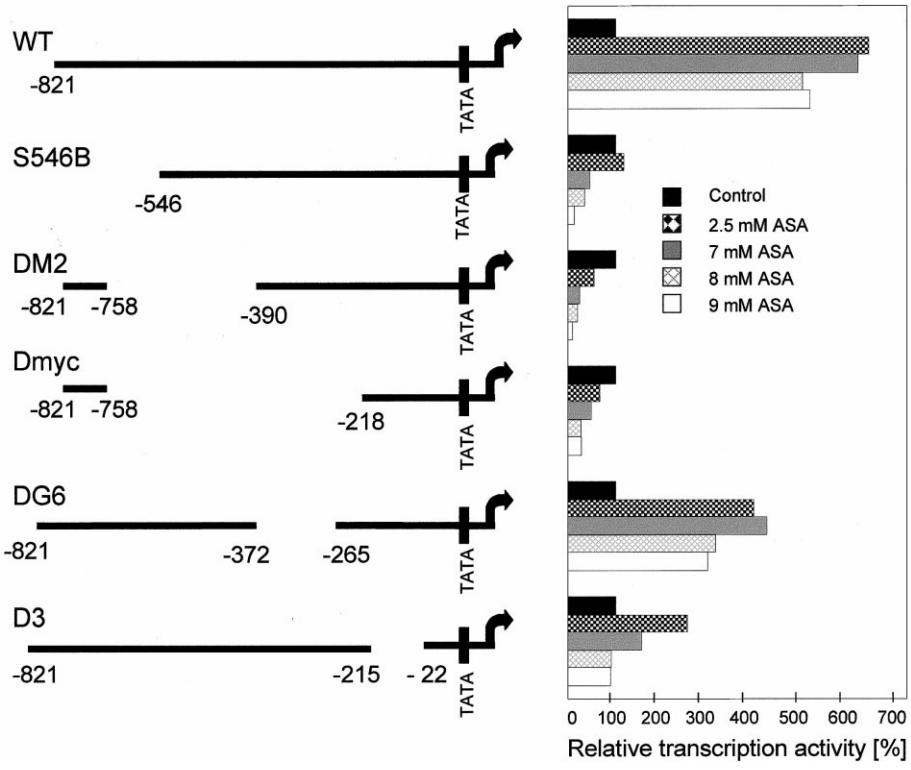

Fig. 4. Localization of the 5′-flanking region of *TFF2* responsible for aspirin activation. Various reporter gene constructs are tested for their ability to respond to different ASA concentrations. For each construct the effect of aspirin is displayed in % of transcriptional activity relative to the control.

cancers [7]. Although *TFF3*$^{-/-}$ mice do not develop colon cancer, overexpression of TFF3 has been shown to inhibit colon tumor cell proliferation in vivo and in vitro [13]. TFF2 may also suppress tumor growth, since recombinant TFF2 substitutes the cytoprotective function of TFF1 and TFF3 [9,10,12,28].

The widely used drug aspirin is known to have harmful side effects leading to gastric lesion and ulceration. However, epidemiological data, animal models and clinical studies all suggest that aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) are potent preventive agents for colon cancer [18] and there is also recent evidence that regular aspirin users are at reduced risk of gastric and esophageal cancer [24,25].

One putative biochemical target of the cancer chemopreventive activity of aspirin is cyclooxygenase (COX) inhibition. ASA is reported to inhibit both cyclooxygenases with a preference for COX-1 (IC_{80} = 8 μM) over COX-2 (IC_{80} = 30 μM) [26]. However, reports of chemopreventive activity of NSAID derivatives lacking COX inhibition suggest that there are other biochemical targets as well [27].

