Gene Regulation of the Human Trefoil Factor Family (TFF) in Gastrointestinal Cell Lines

Dissertation
zur Erlangung des Grades eines Doktors
der Naturwissenschaften

der Fakultät für Biologie
der Eberhard-Karls-Universität Tübingen

vorgelegt von

Ezzal-din Al-azzeh
aus Kuwait

2001

Tag der mündlichen Prüfung:	11. Januar 2001
Dekan:	Prof. Dr. Schnitzler
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ACKNOWLEDGMENTS

I would like to thank PD Dr. Peter Gött, Division of Molecular Genetics, University of Tübingen, for long discussions and important advice during this study.

I am very grateful to Prof. Dr. Nikolaus Blin, Division of Molecular Genetics, University of Tübingen, for his constructive scientific advice. His attention and encouragement have contributed decisively to the success of this study.

I acknowledge Prof. Dr. J. Kömpf and B. Lüscher for discussions and valuable counsels over the extent of the study.

I express my gratitude to Dr. Dieter Kube for his sustainment and motivation during my work.

I express my great gratitude to Mrs. Petra Fegert for her kind support as a friend and for her help throughout this study.

I offer my warmest thanks to Tessa Haug, Pia Azarschab, Veronika Dossinger, Ulrike Zeissler and Reinhard Drews for helpful advice and friendly atmosphere during my studies.

I am especially thankful to Dr. Kristina Schröder, Susan Kupka, Peter Leistenschneider, Simone Braun, Birgit Haack, Susanne Aberle and Souzan Salemi, for their support at any time and the pleasant atmosphere in the laboratory.

I express my gratitude to Tuncay Kayademir, Timea Toth, Fahrad Mirghomizadeh and Mirela Baus-Loncar for their prompt help and invaluable advice as friends.

I am very grateful to Wolfgang Kornberger for technical assitance and Reinhard Reuß for his help during my study.

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Abbreviations

bp	Basepair(s)
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
Da	Dalton(s)
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
DTT	Dithiothreitol, threo-1,4-Dimercapto-2,3-butandiol
EDTA	Ethylendiamin-N,N,N´,N´-tetra acid
EGF	Epidermal growth Factor
EGFR	Epidermal Growth Factor Receptor
EtOH	Ethanol
F	Forward
FCS	Fetal Calf Serum
GI-tract	Gastrointestinal Tract
h	hour
H_2Odd	Double Distilled Water
hITF	Human Intestinal Trefoil Factor
hSP	Human spasmolytic Polypeptide
IM	Intestinal Metaplasia
kb	Kilobase
kbp	Kilobasepair(s)
kDa	Kilodalton(s)
LOH	Loss of Heterozygosity
M	Molar
Mb	Megabase
min	Minutes
O.D.	Optic Density
PAA	Polyacrylamide Gel
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
rev	Reverse
RNA	Ribonucleic acid

RNAse	Ribonuclease
rpm	rotations per minute
RT	Room temperature
RT-PCR	Reverse Transcription-polymerase Chain Reaction
s	seconds
SSC	Sodium Saline Citrate
TAE	Tris-acetat-EDTA
TBE	Tris-borate-EDTA
TBS	Tris-buffered Saline
TE	Tris-EDTA
TEMED	N,N,N`,N´-Tetramethylethylendiamin
TFF	Trefoil Factor Family
TGF	Transforming Growth Factor
UACL	Ulcer Associated Cell Lineage
Vol	Volume

1. INTRODUCTION

1.1. Foreword

Gastric carcinoma is one of the most common type of cancer in the world. In a very simplified way, it is generally accepted that there are two main pathways of malignant transformation of the gastric mucosa: one pathway is starting from intestinal metaplasia and adenomatous dysplasia, leading to intestinal carcinoma and the other pathway is initiated by hyperplastic polyps or *de novo* changes, with or without concurrent non-metaplastic dysplasia, leading to diffuse carcinoma and to a subset of intestinal carcinoma (Correa, 1992; Solcia et al., 1996; Carneiro, 1997)

In the diffuse type, poorly cohesive cells invade the gastric wall with an infiltrative pattern. This occurs commonly in the body of the stomach, is not associated with intestinal metaplasia, and may be determined by genetic predisposition (Bocker et al., 1997)

In the intestinal type, malignant cells, resembling intestinal columnar epithelia, form glandular structures and display an expansive growth pattern. This type is found most frequently in the antrum associated with intestinal metaplasia and appears to be environmentally related (Bocker et al., 1997)

Gastric carcinomas display different genetic alterations described in other carcinomas, for example point mutations of the ras oncogene, p53 tumour suppresser gene, gene amplification of epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), platelet derived growth factor (PDGF) and des-regulation of peptides belonging to the trefoil factor family (TFF). In several studies the up-regulation of *TFFs* in epithelial cells around areas of damage in conditions such as gastritis, peptic ulceration, meta- and neoplasia of the gastrointestinal tract were observed. Gastric carcinoma exhibit loss of heterozygosity (LOH) in chromosomes 1, 5, 7 and 17 (Lemoine et al., 1992; Poulsom et al., 1993; David et al., 1994; Gött et al., 1996; Machado et al., 1997).

During the last 10 years several reports have elucidated the impotent role of trefoil peptides in the development and repair of the gastrointestinal mucosa suggesting that TFFs may be involved in gastric carcinogenesis and in the biopathology of these tumours.

The molecular background responsible for the development of gastric tumours is complex and not well elucidated to date. A complicated network of different components is necessary to keep the gastrointestinal mucosa intact. Thus, the influence of different transcription factors on the regulation of the *TFF* genes has to be elucidated. Therefore, the transcriptional regulation of the TFF healing peptides was investigated in my Ph.D. thesis work.

1.2. Trefoil Factor Family (TFF)-Domain Peptides

1.2.1. General aspects

Trefoil peptides are a group of small stable secreted peptides characterised by the presence of one up to six cystein-rich domains (Thim, 1989).

The trefoil motif consists of six conserved cysteine residues that permit the formation of three intrachain disulphide bonds (Cys1-Cys5, Cys2-Cys4, Cys3-Cys6). The spacing between these cysteine residues is almost invariant. The proposed structure of this domain, with its three disulphide loops, resembles a trefoil, which has given the peptide family its name. This conformation appears to result in marked proteolytic stability and resistance to acid digestion (Thim, 1989; Babyatsky et al., 1996).

In 1988 trefoil motifs have been discovered in different sub-species of *Xenopus laevis* (FIM-A.1) (Hauser et al., 1990). In this study, additional proteins containing six trefoil domains (FIM-C1) have been identified, which are secreted by the amphibian dermal glands (Hauser and Hoffmann, 1992). Further investigations have identified two proteins expressed by the mucous surface cells of *Xenopus* gastric mucosa (Hauser and Hoffmann, 1991). These proteins contain either one trefoil motif (xP1) or four motifs (xP4). The trefoil domain can be observed in other species such as porcine and rodent peptides from the gastrointestinal tract (Masiakowski et al., 1982; Tomasetto et al., 1990; Hoffmann and Hauser, 1993).

Three human trefoil peptides have been identified to date: TFF1 (formerly named pS2) and TFF3 (formerly named hITF) with one trefoil domain each, and TFF2 (formerly named hSP) with two trefoil domains (Fig. 1).

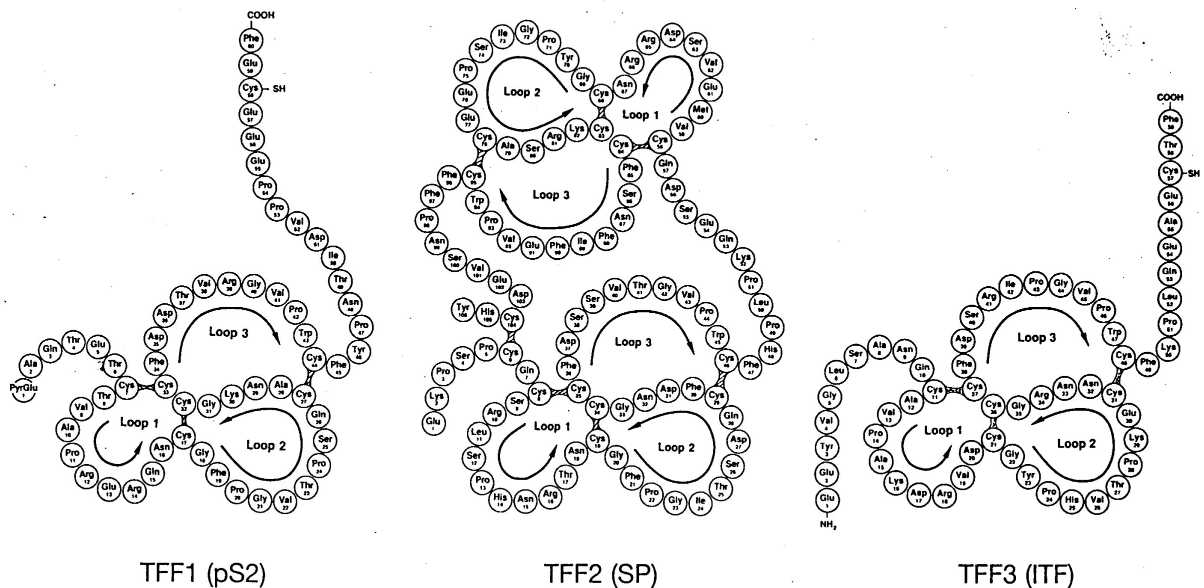


Fig.1: Secondary structure of the three human trefoil peptides.
The cystein residues form disulfidebridges in a 1-5, 2-4 and 3-6 fashion (Thim, 1997).

TFF1 was originally identified in 1982 as the product of an estrogen-responsive gene in the breast carcinoma cell line MCF-7 (Masiakowski et al., 1982). TFF2 was discovered in 1990 (Tomasetto et al., 1990) as a human homologue of a peptide isolated from porcine pancreas, the porcine TFF2 (Rose et al., 1989). TFF3 was identified in 1993 (Hauser et al., 1993; Podolsky et al., 1993) as a human homologue of rat TFF3 (Suemori et al., 1991).

1.2.2. Structure of trefoil peptide genes

The three human trefoil peptide genes were mapped to the same chromosomal region 21q22.3 (Theisinger et al., 1992; Tomasetto et al., 1992; Chinery et al., 1996; Gött et al., 1996; Schmitt et al., 1996) in a tandemly oriented fashion within a 50 kbp genomic fragment (Gött et al., 1996; Seib et al., 1997). The order and distances of the corresponding genes are presented in figure 2A. Analysis of the exon structure of the three trefoil peptide genes revealed a very similar organisation. The first exon encodes the secretion signal sequence, the second, respectively the third exon (*TFF2*) encodes the trefoil domain, and the last exon encodes three to four residues of the carboxyl terminus (Gött et al., 1996). The genomic organisation of the mouse *TFF* gene cluster is similar to the human *TFF* genes (Kayademir et al., 2000)

suggesting that the conserved structural organisation may have evolved by gene duplication and exon shuffling (Gött et al., 1996).

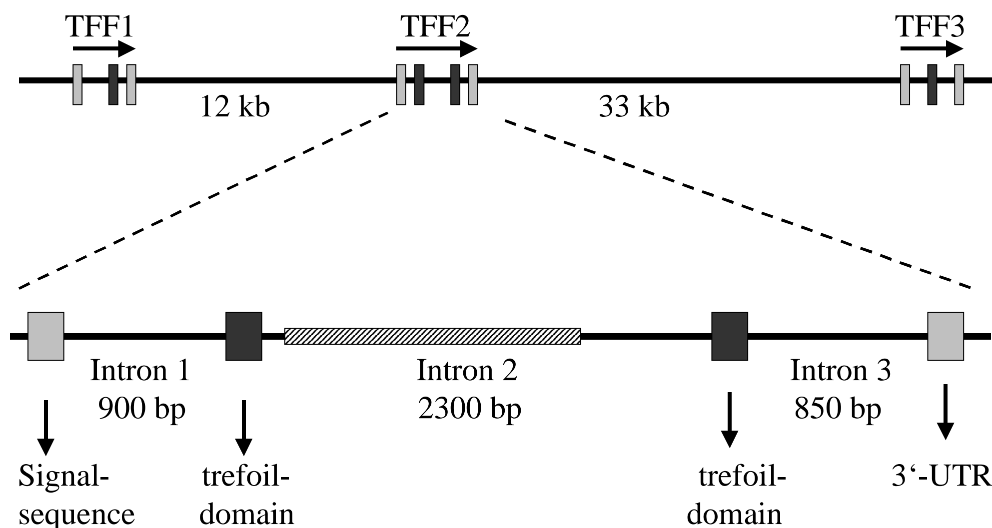


Fig. 2A: Genomic organisation of the human trefoil gene locous harbouring the genes *TFF1, TFF2* and *TFF3.*

The *TFF1* gene, formerly named as *breast cancer oestrogen inducible* (BCEI) gene, is formed by three exons (Rio and Chambon, 1990), the single trefoil domain of *TFF1* is encoded by exon 2. The 5´-flanking region of *TFF1* contains several control elements all located within 1.000 bp, which are responsive to estrogen (ERE), phorbol esters, c-H-ras and c-jun (Nunez et al., 1989) direct DNA binding of jun- and fos-like proteins has been identified as well (Schuh and Mueller, 1993).

TFF2 is located centrally in the *TFF*-cluster and it is the only gene encoding two TFF-domains. The genomic structure of *TFF2,* Previously was denominated *SML1*, contains four exons (Tomasetto et al., 1990). Exon 2 and 3 encode the two trefoil domains (Gött et al., 1996). They are separated by an intron containing an unique 25 bp tandem repeat cluster, repeated approximately 48-53 times (Kayademir et al., 1998).

TFF3 is the most recently described member of the mammalian trefoil family (Podolsky et al., 1993; Hauser et al., 1993). Analysis demonstrated that human *TFF3*

displays a high homology to human *TFF1* due to the three exon structure (Seib et al., 1995)
The promoters of all *TFF* genes share four motifs with high sequence homology. Motif I and IV were identified in all human trefoil genes and also in rat and mouse (Gött et al., 1996). In contrast, motif II and III are exclusively shared be the stomach-specific genes *TFF1* and *TFF2* in several species (Fig. 2b).

The study of the 5´-flanking motifs of the *TFF* genes in several gastrointestinal cancer cell lines using different *TFF* reporter gene constructs demonstrated the following facts:
(1) The truncation of sequences in *TFF1,* which are located downstream of motif I as well as the mutation in motif IV provoke the impairment of transcriptional activity.
(2) *TFF2* was not affected by the deletion of motif I.
(3) A deletion in *TFF1* affecting motif II as well as the ERE resulted in a significant reduction of transcriptional activity in all tested tumour cell lines. These results indicate that the region downstream of motif II harbours essential targets for a positive regulation (Beck et al., 1998).
(4) Motif III, present within the *TFF1* and *TFF2* promoters, is a general enhancer element of both genes.
(5) Motif IV, present in all three *TFFs*, has an enhancer function in *TFF1* and *TFF3*, and was shown to be a binding site for the Hepatocyte Nuclear Factor 3 (Beck et al., 1999) (Fig 2b).

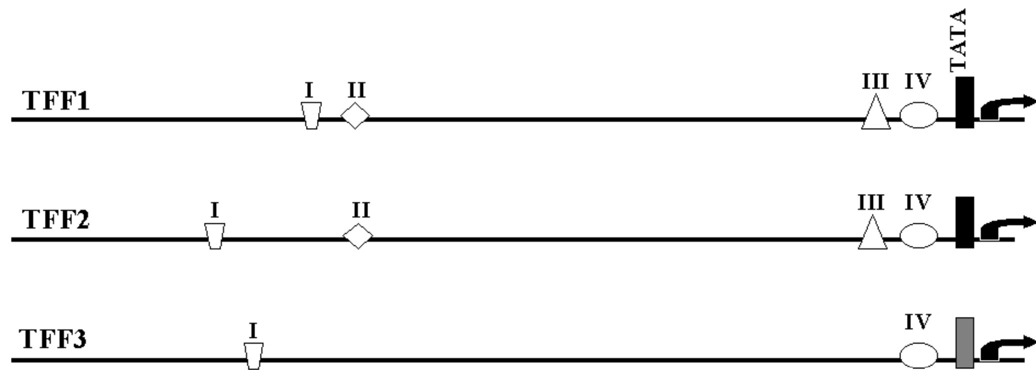


Fig. 2b: *TFF1*, *TFF2* and *TFF3* reporter gene constructs. The grey box indicates the TATA box in *TFF3* (CATAAA).

1.2.3. Structure of trefoil peptides

In its immature form TFF1 is 84 amino acids long and proceed to a peptide of 60 amino acids (Mori et al., 1988). The immature TFF2 is 129 amino acids long and its mature form is 106 amino acids long (Tomasetto et al., 1990). TFF3 has a sequence of 80 amino acids in the immature form and 59 amino acids in the mature form (Thim et al., 1995).

The characteristic trefoil domain of these peptides displays a compact tri-dimensional three-looped structure that appears to confer marked resistance to proteolysis and acid digestion (Jorgensen et al., 1982a; Mori et al., 1988; Playford et al., 1995; Thim et al., 1995).

There is some evidence that trefoil peptides can naturally exist as dimers, TFF1 can bridge through the seventh cysteine residue either to another TFF1 molecule (Chadwick et al., 1995; Chinery et al., 1995) or perhaps to TFF3 (Chinery et al., 1995); likewise, for TFF3 the possibility of homo-dimerisation was also demonstrated (Chinery et al., 1995; Thim et al., 1995). These data show that dimerisation might be essential for some biological properties of trefoil peptides and raise the intriguing possibility that a spectrum of biological activities may be generated by combining different single-trefoil peptides.

1.3. Trefoil Peptides in mammals

1.3.1. TFF1

TFF1 was originally discovered in 1982 during a screening of a cDNA library obtained from the human breast cancer cell line MCF-7 induced by estrogen (Masiakowski et al., 1982; Jakowlew et al., 1984). The molecular mass of TFF1 is 6674 Da and this protein can be observed in monomeric and homodimeric form. There is evidence that TFF1 has the ability to dimerize via a disulphide bond. The ability to form dimers through Cys58 is be functionally important. One of the roles that have been suggested for TFFs is the biosynthesis and dimerization of single trefoil proteins in combination with mucins (Chadwick et al., 1997; Polshakov et al., 1997).
TFF1 is expressed by the mucosal cells of the normal stomach, where it is abundantly expressed in the superficial and foveolar epithelium (Rio et al., 1988; Luqmani et al., 1989) and in the upper ducts and surface of Brunner´s glands in the duodenum (Hanby et al., 1993). Weak expression of this protein has also been reported in different parts of the gastrointestinal tract such as the tips of microvilli in the jejunum and ileum (Piggott et al., 1991a), salivary glands (Rio et al., 1988) and pancreas (Wright et al., 1990a) (table 1).

Besides the GI tract, TFF1 protein is expressed in low levels in other mucinous epithelia (table 1) such as the respiratory tract (Dos Santos Silva et al., 2000), normal breast epithelium, more precisely in the lumenal ductal cells (Brown et al., 1984; Piggott et al., 1991b; Racca et al., 1995) and it is found at high levels in almost 50% of breast carcinoma, where it is a marker of hormonal responsiveness and good prognosis (Henry et al., 1991a; Poulsom et al., 1997). Other tumours found to express TFF1 include carcinomas occurring in different organs: pancreas (Welter et al., 1992); lung (Higashiyama et al., 1994), endometrium (Henry et al., 1991b), ovary (Mucinous carcinoma) (Dante et al., 1994), prostate (Bonkhoff et al., 1995), urinary bladder (Lipponen and Eskelinen, 1994), biliary tract (Seitz et al., 1991), colorectum (Welter et al., 1994; Labouvie et al., 1997), oesophagus (Labouvie et al., 1999) and skin (mucinous carcinoma) (Hanby et al., 1998a) In gastric carcinoma the expression of *TFF1* has been described by several authors (Luqmani et al., 1989; Henry et al., 1991b; Theisinger et al., 1991; Muller and Borchard, 1993) in a frequency ranging from 48% to 57%. (Theisinger et al., 1991). An association between the expression

of *TFF1* and the diffuse type of gastric carcinoma has been reported (Muller and Borchard, 1993), in terms of a correlation between *TFF1* expression and the extent of tumour growth.

1.3.2. TFF2

TFF2 was formerly named spasmolytic polypeptide (SP) or spasmolysin (SML1). Porcine spasmolytic polypeptide (pSP) was first isolated by Jorgensen and co-workes in 1982, during a purification of insulin from porcine pancreas (Jorgensen et al., 1982a).

In 1990, TFF2 was identified as a human homologue of the porcine spasmolytic polypeptide (pSP) (Tomasetto et al., 1990). The human variant was then named human spasmolytic polypeptide (hSP). In man and rodents, the predominant site of expression is the deep foveolar pit of the gastric antrum. Smaller amounts of TFF2 have also been observed in Brunner´s glands of the duodenum (Hanby et al., 1993; Jeffrey et al., 1994) and some focal expression is found in the ductal epithelium of pancreas (Wright et al., 1990a) and in gall bladder epithelium (Seitz et al., 1991) (table 1).

The expression of *TFF2* in other species has been shown for example in pig in the acinar cells of the pancreas, the mucous cells of the stomach and duodenum, and the epithelial cells in jejunum and ileum. In rat, TFF2 has been found mainly in the antrum, whereas low expression has been observed in the small intestine. In mice, its expression has been proved in stomach and pancreas (Tomasetto et al., 1990; Hanby et al., 1998a; Lefebvre et al., 1993).

An increased expression of *TFF2* was observed at sites of mucosal injury such as peptic ulceration and inflammatory bowel disease (Rio et al., 1991; Poulsom et al., 1992; Wright et al., 1993), in some types of neoplasia of the gastrointestinal tract (Hanby et al., 1993; Taupin et al., 1996), in pancreatic carcinoma (Welter et al., 1992), stomach carcinoma (Theisinger et al., 1991), hyperplastic polyps of the colon (Hanby et al., 1993; Taupin et al., 1996), in Barrett´s metaplasia of the esophagus

(Hanby et al., 1994; Labouvie et al., 1999) and in gall bladder carcinoma (Seitz et al., 1991).

1.3.3. TFF3

In 1987, TFF3 (formerly known as intestinal trefoil factor ITF) was identified by Suemori and co-works by a screening of cDNA library obtained from isolated primary rat enterocytes.

The rat, ITF exhibits a pattern of tissue-specific expression within the gastrointestinal tract that is complementary to TFF1 and TFF2 as described previously. TFF3 in rat was present in all regions of the small intestine and colon, but appeared to be most abundant in the distal small intestine. It was absent in rat stomach, liver and pancreas. *ITF* appeared to be expressed predominantly within goblet cells and secreted into the luminal surface.

The latter study suggests the presence of a protein in human colonic and intestinal mucosa highly homologous to the rat ITF. These findings were confirmed by Northern blot analysis and cDNA cloning of a library prepared of normal human colon (Hauser et al., 1993; Podolsky et al., 1993).

Mass spectrometry analysis of the human TFF3 showed a monomeric and a dimeric form. The molecular weight of the monomer is 6694 Da and the dimer form is 13146.8 Da. The homodimeric form is built of two monomers linked by a disulphide bond between Cys-58 residues (Thim et al., 1995).

In humans, the principal expression of *TFF3* occurs in the goblet cells of small intestine and colon and in glands acini and distal ducts of Brunner´s glands. Low expression has been identified in the human uterus (Hauser et al., 1993), normal breast (Poulsom et al., 1997), hypothalamus, pituitary gland (Probst et al., 1995; Schwarzberg et al., 1999) and lung (Wiede et al., 1999) (table 1).
In another species TFF3 has been found in the cardiac glands of the rat stomach (Chinery et al., 1992), rat intestine, rat kidney, rat hypothalamus (Probst et al., 1995),

mouse intestine (Suemori et al., 1991) and in the porcine conjunctival goblet cells (Jagla et al., 1999).

The up-regulation of *TFF3* expression was observed in some neoplastic tissues, such as in skin mucinous carcinoma (Hanby et al., 1998b), breast carcinoma (Theisinger et al., 1996; Poulsom et al., 1997; May and Westley, 1997; Poulsom et al., 1997) and colon carcinoma (Taupin et al., 1996; Efstathiou et al., 1998).

Table 1: sites of physiological expression of trefoil peptides in normal human tissues.

Trefoil peptide	Site	Description	References
TFF1	Stomach	Superficial and foveolar mucosa	(Rio et al., 1991; Luqmani et al., 1989; Hanby et al., 1993).
	Duodeneum	Upper ducts and surface cells of Brunner´s glands	(Hanby et al., 1993)
	Colon	Focal goblet cells of distal regions	(Singh et al., 1998).
	Pancreas	Focally in duct epithelium	(Wright et al., 1990a).
	Gall bladder	Patchy epithelium expression	(Seitz et al., 1991).
	Salivary glands	Patchy epithelium expression	(Rio et al., 1988).
	Breast	Focal in duct lumenal cells	(Poulsom et al., 1997).
	Lung respiratory tract	Focal in epithelial cells of trachea and bronchi	(Dos Santos Silva et al., 2000)
TFF2	Stomach	Mucous glands of body an antrum	(Hanby et al., 1993).
	Duodenum	Brunner´s glands acini and distal ducts	(Piggott et al., 1991b).B
	Pancreas	Focal in duct epithelium	(Wright et al., 1990a).
	Gall bladder	Focal in duct epithelium	(Seitz et al., 1991).
TFF3	Duodenum	Brunner´s glands acini and distal ducts, goblet cells	(Suemori et al., 1991; Chinery et al., 1992; Hauser et al., 1993; Podolsky et al., 1993).
	Small intestine	Goblet cells	(Suemori et al., 1991; Chinery et al., 1992; Hauser et al., 1993).
	Colon	Goblet cells	(Suemori et al., 1991; Hauser et al., 1993)
	Breast	Focally in duct lumenal cells	(Poulsom et al., 1997).
	Uterus	Epithelium	(Hauser et al., 1993).
	Lung	Goblet cells of bronchi	(Wiede et al., 1999; Dos Santos Silva et al., 2000)
	Hypothalamus Pituitary gland	Neurons in peri- and paraventricular nuclei Anterior and posterior	(Probst et al., 1997)

1.4. Function of Trefoil Peptides

The first studies on the biological activity of trefoil peptides were performed by (Jorgensen et al., 1982b) on porcine TFF2 in experimental animal models. These studies suggested that these peptides played a role in the inhibition of gastrointestinal motility and pentagastrin-induced acid secretion (Jorgensen et al., 1982b). However, recent studies on the human and porcine peptides could not support these findings (Playford et al., 1995; McKenzie et al., 1997).

TFF1 "knockout mice" present inappropriate cellular differentiation patterns, hyperplasia in the distal part of the stomach and a complete lack of mucus. These data suggest that TFF1 may be involved in the induction of differentiation pathways and cellular migration during gut maturation. In addition, TFF1 "knockout mice" developed gastric tumours and one third of them exhibited neoplasia (Lefebvre et al., 1996). In contrast, TFF3 "knockout mice" revealed a normal phenotype but with increased sensitivity to damaging agents (Mashimo et al., 1996). Another possible explanation for the early expression of the trefoil peptides in the embryonic gut prior to the development of enzymes and acid secretion, is their participation in the protective mucosal barrier. This epithelial protection occurs especially in association with mucus glycoproteins (Kindon et al., 1995; Babyatsky et al., 1996).

Rapid regeneration of mucosal integrity in the gastrointestinal tract is an important step in preventing the progression of small foci of damage into significant ulcers. This repair mechanism is characterised by rapid migration of surviving epithelial cells around the ulcer margins over the denuded basement membrane. The movement of epithelial cells to seal small wounds occurs quickly and without the need for cell division (Lacy et al., 1993; Pignatelli, 1996). Subsequently, cell proliferation and differentiation occur to re-establish normal function and tissue architecture (Poulsom et al., 1996).

The physiological role of trefoil peptides is not completely elucidated. The presence of the peptides in exocrine cells of the GI tract and the association with mucin glycoproteins to form large, highly viscous, and resistant complexes suggests that trefoil peptides are involved in the protection of the GI epithelium against physical and chemical insults. Reports that *TFF* gene activity is only not restricted to

malignant tissues but already noted in gastric ulcers, inflammation and intestinal metaplasia, proved the genes' significance at precancerous stages of cellular irritation (Rio et al., 1991; Machado et al., 1996a; Gött et al., 1999).

Previous studies have demonstrated that the expression of *TFF1* and *TFF2* are normly restricted to the stomach. *TFF1* and *TFF2* are up-regulated in mucosal ulceration elsewhere in the digestive tract, most notably in Crohn´s diseases (Rio et al., 1991). This ectopic over-expression is also observed in epithelial cells undergoing migration across the base of ulcer (UACL-ulcer associated cell lineage) (Wright et al., 1993). UACL develops from the glands and crypts adjacent to the lesions. It is considered as a prototypic repair lineage, which secretes proteins that are potentially relevant in the healing of endodermally-derived tissues, such as epidermal growth factor (EGF), transforming growth factor (TGF-α), TFF1 and TFF2 (Wright et al., 1990b; Wright et al., 1990a; Hanby et al., 1997).

Possible interaction between TFFs and these growth factors were considered. The study of the expression of *TFFs* in the *TGF-α*-gene knockout mouse model demonstrated that the expression of *TFF2* and *TFF3* are suppressed in the late phase of epithelium repair, indicating that *TGF-α* may play a role in the increase of *TFF2* and *TFF3* expression in the later stages of gastric mucosal reconstitution (Cook et al., 1997).

TFF-peptides are also potent motogens and they have been shown to accelerate cell migration of colonocytes (Dignass et al., 1994) and to act as chemotaxins for monocytes (Cook et al., 1997), both participating in reparatory events following damage in inflammatory gut diseases.

Experimental colitis induced in the rectum of rats after application of acetic acid, showed a co-ordinated expression during epithelium repair: *TFF1* up-regulation was noted in the acute phase and *TFF3* overexpression was observed during the recovery stage (Itoh et al., 1996).

The use of recombinant TFF2 and TFF3 peptides in some experiments has demonstrated that they protect against gastric injury induced by ethanol, indomethacin or aspirin in rats (Chinery and Playford, 1995; Cook et al., 1998;

Konturek et al., 1998). In another established model of colitis in rats, the luminal application of recombinant TFF2 peptide promotes rapid repair of the epithelium and reduces inflammatory response (Tran et al., 1999).

Studies using transgenic mice, which overexpress *TFF1* within the jejunum, demonstrated an increased resistance to damage, with no protective effect seen in the non-expressive ileal segment. This suggests that the protecting action of *TFF1* expression was mediated by local effects (Playford et al., 1996).

TFF-peptides exert their main functions by at least two mechanisms. One theory relies on the possibility that TFFs and mucins act in synergetic manner to protect and reconstitute epithelial tissues (Dignass et al., 1994; Kindon et al., 1995). According to this theory, trefoil peptides and mucins would interact and thus enhance the protective functions of the mucus layer (Otto and Wright, 1994). This hypothesis is supported by different preliminary studies which demonstrated that the application of TFF2 and TFF3 increases the viscosity of mucin gels (Babyatsky et al., 1996). After this observations another study showed the co-expression of trefoil peptides and mucins (Gajhede et al., 1993; Carr et al., 1994). TFF1 is produced in gastric surface mucous cells together with the mucins MUC1 and MUC5AC. *TFF2* is expressed in gastric mucous neck cells and cells at the base of antral glands in combination with MUC6. TFF3 and MUC2 are both generated in intestinal goblet cells (Wright et al., 1993; Sands and Podolsky, 1996).

Regarding the premalignant and cancerogenous situation the specific pattern of co-expression of *TFFs* and *mucins* is different. Studies of Barrett´s metaplasia of the oesophagus, for example, has demonstrated a co-expression of trefoil peptides and mucins. It seems that *TFF1, MUC1* and *MUC5AC* are up-regulated during the neoplastic process at the early stage, corresponding to Barrett´s metaplasia. In contrast, all cases of Barrett´s metaplasia and squamous cell carcinoma of the oesophagus remained negative for TFF2 and MUC2 (Labouvie et al., 1999).

A recent study has shown that gastric polyps with foci of malignant transformation exhibited an up-regulation of *TFF1* expression. The same study has identified a co-expression of *TFF1* and *MUS5AC.* All cases of gastric polyps with signs of malignant transformation were negative for *TFF2* expression (Nogueira et al., 1999).

TFF3 expression was detected in colorectal carcinoma, associated with loss of differentiation and co-localisation with MUC2 (Taupin et al., 1996); in skin mucinous carcinoma (Hanby et al., 1998b), as well as in breast carcinoma (Theisinger et al., 1996; Poulsom et al., 1997) where it co-localises with TFF1 (Poulsom et al., 1997).

Moreover, expression of *TFF1, TFF3, MUC1* and *MUC2* was found in several cases of rectum carcinomas, whereat was associated with lack of *TFF2* expression (Labouvie et al., 1997).

According to the second hypothesis, it has been demonstrated that in gastrointestinal cells TFF3 can modulate epidermal growth factor (EGF) receptor on the epithelial transport only when the basolateral surface of the cell is exposed to TFF3 (Chinery and Cox, 1995). TFF3 induces a rapid phosphorylation of ß-catenin, which is associated with perturbation of the functional integrity of the E-cadherin/Catenin system and the promotion of cell motility in association with EGF (Liu et al., 1997).