There is recent evidence that *TFF2* activation by aspirin also occurs in vivo. In the stomach of rats, the application of ASA leads to activation of *TFF2* after 24 h [19]. The activation in vivo as well as the up-regulation of *TFF2* in vitro may be explained by the same molecular mechanisms. Thus our in vitro system may provide an interesting model to explain some observations in vivo. The concentration of ASA (2.5 mM) that activates *TFF2* in vitro, is also reached in the human gastric lumen after intake of 100 mg of aspirin. This suggests that *TFF2* activation also occurs in many individuals who use this drug e.g. for prevention of cardiovascular disease. Whether *TFF2* activation is the relevant molecular keyplayer for the observed gastric chemopreventive effect of aspirin remains to be proven. In rodents and in vitro, however, gastrointestinal cytoprotection through administration of recombinant TFF2 peptide is well established [9,10,12,28].

In KatoIII and MKN45, we observed a *TFF2* up-regulation between 2 and 8 mM and growth reduction and loss of adherence to plastic surface between 15 and 25 mM. In vivo, the chemopreventive and the detrimental (ulcerative) effect of aspirin may also be within a small dose response range.

Various experiments were performed to elucidate the molecular mechanism of TFF2 activation by aspirin. Although several signaling cascades may be involved we favor the model displayed in Fig. 5: Aspirin inhibits COX-1 raising up the intracellular level of arachidonic acid. This would activate protein kinase C eventually modifying (directly or indirectly)

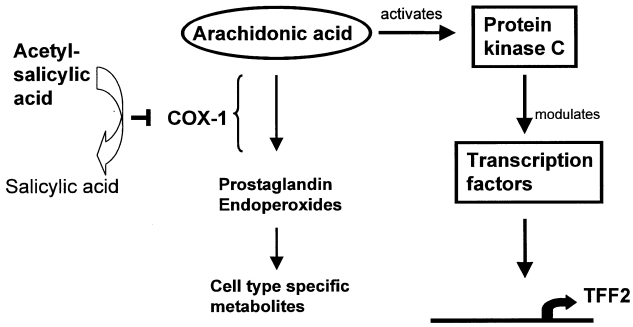

Fig. 5. Proposed signal transduction pathway leading to transcriptional up-regulation of *TFF2*.

transcription factors acting on elements of the 5′-flanking region of TFF2.

Our model that COX-1 inhibition is the primary target of the aspirin response is based on the observation that COX-1 but not COX-2 transcription occurs in our MKN45 cell lines (performed by RT-PCR, unpublished results). Moreover indomethacin, another COX-1 inhibitor, causes up-regulation of *TFF2* (Fig. 3A) at doses known to specifically affect COX-1 [29]. Addition of arachidonic acid – the physiological substrate of COX-1 – also leads to a 6-fold enhancement of *TFF2* transcription. Furthermore, we found no additive effect after co-stimulation of MKN45 cells with arachidonic acid and ASA. This indicates that arachidonic acid and aspirin influence the same signal transduction pathway. Arachidonic acid is known to activate protein kinase C (PKC) [30]. Therefore, inhibition of PKC by staurosporine should lead to a marked reduction in *TFF2* transcription. Indeed, staurosporine treatment resulted in a 10-fold reduction of *TFF2* transcription as displayed in Fig. 3C. Moreover, addition of ASA did not reverse the inhibitory effect of staurosporine, also indicating that a PKC pathway is involved in up-regulation of *TFF2*.

Finally, using deletion analysis on *TFF2* reporter constructs, we mapped enhancing elements on the 5′-flanking region of *TFF2* responsible for the up-regulation by aspirin. Several deletion constructs were impaired in their ability to respond to aspirin. The smallest overlapping region causing a severe transcriptional reduction is located between −758 and −546 bp upstream to the TATA box (Fig. 4). This region does not cover binding sites for transcription factors HNF-3 and GATA-6 that we showed to activate *TFF2* transcription [15,22] and therefore likely represents a new enhancing element.

Taken together, our results suggest that in gastric cell lines aspirin is able to activate transcription of *TFF2* coding for a gastrointestinal healing peptide. We speculate that in vivo, transcriptional activation of *TFF2* as well as other genes may provide an explanation for the chemopreventive activity of this widely used drug.

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