Supplementary *in vitro* experiments have demonstrated that the induction of trefoil transcription after injury of the mucosa was mediated by activation of the Ras/MEK/MAP kinase signal transduction pathway and the activation of EGF-R (Taupin et al., 1999). This involvement of cytoplasmic signal transduction is strongly indicating a receptor-mediated response. The presence of a receptor has been suggested due to *in vitro* investigations on rat intestinal mucosa cell membranes and in frozen sections. Binding of ^{125}I-ITF and ^{125}I-SP was localised in the collecting ducts of the kidney, the neck region, and the surface epithelium of stomach and in the crypt epithelium of the small intestine and the colon (Chinery and Playford, 1995; Tan et al., 1997; Poulsen et al., 1998). These results propose the presence of specific receptors or transported proteins on these cells. However, the putative trefoil peptide receptors have yet to be cloned and characterised.

A more recent study has demonstrated that colon carcinoma cell lines that do not express E-cadherin, a cell-cell-adhesion molecule, are unresponsive to the migratory effects of TFF2 protein (Efstathiou et al., 1999). However, transfection of these cell lines with E-cadherin cDNA increased the cell migration significantly. These findings suggest that the stimulatory effect of TFF2 is associated with E-cadherin (Efstathiou et al., 1999).

Other experiments have also proved the interaction of trefoil peptides with E-cadherin/catenin and adenomatous polyposis coli (APC)-catanin complexes. The experiments have shown that stimulation by rat TFF3 led to decreased cell substratum and cell-cell adhesion in cell line HT-29. This effect is associated with disturbed regulation of the expression and cellular localisation of E-cadherin, catenin and APC.

The restitution of the function of E-cadherin complexes was realised by the use of tryphostin. This study confirms that tyrosine phosphorylation is a mechanism by which rat TFF3-mediated changes are regulated (Efstathiou et al., 1998).

A close relationship was found between *TFF1* expression and gastric carcinomas of the diffuse type. A significant correlation was found between *TFF1* expression and lymph node metastases. On the other hand, *TFF* expression is neither associated with features of tumour aggressiveness nor influences the survival of patients with gastric carcinoma (Machado et al., 1996b)

High serum levels of TFF1 protein were observed in a study of several patients with lung adenocarcinoma, more precisely with a bronchi-alveolar goblet cell subtype (Higashiyama et al., 1996). Expression of *TFF1* was also detected in non-malignant tissue from patients with advanced prostate carcinoma and it was considered as a marker of pre-malignant transformation of the prostate gland (Bonkhoff et al., 1995).

1.5. Transcription factors involved in the regulation of *TFF*

To understand the regulatory machinery, the cell-specific transcriptional mode of the *TFF* gene family was investigated by comparing and modifying the 5´-flanking regions. It is interesting to note that *TFF* expression is developmentally controlled in the GI tract, and appears before mucous cell differentiation (Otto and Patel, 1999). Regulation of the differentiation programs of the gastrointestinal tract is thought to involve lineage-restricted transcription factors that control the expression of terminal-differentiation genes. Some factors play an important role in the differentiation of the intestine and the regulation of intestine-specific genes such as *HNF-1, HNF-3, HNF-4, COUP-TF* and *Cdx-2* (Gao et al., 1998). Recently , while studying the regulatory

mechanism of *TFF* genes in the gastrointestinal tract, we discovered new DNA motifs within the 5´control regions of human *TFF* genes.

One of the putative binding sites present in the 5`control region of all *TFFs* is a target for the transcription factor of the GATA family. The GATA-6 is the only stomach-specific DNA-binding protein identified that belongs to the family of GATA factors (Tamura et al., 1993). GATA proteins recognise the motif (A/T)GATA(A/G) or related sequences. In *Caenorhabditis* a GATA factor is essential for controlling gut development (Egan et al., 1995). Whereas mammalian GATA-1/2/3 isoforms are predominantly expressed in the hematopoietic cells, the more recently identified *GATA-4/5/6* genes are expressed in the cardiovascular system and in endoderm-derived tissues including liver, lung, pancreas and gut. GATA-6 was shown to regulate a gastric parietal cell-specific gene coding for H^+/K^+-ATPase (Nishi et al., 1997). *GATA-6* is strongly expressed the human gastric adenocarcinoma cell line MKN 45 (Yoshida et al., 1997), in rodent gastric endocrine cell fractions, in a human ECL cell tumour, and in a gastric endocrine cell line (Dimaline et al., 1997). GATA-6 mRNA abundance was up-regulated during terminal differentiation of the rat stomach and on feeding after a fast. Such physiological changes also affect *TFF* expression (Lüdeking et al., 1998). In the 5‘-flanking region of *TFF1* and *TFF2* binding sites of GATA-6 are located.

Another transcription factor of potential importance for *TFF* regulation is the Nuclear Factor kappa B (NF-κB), because all *TFF* promoters have putative binding sites for NF-κB. NF-κB is known to be activated via tumour necrosis factor alpha (TNF-α) or other factors (e.g. IL-1 and TGF-β). TNF-α is a 17 kDa cytokine, that is produced by macrophages during inflammation and is essential for the regulation of immune response (Beutler and Cerami, 1988). TNF-α is up-regulated in Crohn’s disease and antibodies against TNF-α promise therapeutic benefit (Asakura and Sugimura, 1999). TNF-α signalling is mediated by cell surface receptors TNFR1 and TNFR2 (Tartaglia and Goeddel, 1992). By a downstream signalling pathway TNFα (and other signals such as IL-1, oxygen free-radicals or UV light) triggers the degradation of the inhibitor (IκB) of nuclear factor κB (NF-κB) thereby allowing NF-κB to translocate into the nucleus (Jobin et al., 1999). NF-κB plays a key role in transcriptional activation of various genes involved in mucosal immune and proinflammatory responses (Jobin

and Sartor, 2000). In most cells, NF-κB is found as a heterodimer composed of p65 and p50 subunits. (Ballard et al., 1992; van Den Brink et al., 2000).

Many genes activated by NF-κB were found to contain the consensus binding site: GGGR(C/A/T)TYYCC (Baeuerle, 1991). Although NF-κB acts generally as transcriptional activators, it also has been reported as a transcriptional repressor (Fontaine et al., 2000; Wissink et al., 1997; Supakar et al., 1995). Many putative binding sites of NF-κB are present in the upstream region of all three human *TFF* genes displaying two or one mismatches to the NF-κB consensus binding site within 900 bp of the 5'-flanking region.
The human *TFF2* gene promoter exhibiting a conserved E box element (Gött et al., 1996). E box are bound by a class of transcription factors, the most prominent of which are c-Myc and USF. USF1 (upstream stimulating factor-1) and USF2 are basic helix-loop-helix leucine zipper (bHLHZip) transcription factors involved in the control of cellular proliferation and embryonic development (Sirito et al., 1998). They display strong similarities with the Myc oncoproteins both in their overall protein structure and DNA specificity, which resembles the core consensus sequence CACGTG or CACATG also termed E box. The major USF species present in most tissues and cell types is the USF1-USF2 heterodimer. USF1 homodimers are less abundant and USF2 homodimers are quite uncommon (Viollet et al., 1996; Sirito et al., 1998). Despite their ubiquitous expression, USF proteins may not be transcriptionally active in all cells. For example, in HeLa cells (cervix carcinoma) the USF proteins were transcribed and overexpression causes growth inhibition, whereas in Saos-2 cells (osteosarcoma) USFs are transcriptionally inactive and overexpression have no effect on proliferation (Qyang et al., 1999). Moreover, partial or complete loss of USF transcriptional activity is a common event in breast cancer cell lines (Ismail et al., 1999) suggesting the existence of a putative USF coactivator and that loss of it and following Myc overexpression favours rapid proliferation.

The targets which are known to be activated by Myc or Myc-Max heterodimers include odc, p53, α-prothymosin, ECA39, eIF4E, cdc25, MrDb and cad, most of which are involved in cell proliferation (Boyd and Farnham, 1997). Generally, it is believed that USF factors antagonise Myc proteins. However, the corresponding molecules of the target genes that are activated by USF such as T-cell adhesion

protein CD2, (Outram and Owen, 1994), murine morphogenetic protein-4 (Ebara et al., 1997), fatty acid synthase (Wang and Sul, 1997) transcription factor C/EBP (Timchenko et al., 1995), lung surfactant protein-A (Gao et al., 1997), mitochondrial ATP synthase (Breen and Jordan, 1997) and MAPK phosphatase 1 (Sommer et al., 2000) seemed to be more heterogeneous in their function. For the mouse *Cad* gene competition between USF and Myc for binding to the E box has been observed in living cells (Boyd and Farnham, 1997; Boyd and Farnham, 1999). Thus, discrimination may be dependent on the core promoter elements and the chromatin structure surrounding the E box.

1.6. Aims of the work

Although the molecular function of trefoil peptides are yet largely unknown, it appears that they act to protect and heal the gastrointestinal mucosa and may suppress tumour growth. Because trefoil peptide expression is regulated on the level of transcription, understanding of the signals and factors regulating transcription of *TFF* genes would potentially offer opportunities for therapy of gastrointestinal diseases.

The first aim of this study was to test whether *TFF* transcription is influenced by the transcription factor GATA-6, an evolutionary conserved factor governing gastrointestinal development. For this purpose, chromosomal *TFF* expression should be monitored by reverse transcription and multiplex PCR (RT-PCR) and *TFF* reporter gene expression by luciferase assays in gastrointestinal cell lines, respectively. A functional analysis of deletions and mutations in putative GATA binding sites, as well as DNA binding by electrophoretic mobility shift assays (EMSA) was also intended.

A second factor thought to be important for *TFF* regulation was the Nuclear Factor kappa B (NF-κB), a key player mediating the response to infection and inflammation, since putative binding sites were observed in the 5’-flanking region of *TFF* genes.

Finally, as an aim to asses the role of TFFs as tumour suppressor it was interesting to define the role of a conserved E box in the *TFF2* promoter. E boxes are bound by a class of transcription factors, the most prominent of which are the oncogene c-Myc and the Upstream Stimulating Factor (USF) that is known to stimulate anti-proliferativ

functions. For this purpose it was intended to use chromatin crosslinking and immunoprecipitation (ChIP) besides the methods mentioned before to prove *in vivo* binding of c-Myc and USF.

2. MATERIAL AND METHODS

2.1. Cell lines

Table 2. List of tested cell lines

Cell line	Type	Culture	Reference
MKN45	Gastric adenocarcinoma	RPMI 1640, 20% FCS	Machado, Porto
KATO III	Gastric adenocarcinoma	DMEM 4500, 10% FCS	G.Stamp, London, ATCC # HTB 103
LS174T	Colon adenocarcinoma	DMEM 4500, 10% FCS	G.Stamp, London ATCC # CL188
HT-29	Colon adenocarcinoma	Waymouth, 10% FCS	ATCC # HTB 38
CAPAN-2	Pancreatic carcinoma	DMEM 4500, 10% FCS	DKFZ, ATCC # HTB 80
IMIM-PC1	Pancreatic ductal carcinoma	DMEM 4500, 10% FCS	Menke, Andre
IMIM-PC1	Pancreatic ductal carcinoma	DMEM 4500, 10% FCS	Menke, Andre
DAN-G	pancreatic carcinoma	RPMI 1640 10% FCS	DKFZ, TZB 610006
HUTU 80	small intestinal carcinoma	DMEM 4500, 15% FCS	ATCC # HTB 40

2.2. Primers

Table 3. List of primer pairs. F and R refer to forward and reverse orientation of the Primers respectively.

Method	Name	Localisation	Sequence	Purpose
PCR	TFF1 F	pos.21, exon 1	TTTGGAGCAGAGAGGAGGCAATG	To test the expression of TFF1
	TFF1 R	pos.166, exon 2	ACCACAATTCTGTCTTTCACGGGGG	
	TFF2 F	pos.158, exon 2	GTGTTTTGACAATGGATGCTG	To test the expression of TFF2
	TFF2 R	pos.258, exon 3	CCTCCATGACGCACTGATC	
	TFF3 F	pos.140, exon 2	AACCGGGGCTGCTGCTTTG	To test the expression of TFF3
	TFF3 R	pos.234, exon 3	GAGGTGCCTCAGAAGGTGC	
	GAPDH F	pos.610	ACCCAGAAGACTGTGGATGG	As control

Method	Name	Localisation	Sequence	Purpose
	GAPDH R	pos.724	GGATGACCTTGCCCACAG	As control
	hG-6 F	pos. 1783	GATGGAAGGGAAGGGCCAG	Determination of the GATA-6 gene expression in different cell lines
	hG-6 R	pos. 1963	GAAGAAGCACATGATTTTGGAC	
Mutagenesis	T2D3	TFF2 (-22 bp -215 bp)	CCAGACCTGCACGTGGCCGGGTGGGACAAACAGAGGGGAG	Deletion of motif III
	T2DMyc	TFF2 (-218 bp -758 bp)	GGGTCCCCTCTGTCCTTCCCGCCGGTTTTCCACGCTGGCAG	Deletion of motifs I, II and the binding site of Myc
	T2DM2	TFF2 (-390 bp -758 bp)	GGGTCCCCTCTGTCCTTCCCTCAGGGAGGGGACTTTTCC	Deletion of motifs I and II
	T2DG-6	TFF2 (-265 bp -372 bp)	CTCAGGGAGGGGACTTTTCCCCCTTCCTGGGTGTGACCC	Deletion of both binding sites of GATA-6
Mutagenesis	T2M NF-κB	TFF2 in Pos. 421	GCTTCCCTTCCTCAGGTACCGGATCCTTCCATGGCTATCTGC	Point mutation in the binding site of NF-κB
	T2mMyc	TFF2 in pos.250	GCCCAGCCCCAGACCTGCAGGGCCGGTTTTCCACGCTGG	Point mutation in the binding site of Myc
ChIP	TFF1 F	pos. 97	GGCCTCTCAGATAGAGTAG	To proof the binding site of E Box in vivo
	TFF1 R	pos.97	TCCTCTGAGACAATAATCTCC	
	TFF2 F	pos.250	TGTGGTCCCTGCCCACTC	To proof the binding site of E Box in vivo
	TFF2 R	pos. 250	TCTCCCTGCTCGGTGATAC	
	TFF3 F	pos. 293	GGCTCTCTTGTCATGGGAC	To proof the binding site of E Box in vivo
	TFF3 R	pos. 293	AAGCGGTAAGGGCGGATTC	
EMSA	G6	pos.400	GCCAGCAGATAGCATGGAAAAG	Binding site of GATA-6
	GM	pos. 400	GCCAGCAGATGCCATGGAAAAG	Point mutation in the binding site of GATA

Method	Name	Localisation	Sequence	Purpose
	P(PEA-3)	pos. 196	GGGCAGGAAGAGGTATCACCG	Binding site PEA-3 for Control
	TFF2	pos. 421	CCTCAGGGAGGGGACTTTTCCATG	Binding site of NF-κB
	TFF3	pos. 97	GTTTGCTTGGGGAAGGCTCTCCCTCC	Binding site of NF-κB
	TFF3	pos. 644	TAATACAAAGAGCCTTTCCTATCCAG	Binding site of NF-κB
EMSA	TFF1	pos. 97	GATGACCTCACCACATGTCGTCTC	Binding site of Myc
	TFF2	pos. 250	CAGACCTGCACGTGGCCGGTTTTC	Binding site of Myc
	TFF3	pos. 293	CTGCCACCCCACATGGCTCCTGCAC	Binding site of Myc

2.3. Plasmids and Reporter gene constructs

Table.4. List of used plasmids and reporter gene constructs. Amp: ampicillin

Plasmid name	Description	Vector	Reference	Plasmid size	Resis t-ance
pRL-CMV	Renilla Luciferase with CMV-Promoter	pRL	Promega	4.079 kb	amp
pGL3-TFF1	TFF1-Promoter 1100 bp	pGL3-basic	Beck, Gött	6.100 kb	amp
pGL3-TFF1-DNF-κB	TFF1-Promoter 1074 bp	pGL3-basic	Al-Azzeh, Gött	6.074 kb	amp
pGL3-TFF2	TFF2-Promoter 821 bp	pGL3-basic	Beck, Gött	5.690 kb	amp
pGL3-T2D3	TFF2-Promoter 630 bp	pGL3-basic	Al-Azzeh, Gött	5.500 kb	amp
pGL3 T2DMyc	TFF2-Promoter 267 bp	pGL3-basic	Al-Azzeh, Gött	5.136 kb	amp
pGl3-T2DM2	TFF2-Promoter 453 bp	pGL3-basic	Al-Azzeh, Gött	5.322 kb	amp

Plasmid name	Description	Vector	Reference	Plasmid size	Resis t-ance
pGL3 T2DG-6	TFF2-Promoter 715 bp	pGL3-basic	Al-Azzeh, Gött	5.584 kb	amp
pGL3 T2MNF-κB	TFF2-Promoter 821 bp	pGL3-basic	Al-Azzeh, Gött	5.690 kb	amp
pGL3-TFF3	TFF3-Promoter 867 bp	pGL3-basic	Beck, Gött	5.700 kb	amp
pCDNA1-hGATA-6	Expression plasmid	pCDNA1	Perlman, Suzuk	6.100 kb	amp
pCDNA1-hGATA6ΔZF	Expression plasmid	pCDNA1	Perlman, Suzuk	6.100 kb	amp
pMT2T-p50,	Expression plasmid	PMT2T	Bours, Burd	~ 6,5 kb	amp
pMT2T -p65	Expression plasmid	PMT2T	Bours, Burd	~ 6,5 kb	amp
CMV-MAD-3 (IκB*)	Resistant mutant IκB* plasmid (serines 32 and 36)	pcDNA3	Henkel, Zabel	5,446 kb	amp
pCMV-hu-c-myc	Expression plasmid	pP2	Sommer, Lüscher	~6,1 kb	amp
pUHD-USF1	Expression plasmid	pUHD	M. Eilers	~3,45 kb	amp
pUHD-USF1mutBR	Dominant negative mutant of USF1	pUHD	M. Eilers	~3,45 kb	amp
psvUSF2	Expression plasmid	psG5	M Sawadogo	4,1 kb	amp
pCMV-mad1	Expression plasmid	pP2	Sommer, Lüscher	~ 6,1 kb	amp
pSP-maxp22	Expression plasmid	pSP	Sommer, Lüscher	~ 6,1 kb	amp
M4-mintk-luc	Reporter plasmid	pCMV	Lüscher	~ 4,1 kb	amp
6xNF-κB	Reporter plasmid	pCDNA1	Henkel	~ 5,1 kb	amp

2.4. Cell culture and reporter plasmids

The different cell lines used in this study are discribed in table. 2. With respect to the TATAA box the 5´-flanking region of *TFF1* gene ranging from -100 to +38 was inserted in front of the luciferase gene that was devoid of its promoter residing in the transient eucaryotic expression vector pGL3-basic. Plasmid pGL3-s821B was constructed by subcloning a PCR fragment of the *TFF2* promoter sequence (pos. -821 to +61) tailed with restriction sites KpnI and BglII into the corresponding sites of pGL3-basic luciferase vector. For *TFF3* a corresponding PCR fragment (pos. -867 to +74) was cloned into the *HinfIII* and *SacI* sites of plasmid pGL3-basic (*TFF3* construct) as described (Beck et al., 1998).

2.5 Deletions and mutations of the 5´-flanking regions of *TFFs*

TFF1 wild type promoter (pos. –1100 bp to +38 bp) inserted into plasmid pGL3 was used for deletion mutagenesis by using the GeneEditor ^{TM} *in vitro* site-directed mutagenesis system (Promega). Deletion of 26 bp, harbouring the putative binding site of NF-κB, was accomplished with the following oligonucleotides flanking both sites of the deletion at pos. -68 bp to –94 as indicated in italics (upstream) and underlined letters (downstream) (table 3). *TFF2* wild type promoter (pos. –821 bp to +61 bp) inserted into plasmid pGL3 (Beck et al., 1998) was used for deletion mutagenesis with the same method of GeneEditor ^{TM} *in vitro* site-directed mutagenesis system (Promega). Deletion of reporter plasmids were made with the following oligonucleotides flanking both sites of the deletion as indicated in table 3 italics (upstream) and underlined letters (downstream). The *TFF*2 wild type reporter plasmid was mutagenized with the same method. Point mutations in *TFF*2 at pos. -421 for the putative binding site of NF-κB were introduced changing the original site GGGAGGGGACTTTTCC as indicated (table 3). A two base pair deletion in the E-Box (-250 bp) of *TFF2* was introduced with same method changing the original site TG**CACGTG**GC to TG**CA-G-G**GC with an oligonucleotide flanking both sites of the point mutation indicated in italics, (table 3). All this constructs were verified by DNA sequencing of both strands.

2.6. 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 polyethylenimine reagent ExGen 500 (MBI, Fermentas). Forty eight hours after stimulation cells were lysed with 20 µl passive lysis buffer (for 96-well plates) for 20 min and assayed immediately for light production in the presence of luciferase substrates using the Dual-Luciferase reporter assay system (Promega). Relative light units were measured by luminescence with a minilight luminometer (Lumat LB9501, Berthold). Luciferase assays was performed in triplicates or quadruplicates. Renilla luciferase (pRL-CMV, Promega) were used to standardise transfection efficiency and calculate specific transcriptional activity. Results were calculated as ratio of firefly luciferase to Renilla luciferase and compared to the non-stimulated control (relative transcriptional activity). The standard deviation was less then 15 %. Each experiment was independently performed 3 times. For cotransfection experiments, 320 ng of the reporter plasmid and 96 ng of the following expression plasmids were used (table 4).

2.7. Reverse transcription

Total RNA was prepared after 48 h using RNA-pure (Peqlap, Erlangen, Germany). This RNA was reverse transcribed in the subsequent cycling conditions: a) 37°C for 1 hour, b) 94°C for 2 min. The RT-PCR reaction was carried out as follows:

RNA	1,0 µg
Oligo-(dt)-15 mer (50 µM)	1,0 µl
10X RT-buffer	2,5 µl
DNTP mix (5mM each)	5,0 µl (dATP+ dCTP+dGTP+dTTP)
RNAse Inhibitor (40 U/µl)	1,0 µl
MMLV-RT (50 U/µl)	1,0 µl
DTT	0,3 µl
RNAse-free H_2O dd	25,0 µl

2.4.1. Multiplex-PCR (RT-PCR)

The multiplex PCR was realised with cDNA corresponding to 0,2 µg RNA. Twenty two, 25 and 28 cycles were used. Primer pools containing TFF1 (146 bp)/TFF2 (101bp)/ glyceraldehyde-3-phosphate dehydrogenase GAPDH (115 bp) or TFF1/ TFF3 (92 bp)/ GAPDH primers were chosen in order to obtain expression ratios relative to GAPDH (table 3).

The oligonucleotides were designed from cDNA or genomic sequences of all three *TFFs*. The choice of PCR primers for amplification of TFF mRNAs was based on comparable sizes of PCR products (approximately 90 bp-150 bp) (TFF1: Genbank acc.no.x52003.1; TFF2: acc. no. x51698; TFF3 according to (Hauser et al., 1993) . To avoid possible false positive results due to genomic DNA contamination, all *TFF* primer pairs were chosen to be located in different exons. The primers pairs are described in table 3. The cycle condition for multiplex PCR is summarised in table 5.

Multiplex-PCR reaction was carried out as follows:

cDNA	4 µl
10X buffer AGS	4 µl
$MgCl_2$	2 µl
dNTP (each 5 mM)	2 µl
Primer pool (each 3,3 µM)	4 µl
H_2Odd	23 µl
Taq-DNA-Polymerase	1 µl
Final volume	40 µl

Table 5. Cycling conditions for multiplex PCR

Step	State	Temperature	Duration
Step I	denaturation	94°C	4 min
Step II (22, 25 and 28 cycles)	denaturation	94°C	45s
	annealing	57°C	1 min
	extension	72°C	1 min
Step III	extension	72°C	5 min
	end	4°C	

Gene products were analysed by 6% polyacrylamide TBE gel electrophoresis and ethidium bromide staining. Semi-quantification of *TFF* mRNA expression levels was performed by co-amplification of the target gene (*TFF1*, *TFF2* or *TFF3*) and the housekeeping gene *GAPDH*, and correction of expression levels relative to *GAPDH* levels. Band intensities were determined digitally by a charged coupled device (CCD) camera (L.T.F., Wasserburg) and displayed as percentage of the *GAPDH* control.

2.8. Western blotting

Nuclear protein extracts were measured by standard Bradford assay (BioRad). Fifteen µg protein was separated by SDS/PAGE (12% acrylamide gels). After transfer to microporous polyvinylidene fluoride membranes (PVDF, Millipore), the membranes were probed with rabbit polyclonal antiserum specific for human USF1 (Pognonec and Roeder, 1991) or with antibodies specific for USF2 (C-20, sc-862, Santa Cruz Biotechnology) diluted 1:3000, followed by secondary antibody (pig anti-rabbit peroxidase) diluted 1:100. Proteins were visualised using the ECL western blotting analysis system (Amersham).

2.9 Chromatin crosslinking and immunoprecipitation (ChIP)

This protocol was provided and used by the Lüscher group (Institut für Molekulargenetik, Hannover) who cooperated with us in the USF project. To harvest HT-29 and MKN45 cells ($5x10^7$ to $5x10^8$), plates were rinsed with PBS, incubated with trypsin–EDTA (GIBCO) and scraped. Cells were collected by centrifugation, washed twice in cold PBS, and adjusted to $5x10^5$-$2x10^6$ cells /ml on ice. During stiring on ice 1/10 volume of a the fixation solution (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-HCl, pH 8, adjusted to 11 % formaldehyde [freshly added from 37 % stock solution] was added and was stopped fixation was stopped after 1 hour by the addition of glycine to a final concentration of 0.125 M. After centrifugation cells were washed once in cold PBS (approx. 20 ml PBS/10^8 cells) and were adjusted to ~5ml/10^8cells in washing solution A (0.25 % Triton X-100, 10 mM EDTA, 0.5 mM EGTA,10 mM Tris-HCl, pH 8) and incubated for 20 min on a rocker at room temperature. After collection at 3000 x g for 20 min cells were adjusted to

~5ml/10^8cells in washing solution B (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl, pH8) and incubated for 20 min on a rocker at room temperature.

After centrifugation 2 x 10^7 cells were resuspended in 1 ml ChIP-RIPA buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholic acid [DOC], 0.1% SDS, 1 mM EDTA, 0.5% aprotinin) and the chromatin was sonicated to an average size of 300 bp. Ten µl of the lysate were used to prepare total input DNA. For this 190 µl of TE-buffer (containing 10 µg of RNAse A) was added and the samples were incubated for 30 min at 37°C. The samples were then adjusted to 0.25% SDS and 500 µg/ml proteinase K and incubated for an additional 6 h at 37°C. Further treatment to reverse the crosslinks and to purify the DNA were performed as described below.

Immunoprecipitations (IP) were carried out over night using 2 x 10^7 cell-equivalents/IP at 4°C, with 1µg of the following antibodies: anti-c-Myc (N-262), anti-c-Myb (H141), anti-USF-1 (C-20), anti-USF-2 (C-20) (all from Santa Cruz). In parallel, 15 µl of protein G-Sepharose beads were incubated with 1 volume of sheared salmon sperm DNA (1 µg/µl) and 1 volume of ChIP-RIPA buffer O/N at 4°C. Blocked beads were washed two times with 1 ml of ChIP-RIPA buffer. Immunocomplexes were recovered by adding 15 µl of washed beads to the samples and incubating for 1 h at 4°C under constant agitation. Beads were then washed sequentially with the following buffers at RT: 2 x ChIP-RIPA, High Salt buffer (10 mM Tris-HCl [pH 7.5], 2 M NaCl, 1% NP-40, 0.5% DOC, 1 mM EDTA), and ChIP-RIPA. Finally, beads were washed with 1 ml of TE and complexes were eluted with 55 µl of elution buffer
(1% SDS in TE) for 10 min at RT. For DNA recovery, 50 µl of the eluate was removed from the beads and 3 volumes of TE (including 10 µg of RNase A) were added. Samples were then incubated for 30 min at 37°C. After addition of 100 µg Proteinase K the samples were incubated for 6 h at 37°C followed by at least 6 h at 65°C to completely reverse the crosslinks. Samples were phenol/isoamylalkohol/ chloroform and isoamylalkohol/ chloroform extracted, followed by a standard ethanol precipitation (using 20 µg of glycogen as carrier). DNA was resuspended in 50µl of TE.

2.9.1. PCR reactions for ChIP assays

PCRs were carried out in 30 µl using 2.5 U of HotStarTaq (Qiagen) in 1x PCR-buffer. Each reaction contained 0.2 mM of each dNTP and 1 µM of each primer. After an initial denaturation step for 15 min at 95°C the reactions consisted of 33-39 cycles of 20 sec at 94°C, 20 sec at 60°C (55°C in the case of the hTFF1-primers), 20 sec at 72°C, and a final elongation step at 72°C for 7 min. As templates, 1/20 of the immunoprecipitated DNA or varying amounts of sonicated total input DNA was used. The following PCR primers were used (table 3).

2.10. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts of gastrointestinal cell lines grown until confluence were prepared as described before (Beck et al., 1999). Double-stranded synthetic oligonucleotides were end-labelled using γ-^{32}P-ATP and T4 polynucleotide kinase. Electrophoretic mobility shift assay was performed by incubating 10 pmol of the oligonucleotide probe termed G6 (putative GATA-6 binding site in *TFF2* at pos. -400bp (table 3) , with 8 µg of nuclear protein in 10 µl of binding buffer (20 mM Hepes pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) and 1 µg of poly (dI-dC) as non-specific competitor for 30 min at 30°C. Competition experiments were performed by addition of 1 nmol (100-fold molar excess) of unlabeled G6, a oligo mutated in the GATA core site termed GM (Table 3), and a oligo harbouring the PEA-3 motif in *TFF2* termed P (at pos. –196; table 3), respectively, prior to addition of the labelled probe. After incubation the DNA-protein complexes were subjected to electrophoresis on a 6% nondenaturating polyacrylamide gel in 0,5 x TBE at constant current (15-30 mA) with a cooling device (4°C) for 3h, and visualised by autoradiography.

For NF-κB EMSA we used another protocol than for GATA-6. Nuclear extract of gastrointestinal cell lines were harvested in 300 µl of F-buffer (10mMTris, pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 5 µM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 units/ml α_2-macroglobulin, 2.5 units/ml pepstain, 2.5 units/ml leupeptin, 150 µM benzamidin, and 2.8 µg/ml aprotinin) per 10-cm dish, incubated on ice for 10 min, vortexed for 45 s, and cleared by centrifugation at 14,000 rpm (Sommer et al., 1998; Vervoorts and Lüscher, 1999).

The following oligonucleotide were used as probes *TFF2* at pos. –421 (table 3), in *TFF3* at pos. –97 (table 3) and in *TFF*3 at pos. –644; (table 3). Competition experiments were performed by addition of 1 nmol (100-fold molar excess) of the corresponding unlabeled oligo, and a non-specific oligo harbouring the PEA-3 motif in *TFF2* (pos. –196; table 3), respectively, prior to addition of the labelled probe. Nuclear extracts were incubated with an antibody against NF-κB p65 and USF-2 (sc-109X, and sc-862, Santa Cruz Biotechnology) for one hour prior before addition of the labelled oligos.

The EMSA for USF was performed by the group of Lüscher, the nuclear extract was prepared as described for NF-κB. The CMD oligonucleotide (5´-TCAGAC**CACGTG**GTCGGG) was ^{32}P-γ-end-labelled with T4-polynucleotid kinase. 1-3 µl of COS-7 F-buffer lysate (1/300 to 1/100 of a 10 cm dish) (see above) was incubated with 0.1 to 0.5 ng of labelled probe in 15 µl of GS buffer (20 mM HEPES, pH 7.3, 50 mM KCl, 3 mM $MgCl_2$, 1mM EDTA, 8% glycerol, 1 mM β-mercaptoethanol, 10 mM dithiothreitol) in the presence of 1 µg of sonicated salmon sperm DNA at 30°C for 30 min. The DNA-protein complexes were separated on 5% polyacrylamid gels in 0.5xTBE (25mM Tris base, 25 mM boric acid, 0.5 mM EDTA) at 20 V/cm and 4°C. For competition experiments unlabeled oligonucleotides (TFF1; TFF2; TFF3: table 3) were used as specified in the figure legends.

3. RESULTS

3.1. Transcription factor GATA-6 activates the expression of gastroprotective trefoil genes *TFF1* and *TFF2* (Al-azzeh et al., 2000)

3.1.1. Putative GATA binding sites in the upstream region of *TFF* genes

Since the *TFF* genes are expressed very early in the development of mammalian gut, I asked whether this could arise from transcription factors mediating lineage and tissue-restricted differentiation programs. Using the sequence (A/T/C)GAT(A/T)(G/A), representing a combined consensus binding sequence of GATA proteins and GATA-6 protein (Sakai et al., 1998), we found several elements in the 800 bp of 5'-flanking region of *TFF* genes, displaying 100 % identity to this consensus sequence (Fig. 3). Interestingly, the putative GATA binding site located 32 bp upstream to the TATA box (AGATAA) is conserved in its flanking sequences and position between the two stomach-specific genes *TFF1* and *TFF2*. The orientation and distribution of the putative GATA sites in respect to transcription of *TFF1* and *TFF2* suggests that both orientations may be considered to be relevant.

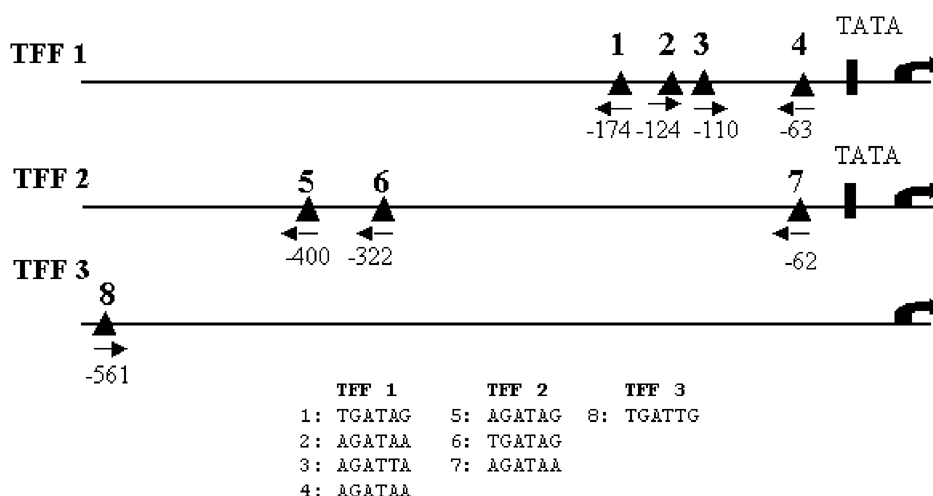


Fig. 3: Putative GATA binding sites in the 5'-flanking region of *TFF* genes. The sequence of the GATA consensus binding sites (A/T/C)GAT(A/T)(A/G) of all three *TFF* genes are displayed by numbers. Arrows and small numbers indicate the orientation and position of the motif with respect to transcription.

3.1.2. Endogenous *expression of GATA-6 in gastrointestinal cell lines*

I then explored the ability of several human gastrointestinal cell lines to express GATA-6 by performing comparative RT-PCR assays using GAPDH as an internal standard (Fig. 4). High amounts of GATA-6 transcripts were detected in the adenocarcinoma derived cell lines from stomach (MKN45 and KATOIII) and from colon (LS174T). The pancreatic carcinoma derived cell lines displayed variable but low levels of GATA-6 transcripts. The order of gene expression was IMIM-PC2 > IMIM-PC1 > CAPAN2. Compared to the expression of GAPDH mRNA, in HeLa cells, no transcripts of GATA-6 were detected (data not shown). Thus GATA-6 mRNA is present in tissue as well as adenocarcinoma-dervied cells of gastrointestinal origin that were previously shown to express *TFF* genes (Beck et al., 1998).

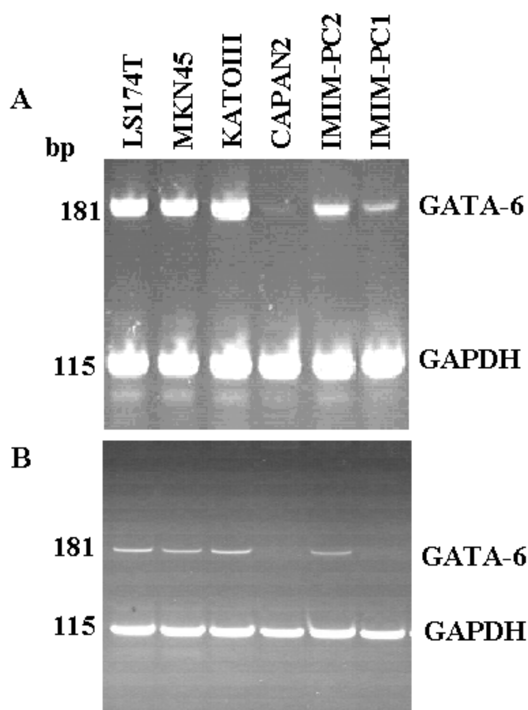


Fig. 4: Expression of GATA-6 mRNA in gastrointestinal cell lines. Ethidium-bromide staining after gel-electrophoresis of multiplex RT-PCR exhibits transcription products of GATA-6, GAPDH (internal control). Tumour cell lines are from gastric origin (MKN45, KATOIII), intestinal (LS174T) and pancreatic origin (Capan-2, IMIM-PC1, IMIM-PC2). **(A)** Twenty five cycles of PCR amplification; **(B)** 22 cycles of PCR amplification. Using GAPDH primer together with primers hG-6F and hG-6R (table 3).

3.1.3. Activation of *TFF*-reporter constructs by GATA-6

To investigate the endogenous expression of *TFF* genes in cell lines, we determined the ratio of *TFF* to *GAPDH* mRNA levels after multiplex RT-PCR (Fig.5). *TFF2* is expressed predominantly by KATOIII, whereas *TFF1* is predominantly expressed by cell lines MKN45 and LS174T. The latter cell line also expressed high amounts of *TFF3*. However, after transfection of GATA-6 expression vectors, no stimulation of endogenous *TFF* transcription was observed, presumably due to the low transient transfection efficiency (less than 2 %). Thus, to analyse only the fraction of transfected cells, we used TFF reporter genes and performed cotransfection experiments. A GATA-6 vector deleted in the zinc finger domain (ΔZF) served as a control to normalise for transfection differences due to cell lines and reporter constructs. Strikingly, cotransfection of TFF-luciferase constructs with GATA expression vectors stimulated transcription of *TFF1* and *TFF2* several fold (Fig. 6). A four to six fold enhancement of *TFF1* and *TFF2* transcription was observed in all three cell lines. In contrast, *TFF3* transcription was not enhanced in the colon cell line LS174-T and KATOIII and only slightly in MKN45. Similar extend of *TFF1* and *TFF2* activation was obtained with another human GATA-6 expression vector that was previously used to activate the H^+/K^+-ATPase (Nishi et al., 1997). Surprisingly, GATA-6 cotransfection influenced neither *TFF1* nor *TFF2* reporter expression in any of the three pancreatic cell lines that we have tested (Capan-2, IMIM-PC1, IMIM-PC2) (data not shown). These results strongly suggested that GATA-6 is a transcriptional activator of *TFF1* and *TFF2* but not of *TFF3* in gastric and intestinal cell lines.

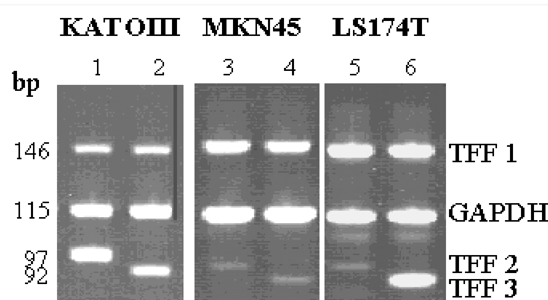


Fig. 5: Endogenous expression of *TFF* genes in three cell lines. Ethidium-bromide staining after gel-electrophoresis of multiplex RT-PCR (25 cycles) exhibits transcription products of TFF1, GAPDH, and TFF2 (odd numbered lanes), versus TFF1, GAPDH and TFF3 (even numbered lanes).

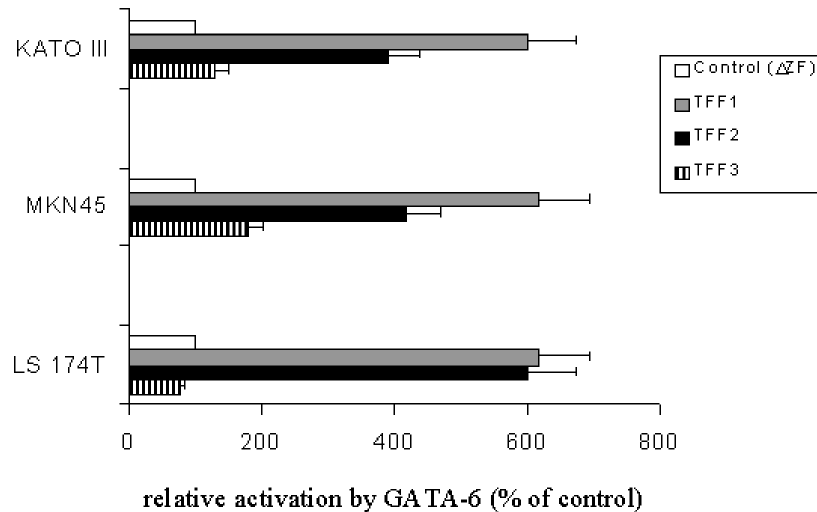


Fig. 6: Activation of *TFF*-reporter gene constructs by GATA-6. For each *TFF*-reporter construct, transient cotransfection together with a *GATA-6* expression vector or cotransfection of a mutated GATA-6 vector (ΔZF; control) was performed, respectively. Transcriptional activation by *GATA-6* is expressed in % of the control.

3.1.4. Characterisation of functional GATA-6 motifs in the promoter of *TFF2*

Having demonstrated activation of *TFF1* and *TFF2* by GATA-6, we wanted to characterise in more detail the corresponding functional motifs in the promoter of *TFF2* displaying three putative GATA binding sites in reverse orientation. Thus, internal deletions in the promoter of the TFF2 reporter plasmid were constructed, and these derivatives were used in transient GATA-6 cotransfection experiments (Fig. 7). Using the gastric cell line KATOIII, these experiments indicated that the activation by GATA-6 is mainly associated with the presence of the two GATA-6 binding sites located in tandem at positions –400 and –322, since a deletion of this region resulted in a decrease of activation from 380 % to 155 % (Fig. 7, T2DG6). In addition, a minor part of the activation by GATA-6 was associated with the presence of the conserved binding site at position –62. Using these reporter constructs, similar activation profiles were obtained also in the other gastric cell line MKN45 (data not shown). Taken together these experiments indicated that all three GATA motifs are functionally relevant in terms of *TFF* gene activation by the transcription factor GATA-6.

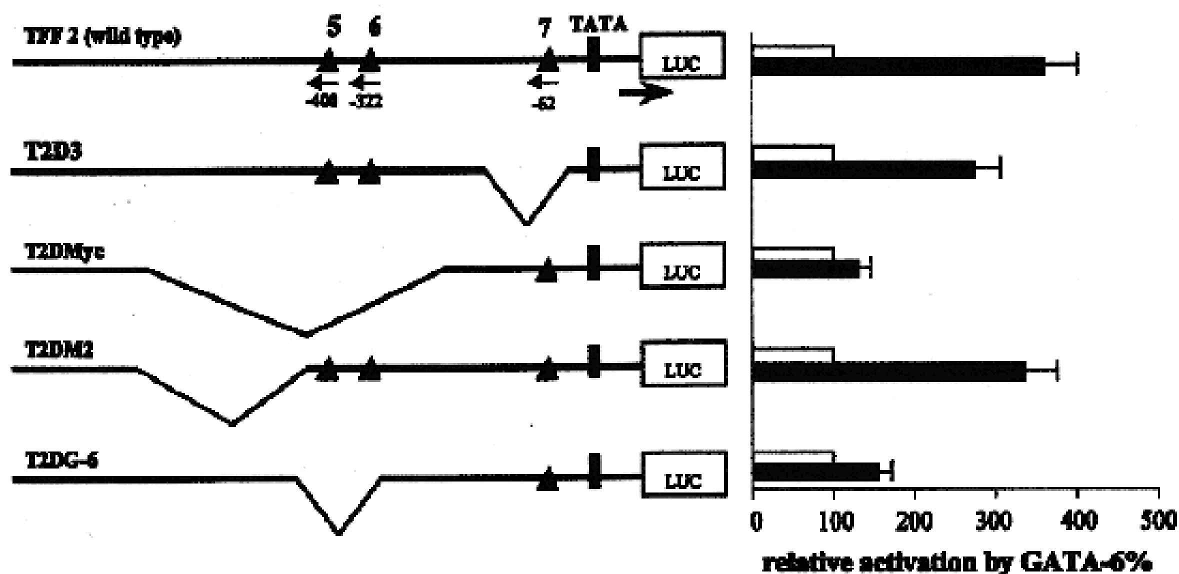


Fig. 7: Functional GATA motifs in the 5'-flanking region of *TFF2*. Deletion in the 5'-flanking region of T2D3 (192 bp), T2DMyc (554 bp), T2DM2 (383 bp) and T2DG6 (104 bp) are as indicated in scale. Activation by GATA-6 is displayed in % in relation to the control (open bars) in cotransfection experiments (see Fig. 6).

To further demonstrate the binding of GATA-6 to the motifs in *TFF2,* nuclear protein extracts were isolated from cell lines and analysed by electrophoretic mobility shift assays (EMSA) using double stranded oligos corresponding to one of the functional GATA sites in *TFF2* termed G6 (Fig. 1, no. 5; AGATAG). The appearance of a shifted band indicated binding of a nuclear protein from KATOIII (Fig. 8, lane 4). The specificity of this band was proved by competition experiments using a 100-fold excess of unlabeled G6 oligo (lane 1). No competition was found using an oligo of unrelated sequence termed P (lane 2). An oligo with a 2 bp change in the core region of the G6 oligo (GATGC instead of GATAG) did not efficiently compete (lane 3). Similar band shifts were found with nuclear extract of MKN45 and LS174T (data not shown). These binding experiments together with decreased transcriptional activation of mutated biding sites (Fig. 7) and the expression of GATA-6 in the gastrointestinal cell lines (Fig. 4) strongly suggested binding of GATA-6 motifs in the 5'-upstream sequence of *TFF2*.

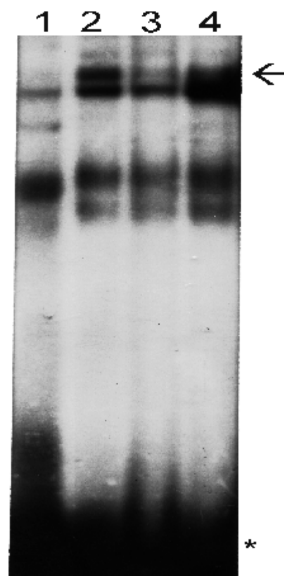
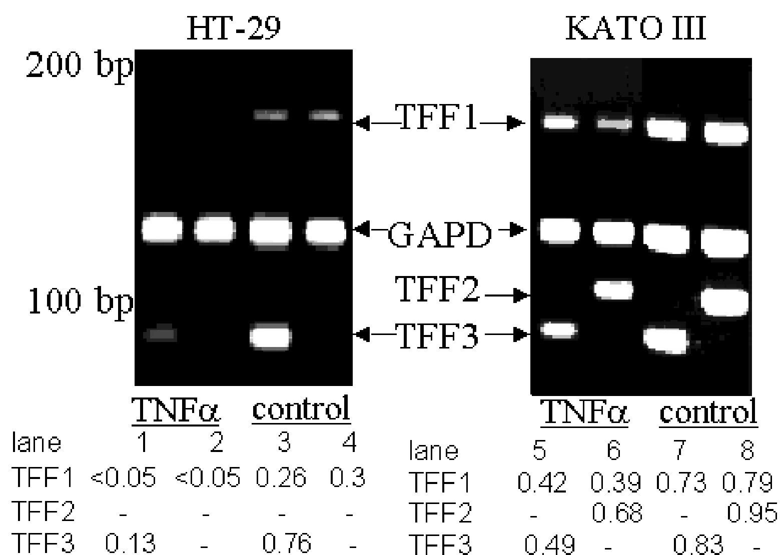


Fig. 8: Binding of KatoIII nuclear extracts to the GATA motif in TFF2. EMSA with ^{32}P-double stranded oligo G6 (sequence at pos. –400 in *TFF2*) using nuclear extract of KATOIII (lane 4), and competition with a100-fold excess of unlabelled oligos G6 (lane 1), P (PEA-3) (lane 2) and GM (lane 3), respectively. Arrow indicate the position of the specific shifted band, the asterisk indicate the free probe.

3.2. TNF-α and NF-kappaB inhibit the transcription of human *TFF* genes encoding gastrointestinal healing peptides

3.2.1. TNFα down-regulates *TFF* reporter gene and endogenous *TFF* gene expression

Trefoil peptides encoded by *TFF* genes have been demonstrated to be upregulated during repair and healing. Thus, it was important to investigate whether *TFF* gene expression would be modulated by the proinflammatory cytokine TNF-α. RNA from the colon cell line HT-29 and the gastric cell line KATOIII was analysed by semi-quantitative multiplex PCR (Fig. 9A). Compared to GAPDH mRNA expression TNF-α evoked down-regulation of *TFF1* (17 to 19 %) and *TFF3* (17 %) in HT-29 cell lines (lanes 1 and 3). This cell line does not express endogenous *TFF2* (lanes 2 and 4). In the gastric cell line KATOIII TNF-α evoked down-regulation of *TFF1* (49 to 57 %, lanes 5-8), *TFF2* (71 %, lanes 6 and 8) and *TFF3* (59 %, lanes 5 and 7).



lane	1	2	3	4
TFF1	<0.05	<0.05	0.26	0.3
TFF2	-	-	-	-
TFF3	0.13	-	0.76	-

lane	5	6	7	8
TFF1	0.42	0.39	0.73	0.79
TFF2	-	0.68	-	0.95
TFF3	0.49	-	0.83	-

Fig. 9: Down-regulation of endogenous *TFF* genes and reporter genes by TNF-α

A: Endogenous expression of *TFF* genes stimulated with TNF-α for 48 h as determined by multiplex RT-PCR, electrophoresis and ethidium bromide staining. Odd lanes display *TFF1*, *GAPDH* and *TFF3*, even lanes display *TFF1*, *GAPDH* and *TFF2* multiplex amplification. Densitometric analysis of band intensities are calculated and displayed as *TFF* to *GAPDH* ratios.

To analyse this down-regulation further we used luciferase reporter genes driven by the 5'-flanking regions of the corresponding *TFF* genes. These constructs were previously shown to resemble endogenous *TFF* gene expression in a variety of gastrointestinal cell lines (Beck et al., 1998). Compared to the un-stimulated control, TNF-α also evoked down-regulation of all three *TFF* reporter genes in HT-29 and KATO III (Fig. 9B). The most severe effect was a down-regulation of *TFF3* to 11.5 % in HT-29 (24.7 % in KATOIII). In other gastrointestinal cell lines (MKN45, LS174T, HUTU80, and DAN-G) *TFF* reporter genes were also down-regulated by TNF-α (data not shown). To verify that TNF-α signalling cascade is active and mediates a signal to transcriptional activator nuclear factor kappa B (NF-κB), I used a NF-κB-responding luciferase vector (6x NF-κB-luc; Fig. 9C). As was expected, TNF-α stimulated transcription up to 11-fold, indicating a functional NF-κB signalling pathway in HT-29 and KATOIII.

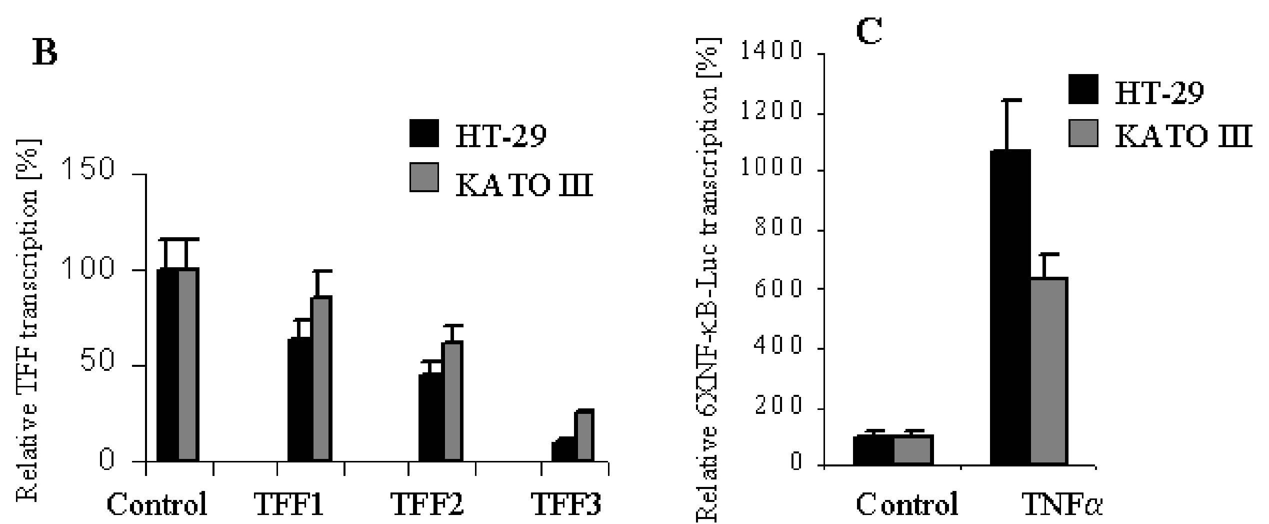


Fig. 9: B: *TFF* reporter gene activities 48 h after TNF-α 20 ng/ml) in the intestinal cell line HT-29 and gastric cell line KATO III expressed in % of the non-stimulated control. Two independent experiments were carried out in triplicate and firefly luciferase activities were normalised by renilla luciferase. C: Stimulation of a NF-κB responding reporter gene (6X NF-κB-Luc) by TNF-α

These results prompted me to focus on this transcription factor, also because NF-κB is a key player in signalling inflammatory processes and governing the immune response. In fact, a computer search for putative NF-κB binding sites revealed a couple of motifs in the 5'-flanking region of all three human *TFF* genes (Fig. 10)

sharing 80 to 90 % identity to the consensus NF-κB binding site GGGR(A/T/C)TYYCC.

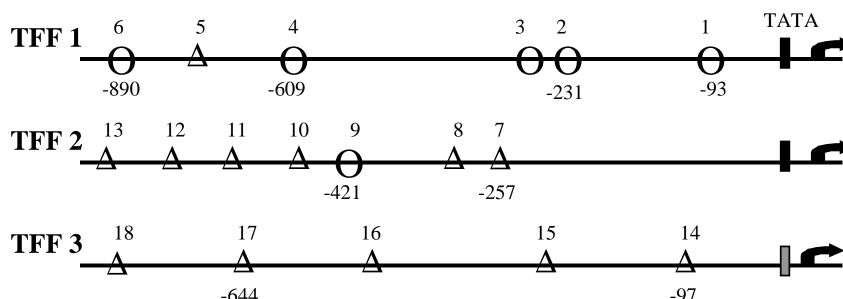


Fig. 10: Putative NF-κB binding sites in the proximal 5'-flanking region of the three *TFF* genes. The sites are displaying 90 % (O), and 80 % (Δ) identity to the consensus NF-κB binding site (GGGR(A/T/C)TYYCC). The sequence of putative binding site the NF-κB by TFF1: 1:GGGGATCC*T*C; 2: GGGACTTTC*T*; 3: GGGA*G*TCTCC; 4: GGGGTTTC*G*C; 5: *A*G*T*GATTCTC; 6: GGGGCTCCC*T* . By TFF2 : 7: *CC*GGTTTTCC; 8: GGG*T*CAC*A*CC; 9: GGGACTTT*T*C; 10: GGGGCT*G*C*T*C; 11: GGGAAT*G*C*T*C; 12: GGGAA*C*TTC*G*; 13: GGGG*G*TT*G*CC. By TFF3: 14: *AA*GGCTCTCC; 15: G*C*GGATTCC*A*; 16: GGGGT*G*TTC*T*; 17: G*A*G*C*CTTTCC; 18: GGG*T*CTT*G*CT. All underlined letters are not identical to consensus NF-κB binding site.

3.2.2. NF-κB (p50/65) directly affects *TFF* gene expression

Experiments in Fig. 9B and C suggested a functional NF-κB signalling pathway. Therefore, I directly tested the effect of NF-κB by cotransfecting expression vectors encoding the NF-κB subunits p50 and p65. Reporter gene activities of all three *TFF* genes were considerably reduced by p50, p65 and the combination of both (p50/65) in HT-29 (Fig. 11A). A similar result was obtained in KATOIII, with the exception of p50 that only slightly affected expression of *TFF*2 and *TFF*3 (Fig. 11B). If NF-κB is responsible for TFF down-regulation, activation of endogenous NF-κB through TNF-α together with cotransfecting p50/65 should further down-regulate *TFF* reporter genes. This idea was proved in KATOIII cells with *TFF*2 (Fig. 11C). Indeed, TNF-α together with NF-κB cotransfection was able to reduce *TFF2* expression more efficiently.

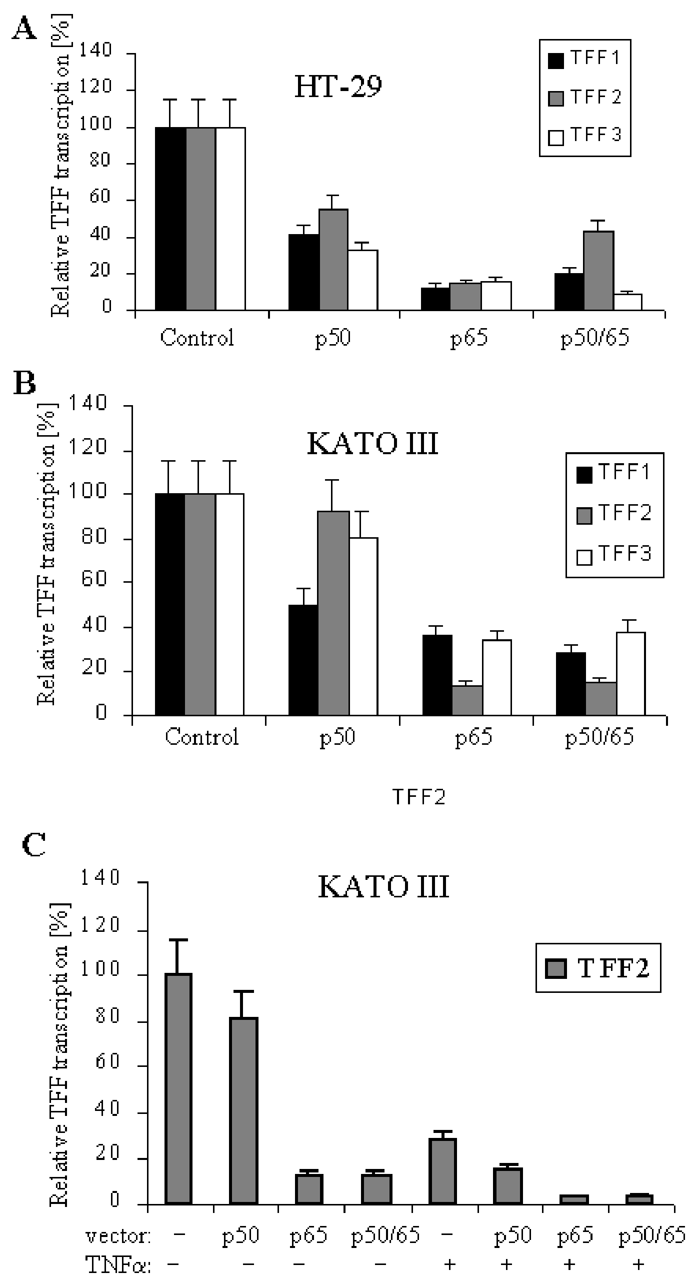


Fig. 11: The effect of NF-κB on the expression of *TFF* reporter constructs in HT-29 **A:** and KATO III **B:** by cotransfection of p50, p65 and p50/65 expression vectors. Transcriptional down-regulation by NF-κB is expressed in % of the control (cotransfection with pUHD). **C:** Effect of TNF-α in combination with p50, p65 and p50/65 cotransfection of *TFF2* in KATO III.

3.2.3. Constitutive repressive IκB neutralises NF-κB-mediated down-regulation of *TFF3*

Having established *TFF* down-regulation by TNF-α and NF-κB, I used the constitutive repressive inhibitor of kappa B (IκB*) also called super-repressor (Henkel et al., 1992) that is able to bind and inactivate NF-κB. After cotransfection of an IκB* expression vector together with p50, p65 and p50/65 we actually found that the down-regulating effect on *TFF3* was neutralised (Fig. 12A). Similar results were obtained with *TFF1* and *TFF2* (data not shown). In a separate experiment we verified the inhibition of the NF-κB function through the IκB vector in HT-29 (Fig. 12B). Stimulation of a NF-κB responding reporter gene by TNF-α was abrogated in the presence of IκB, strongly indicating a functional NF-κB signalling pathway through TNF-α and IκB in this cell line.

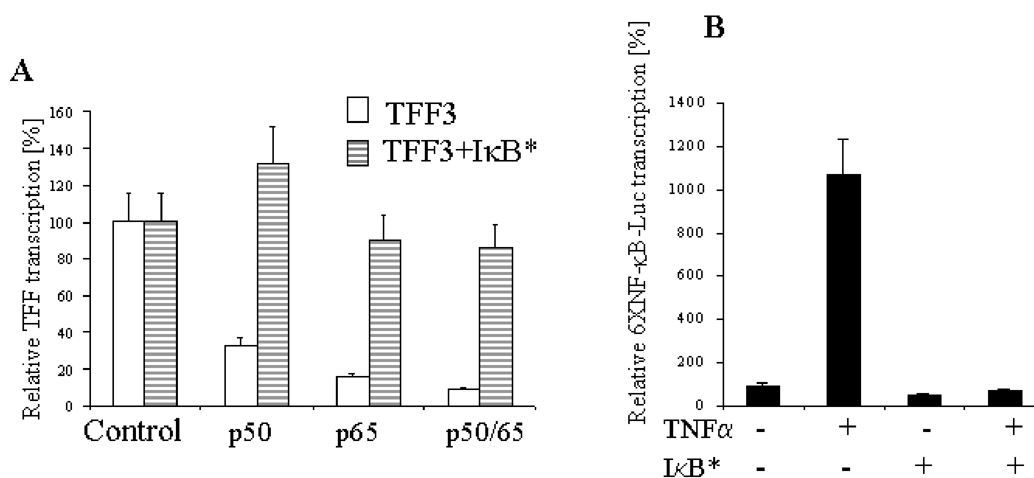


Fig. 12: IκB* stopped NF-κB-mediated down-regulation of *TFF3*.in HT-29 **A:** Transient cotransfection of *TFF3* reporter gene with a p50, p65 and p50/65 and together with or without expression plasmid encoding a constitutive repressive IκB (IκB*) in HT-29. **B:** To test the efficacy of IκB, 6X NF-κB-luc reporter was transiently cotransfected with or without IκB* in combination with stimulation by TNF-α to induce the NF-κB pathway.

3.2.4. Functional analysis of NF-κB binding sites in *TFF* genes

Because NF-κB seemed to act on the 5'-flanking regions of *TFF* genes, we deleted 26 bp (pos. -93) in the *TFF1* promoter covering a putative NF-κB binding site that was close to the TATA box (Fig. 10 and 13). This deletion is located very close to the functional HNF-3 binding site (Beck et al., 1999). After cotransfection of p50, activity of the wild type *TFF1* promoter was reduced to 40 % whereas activity of the mutant *TFF1* promoter remained, suggesting that the motif at position –93 in *TFF1* respond to interaction with p50 (Fig. 13). However, there was no significant difference in response to p65 and p50/65 regarding wild type and mutant promoter.

To demonstrate the functionality of another putative NF-κB binding site we chose the motif at pos. –421 in *TFF2*, since it is 90 % identical to the NF-κB consensus site. After cotransfection of p65, activity of the wild type *TFF2* promoter was reduced to 15 % whereas activity of the mutant *TFF2* promoter remained 100 % (Fig.13). In addition, p50/65 cotransfection also revealed a considerable difference between wild type and mutated construct, suggesting that this site in *TFF2* is functionally important for NF-κB-mediated gene silencing.

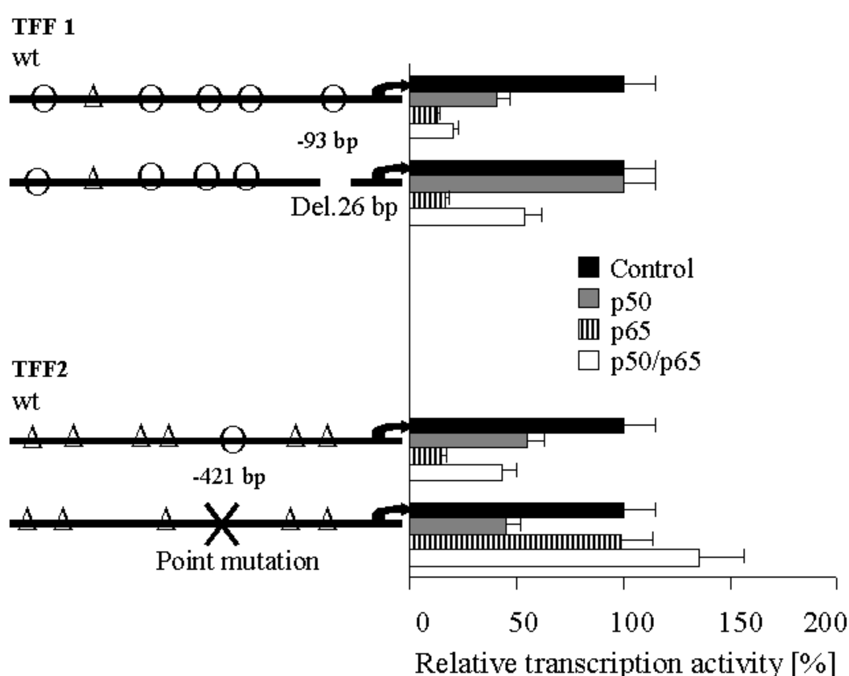


Fig. 13: Functional test of putative NF-κB motifs in the 5`-flanking region of *TFF1* and *TFF2*. Deletion of 26 bp at the position -93 of *TFF1*, and point mutation at position -421 of *TFF2*. The effect of NF-κB is displayed in % in relation to the control (see Fig. 11).

3.2.5. Binding of NF-κB to elements in the promoter of *TFF2* and *TFF3*

Since the –421 NF-κB motif is functionally involved in mediating *TFF2* down-regulation we determined specific binding of NF-κB to this motif. Nuclear protein extracts were prepared from HT-29 and analysed by EMSA using double-stranded 26 bp oligos covering the putative binding site (Fig. 14). A specific band shift of the motif in *TFF2* (lane 1) was detected that was absent by addition of unlabelled oligo (lane 2), but not by addition of a non-specific oligo (lane 3). Moreover, disappearance of the specific band occurred by an antibody against p65 (lane 6) but not after adding an antibody against transcription factor USF (lane 5), suggesting binding of NF-κB to this motif. Among the four putative binding motifs that exhibited only 80 % identity to the consensus NF-κB site, we focussed on two motifs proximal to the promoter (pos. -97 and –644). The first putative element in *TFF3* (pos. –97) exhibited weak specific binding while the other motif in *TFF3* (pos. –644) showed no specific binding at all (data not shown).

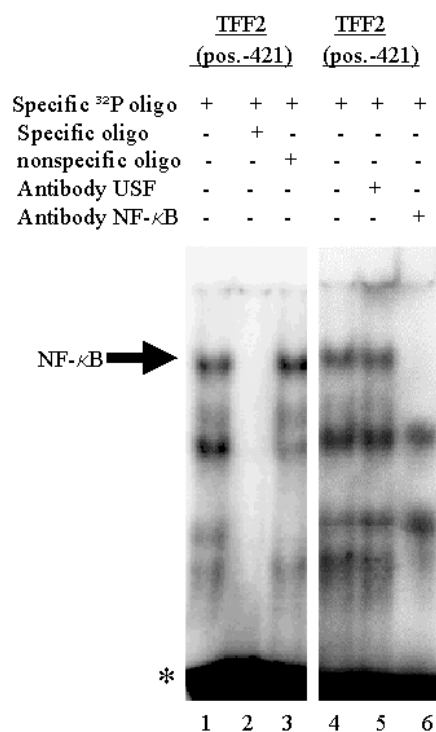


Fig. 14: DNA binding activity of HT-29 nuclear extract to putative NF-κB motifs in *TFF2*. Electromobility shift assays using ^{32}P-labelled double-strand oligos derived from the position -421 in *TFF2* (lane 1-6). Competition experiments were carried out by a 100-fold excess of the corresponding unlabeled oligos (lane 2) and with a non specific oligo (lane 3). In lane 5 and 6 the extract was incubated with anti-USF and anti-p65 antibodies, respectively. Arrow indicates the position of the NF-κB complex, the asterisk indicates the free probe.

3.3. Regulation of the gastrointestinal healing peptide *TFF2* gene promoter by USF but not by c-Myc

3.3.1. Activation of *TFF* reporter genes by USF

Sequence analysis of the three human *TFF* genes revealed a single E box in each promoter (Fig. 15). The E box of the *TFF2* promoter corresponds to the consensus 5'-CACGTG sequence whereas the E boxes of the other two *TFF* genes fit to an extended consensus sequence. All three of then have suboptimal flanking sequences (for review see (Luscher and Larsson, 1999).

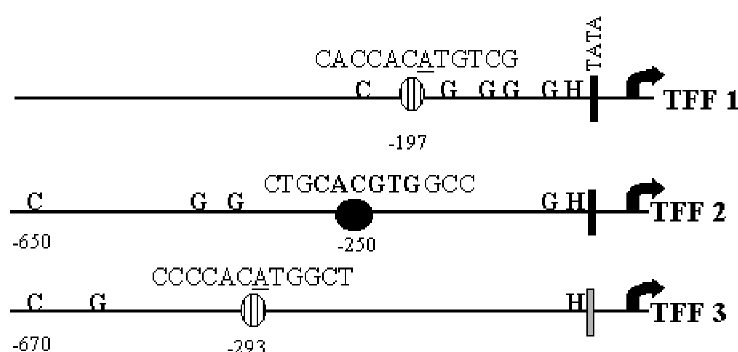


Fig. 15: Schematic representation of the promoters of the three *TFF* genes. The locations of E box elements are indicated and the sequence is shown. In addition binding sites for C/EBP factors (C), HNF-3 (H), and GATA-6 (G) are indicated. The grey box indicate a degenerated TATA box (CATAAA).

We analysed whether c-Myc or USF could activate a *TFF2* reporter gene constructs in gastrointestinal cell lines. In MKN45 cells and to a lesser extend in LS174T cells a strong activation by USF was observed whereas c-Myc showed little activity (Fig. 16A and B). In both cell lines USF1 activated less efficiently than USF2 while coexpression of both USF proteins resulted in higher activation as either factor alone.

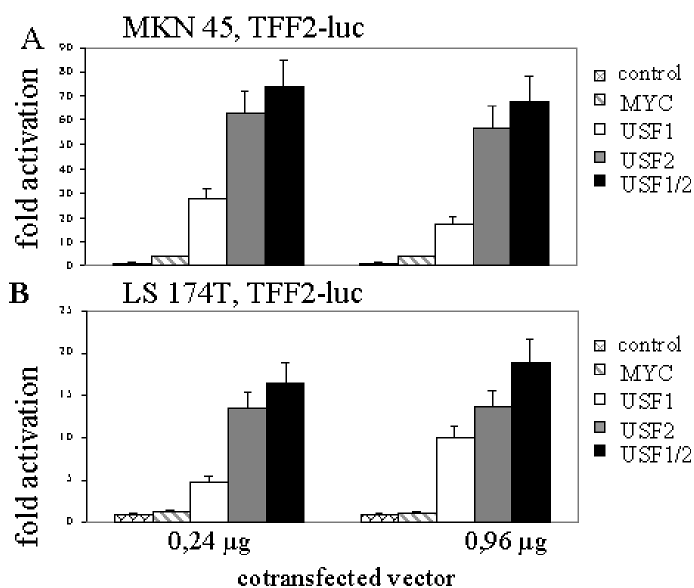


Fig. 16: USF factors activate a *TFF2*-luc reporter gene construct.

A. The gastrointestinal cell line MKN45 was cotransfected with the *TFF2*-luc reporter gene construct and different amounts of expression plasmids encoding USF1, USF2, USF1/USF2, or c-Myc as indicated. A typical experiment performed in triplicate is shown.

B. As in A but the cell line LS174T was used

Similar findings were made in several other gastrointestinal cell lines mentioned in methods (Fig. 16C).

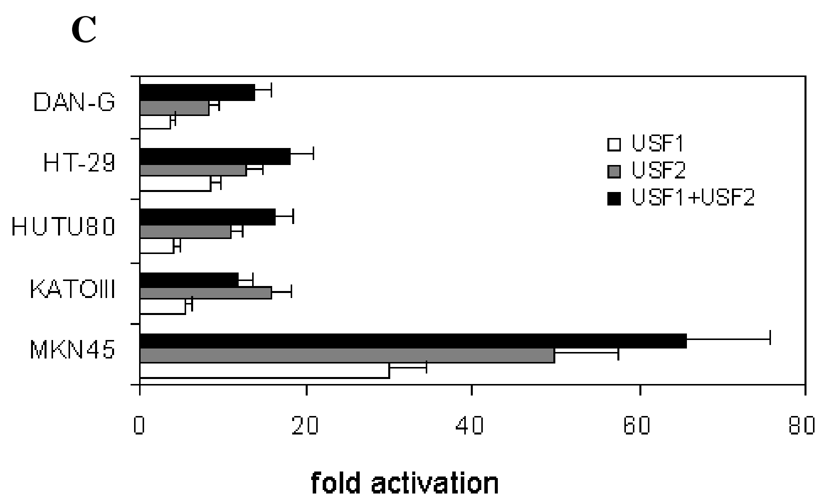


Fig. 16: C. Activation of the *TFF2* reporter gene by ectopically expressed USF1 and USF2 in different cell lines

To determine wether the difference between the activation of TFF2 by USF and by c-Myc was promoter specific or cell line dependent, we measured the activity of these factors on M4-mintk-luc, a reporter gene with 4 high affinity Myc/Max binding sites (equivalent to the CMD oligonucleotide, see below). In MKN45 cells activation by USF and c-Myc was comparable while in LS174T cells USF was more active than c-Myc (Fig. 16 D and E). This suggested that c-Myc is capable of activating transcription in these cells to an extend that is comparable to other cell systems (Vervoorts and Luscher, 1999); (Sommer et al., 2000) and that strong activation by USF appears specific for the *TFF2* promoter.

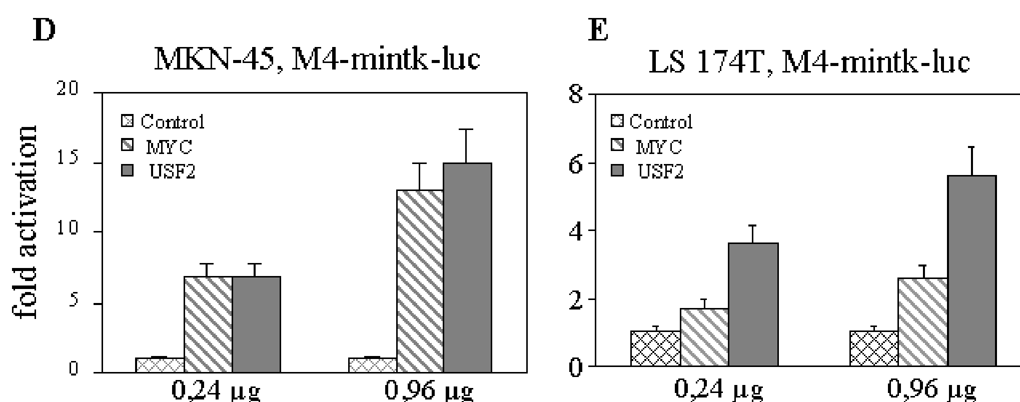


Fig. 16 D,E: Activation of high affinity Myc/Max-binding element by c-Myc and USF. As in A but the M4-mintk-luc reporter gene construct with 4 high affinity Myc/Max binding sites was used. E. As in B but with the M4-mintk-luc reporter gene.

The E box element is important for activation by USF since a *TFF2* reporter gene construct with a mutated E box was poorly activated (Fig. 16F). Furthermore a mutant of USF1 that can not bind DNA did not activate the *TFF2* reporter (Fig. 16F). These findings support the notion that transcriptional activation of this reporter gene by USF is E box dependent.

In addition to *TFF2* we tested whether the two other *TFF* promoters were responsive to USF and Myc/Max/Mad proteins. Moderate activation of the *TFF3* reporter gene was observed in three different cell lines (MKN45, LS174T, and HT-29) with USF1 while the *TFF1* reporter gene was not activated (Fig. 17).

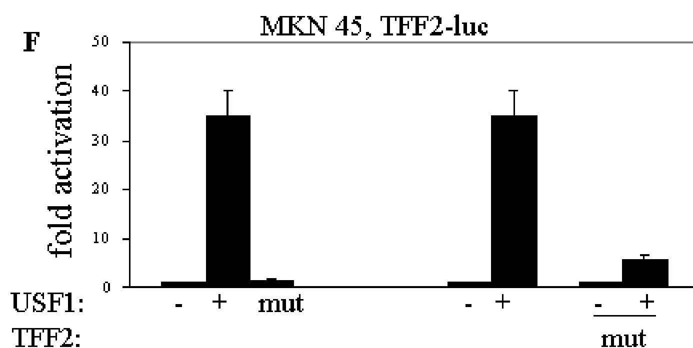


Fig. 16: F. Mutation in either USF1 or the E box of *TFF2* abrogate transcriptional activation. MKN45 cells were cotransfected with the *TFF2*-luc or mE-*TFF2*-luc, a reporter gene with a mutated E box, and with expression plasmids encoding USF1 or USF1mutbr, a mutant USF protein that can not bind DNA, as indicated.

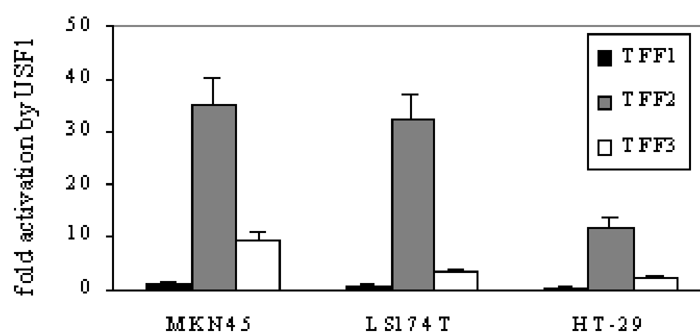


Fig. 17: USF1 predominately activates TFF2: Effects of USF1 on *TFF1*-luc, *TFF2*-luc and *TFF3*-luc. MKN45, LS174T, and HT29 cells were cotransfected with the indicated *TFF* reporter gene constructs in the presence or absence of USF1. The fold induction by USF1 is shown.

The c-Myc was unable to activate the *TFF1* or *TFF2* reporter genes (Table 6). Furthermore other components of the Myc/Max/Mad network, including Max and Mad1, did not affect any of the *TFF* reporter genes (Table 6). Together these findings identify USF proteins as potential activators of the *TFF2* gene, possibly of *TFF3* but not of *TFF1*. None of these genes appears to be regulated by the Myc/Max/Mad network.

Table 6: Influence of cotransfection by Myc, Max and Mad expression vectors on *TFF* reporter gene activity . Ratio of specific reporter gene activities of expression vector /control vector (=cotransfection by vector pUHD); n.d. = not determined. SE was $\leq$ 15 % in triplicate experiments.

	TFF 1		**TFF 2**		**TFF 3**	
	MKN45	**LS174T**	**MKN45**	**LS174T**	**MKN45**	**LS174T**
c-Myc	0.59	0.79	1.56	1.00	0.83	0.87
Max	1.08	n.d.	0.90	0.57	0.51	0.67
Mad1	1.65	0.59	1.29	0.93	n.d.	n.d.
c-Myc + Max	0.45	n.d.	1.38	1.00	0.56	0.95
control	1.00	1.00	1.00	1.00	1.00	1.00

3.3.2. Distinct reporter gene activation is not due to differences in DNA binding

The difference in regulation of the three *TFF* reporters by USF and Myc/Max/Mad network members might be the result of distinct binding to the respective E boxes. Therefore we compared the binding of USF1/USF1, c-Myc/Max, and Max/Max complexes to the three E boxes of the *TFF* genes. COS-7 lysates expressing the relevant proteins were generated and used in electrophoretic mobility shift assays with CMD, a high affinity Myc/Max binding site, as probe. The binding was then competed with unlabeled CMD or oligonucleotides encompassing the respective *TFF* E boxes (Fig. 18). These competition experiments revealed comparable binding affinities of USF to the three *TFF*-E box sequences (Fig. 18A). However binding to CMD was slightly stronger suggesting that DNA binding is unlikely to be the reason for the differences in *TFF* reporter gene and M4-mintk-luc activation.

Both c-Myc/Max and Max/Max complexes bound less efficient to any of the three *TFF*-derived E box elements than CMD (Fig. 18B). Thus weak binding of these complexes might explain in part the inability of c-Myc to regulate the *TFF* reporter genes.

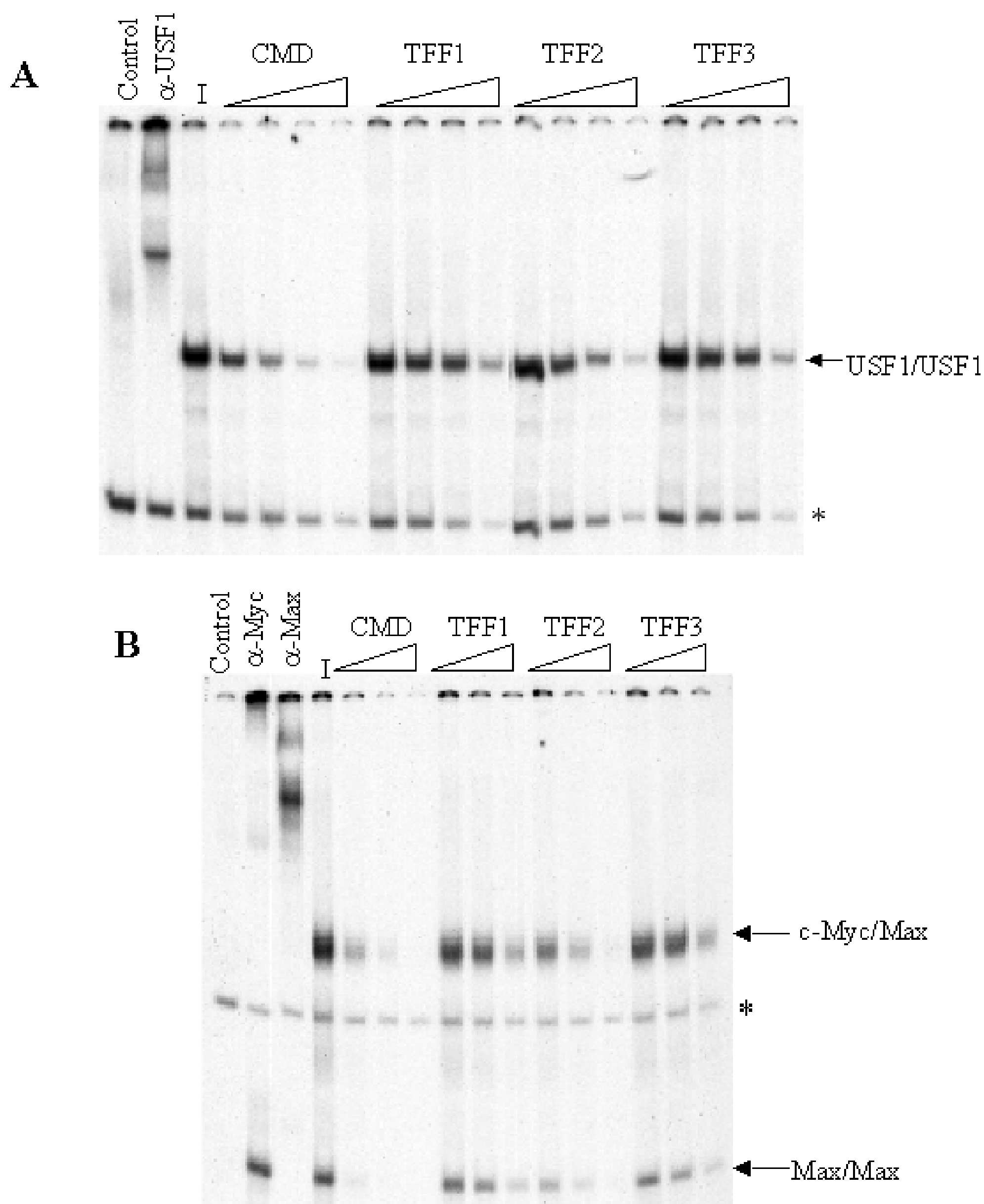


Fig. 18: Binding of USF, c-Myc/Max, and Max/Max complexes to *TFF*-derived E box sequences in vitro. COS7 cells were transiently transfected with plasmids encoding USF1 or c-Myc/Max. Whole cell lysates were used in EMSA experiments with radiolabeled CMD as probe. **A.** Binding of USF1/USF1 homodimers to CMD was competed with a 3-, 10-, 30-, or 100-fold excess of unlabeled CMD, or oligos spanning the E boxes of either *TFF1*, *TFF2*, or *TFF3* as indicated. Supershift experiments were performed with an antibody specific for USF1 (α-USF1). In the control lane whole cell extract of mock transfected cells was used. * indicates a non-specific complex. **B.** Binding of c-Myc/Max and Max/Max complexes were analysed as in A. Competition experiments were performed with a 3-, 10-, or 30-fold excess of unlabeled oligonucleotides. Supershift experiments were done with specific antibodies to c-Myc or Max (α-Myc or α-Max, respectively). The control is as in A. * indicates a non-specific complex. (The EMSA results were kindly provided by J. Vervoorts, Inst. of Molecular Biology, Medical University of Hannover.)

3.3.3. The *TFF2* promoter is occupied and regulated by USF but not by c-Myc *in vivo*

The data described above identify the *TFF2* gene as a USF target in transient transfection experiments. To determine whether endogenous USF factors are associated with the chromosomal *TFF2* promoter, we performed chromatin immunoprecipitation (ChIP) experiments using formaldehyde crosslinking in MKN45 and HT-29 cells. In both lines activation of the *TFF2* reporter gene by USF was observed (Fig. 16 and 17). Immunoprecipitations were performed using antibodies specific to USF1, USF2, c-Myc, and c-Myb, that have been shown to work in ChIP with appropriate target genes (OD and BL, unpublished observation). We observed a strong signal for both USF1 and USF2 on the *TFF2* promoter in the two cell lines analysed (Fig. 19A). The USF1- and USF2-specific signals obtained from the *TFF1* and *TFF3* promoters were considerably weaker (Fig. 19A). Rough quantification was obtained by comparing the signals from the ChIP with a dilution series of sonicated total input DNA. USF1 binding to TFF2 was about 8-fold higher than binding to TFF1 and TFF3 whereas USF2 binding was about 6 to 10-fold stronger (Fig. 19B). No binding of USF factors was detectable to the locus control region of the ADA gene which does not contain an E box element (Cerni et al., 1995)(Fig.19A). The binding of c-Myc to either of the three *TFF* promoters was significantly lower than USF binding and was only marginally above the non-specific c-Myb control (Fig. 19B). This defines specific *in vivo* interaction of USF factors, but not c-Myc, with the *TFF2* promoter and, to a considerably lower degree, with the *TFF1* and *TFF3* promoters.

The findings that USF factors can activate a *TFF2* reporter gene and bind to the *TFF2* promoter in vivo suggests that USF proteins are involved in *TFF2* regulation in vivo. Therefore we transiently expressed USF1 and USF2 in LS174T cells and monitored the expression of the endogenous *TFF2* by multiplex RT-PCR. USF1/USF2 were sufficient to activate the chromosomal *TFF2* gene while no effect on either *TFF1* or *TFF3* was observed (Fig. 20). Together our findings identify USF as a critical and specific modulator of *TFF2* transcription.

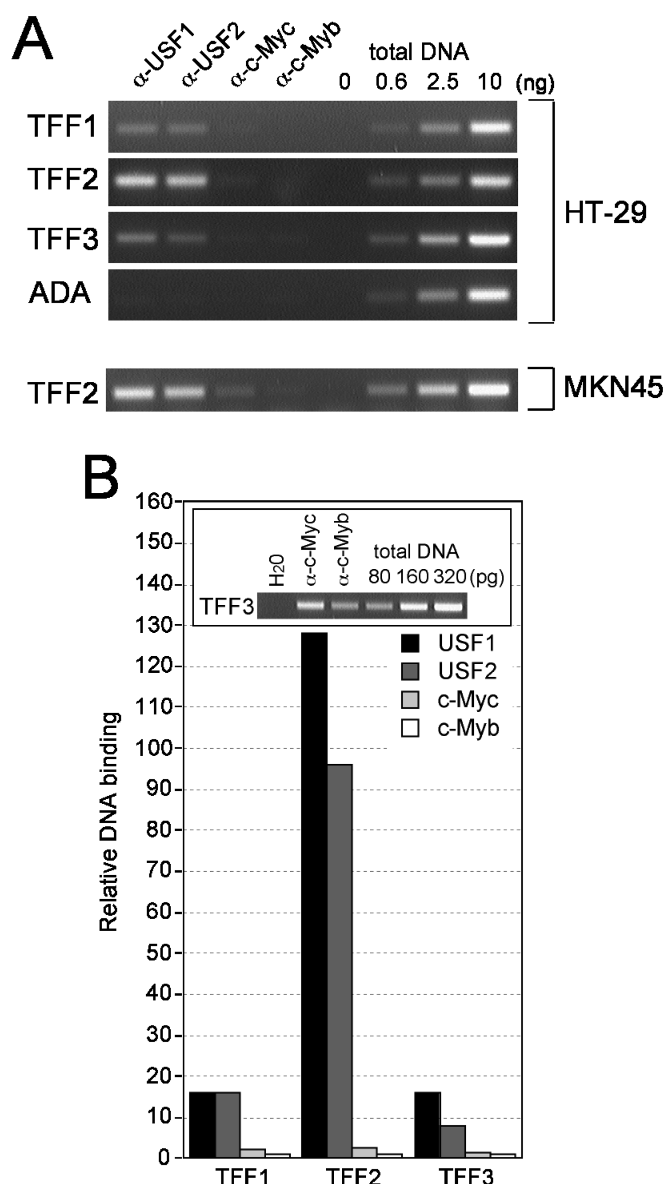


Fig. 19: Binding of USF to the *TFF2* promoter in vivo.

A. ChIP analyses were performed on lysates of formaldehyde-crosslinked HT-29 and MKN45 cells. 1/20 of the purified DNA from the IP reactions with antibodies specific for USF1, USF2, c-Myc, or c-Myb as indicated was subjected to a 34 cycle PCR analysis. Four-fold dilution series of purified input DNA served as PCR templates for normalisation.

B. Quantification of HT-29 ChIP reactions as shown in A. therefore the product intensities of samples and adequate 2-fold dilution series of purified input DNA were compared. The graph shows the mean value of two independent determinations. For samples with little DNA the dilution series of purified input DNA was adjusted so that the amount of bound DNA could be estimated. As an example a PCR analysis with 39 cycles performed in doublicate is shown (insert). Relative DNA binding defines the amount of PCR product obtained from immunoprecipitates compared to total input DNA.

(The ChIP results were kindly provided by O. Dittrich, Inst. of Molecular Biology, Medical University of Hannover)

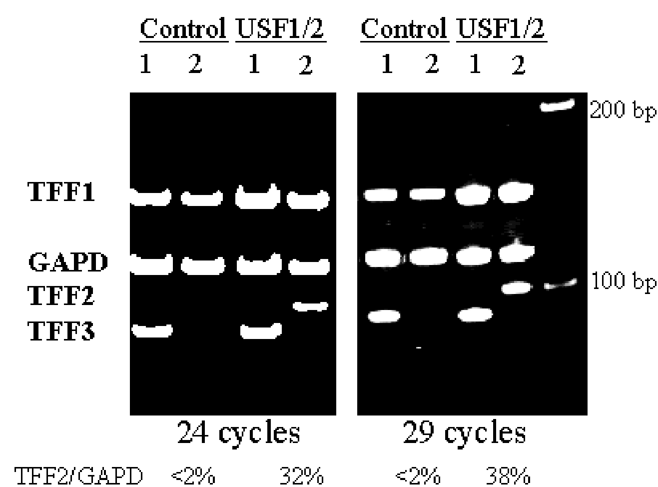


Fig. 20: Exogenous USF stimulates the expression of the chromosomal copy of *TFF2*.
LS174T cells were transiently transfected with empty vector or with plasmids encoding USF1 and USF2 as indicated. Endogenous *TFF* mRNA expression was analysed by multiplex RT-PCR. Lanes 1: TFF1-, GAPDH-, TFF3-PCR reactions; lanes 2: TFF1-, GAPDH-, TFF2-PCR reactions.

Finally, the nuclear extracts were prepared from KATOIII, HT-29, MKN45, LS174T, CAPAN2, HATA and HUTU 80 cell lines and identical protein amounts were separated by electrophoresis and probed by Western blotting (Fig. 21). These analysis revealed that USF1 and USF2 proteins were present in the nuclei of all cell lines tested, except the lack of USF2 in HUTU-80.

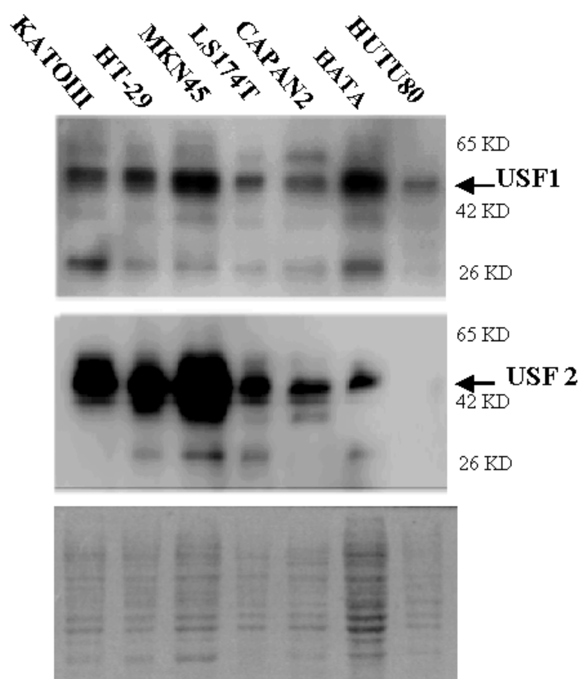


Fig. 21: Expression of USF1 and USF2 in cell lines. Western blotting of equal amounts of nuclear extracts using antibodies against USF1 (upper panel) and USF2 (middle panel). Lower panel: Total amount of nuclear protein transferred to the membrane visualised by Ponceau S staining

4. DISCUSSION

The trefoil factor family (TFF) is a relatively new family of peptides which bear a three-loop trefoil domain. The role of TFF domain peptides has been gradually elucidated since their discovery in 1982. They appear to play a central role in gastric and luminal mucosal defence, in association with luminal mucins, and they are involved in the healing process following ulceration. They are highly conserved during evolution and are heat, acid and enzyme resistant. Their expression and biological action are closely associated with hormonal control, growth factors and oncogenic stimuli. The underlying molecular mechanism of TFF peptide action is still unknown, but their physical properties, and the biological activities of these peptides as motogens may prove to be useful as therapeutic agents in ulcerative conditions, including inflammatory bowel disease where present treatment is far from ideal.

4.1. The effect of GATA-6

The present study provides evidence that the transcription factor GATA-6 activates *TFF1* and *TFF2* coding for gastroprotective peptides predominantly expressed in stomach. GATA-6 exerts no activation of TFF3 that is predominantly expressed in the in testine. The finding of functional GATA sequence motifs in the 5'-flanking region of *TFF1* and *TFF2* in MKN45, KATOIII and LS174T is the second example of activation of a (gastric) gene by transcription factor GATA-6. Previously, GATA-6 has been shown to activate the gene coding for the parietal cell-specific H^+/K^+-ATPase (Tamura et al., 1993; Nishi et al., 1997). Genes encoding gastric pepsingogen, histamine H2 receptor, and histidine decarboxylase genes also contain putative GATA binding sites in their upstream regions. GATA-6 is one of three related genes (including GATA-4 and GATA-5) (Dimaline et al., 1997). Therefore, it seems possible that GATA-4 and GATA-5 may also be relevant for the regulation of the *TFF* genes, which appear to be early in gasrointestinal development (Otto and Patel, 1999).

GATA molecules have a conserved DNA binding domain of 118 amino acid residues, which is also called zinc finger region, since it is composed of tandem zinc fingers containing cycsteine residues, (CX2CX17CX2C)-X-29(CX2CX17CX2C), followed by basic residues (Tsai et al., 1989). GATA DNA-binding proteins conserved in different species are transcriptional regulators which are supposed to be important for development, differentiation of the stomach, intestine and testis, and for cell specific transcription (Maeda et al., 1996; Tsai et al., 1989; Dorfman et al., 1992).

Regulated expression of GATA-6 was reported in gastric endocrine cells (Dimaline et al., 1997), in the human gastric adenocarcinoma cell line MKN45 (Yoshida et al., 1997) and in human colon cancer cell line HT-29 (Gao et al., 1998). Our results obtained with tumour cell lines support these these findings, since both gastric cell lines, MKN45, KatoIII, and the colon cell line LS174T exhibit GATA-6 expression. In gastrointestinal epithelia, GATA-6 is thought to be expressed primarily within the proliferating progenitor population (Gao et al., 1998).

The upregulation of *TFF* genes by the conserved developemental transcription factor HNF-3 has been shown recently (Beck et al., 1999). HNF-3 and GATA-6 are transcription factors governing the development and differentiation of gastrointestinal tract in *C. elegans, D. melanogaster* and mammals (Zaret et al., 1999).

Putative GATA binding motives are also present in the upstream region of *TFF1* and *TFF2* genes in mouse (Genbank acc. no. U78770, O. Lefebvre, personal communication). In addition, in murine *TFF2* one of the GATA consensus sequences (32 base pairs upstream of the TATA box) is also conserved with respect to position and orientation. This GATA consensus sequence overlaps with a putative regulatory motif which we previously termed motif III and is shared among human *TFF1* and *TFF2* (Gött et al., 1996; Beck et al., 1997). Actually, this motif found in *TFF1* and *TFF2* (Fig. 3, numbers 4 and 7) resemble a sequence exhibiting high GATA-6 binding affinity (Sakai et al., 1998). In contrast, the motif found in *TFF3* (Fig. 3, number 8) resembles a sequence of low GATA-6 binding affinity (Sakai et al., 1998).

A closer inspection of the arrangement of the GATA consensus binding sites in *TFF2* revealed that the reverse orientated (with respect to transcription) is also functional (Fig. 6). This new feature of gene activation by GATA-6, suggest that both orientations can activate transcription, since *TFF1* as well as human and rat genes for H^+/K^+-ATPase are activated through a element with forward orientation (Yoshida et al., 1997). In *TFF2,* deletion of either the proximal GATA motif or the two distal motifs resulted in reduction of GATA-6 activation, suggesting that both *cis*-acting motifs function co-operatively in *TFF2*. In *Caenorhabditis elegans* a 36-bp region containing a tandem GATA binding site is critical for directing gut-specific transcription (Egan et al., 1995).

One mechanism to achieve cell-specific transcriptional control would be a crosstalk between GATA-6 and other transcription factors, similar to the oligomer complex of GATA-1 (Sakai et al., 1998). A candidate for such a complex would be the

endoderm-specific transcriptional activator HNF-3 that is able to bind nine nucleotides downstream to the proximal GATA motif and was shown to activate *TFF1* (Beck et al., 1999). Further activating factors might bind to the yet uncharacterised motifs I and II. (Gött et al., 1996; Beck et al., 1998).

The precise reason for the seemingly tissue-specific GATA-6 activation of *TFF* reporter genes in gastric and intestinal cell lines, and the lack of activation in pancreatic cell lines is not known. It may be connected with the fact that *TFF* genes are abundantly expressed in normal pancreas (although *TFF* expression in pancreatic cancer was observed). We hypothesise that GATA-6 may interact with other cell- or tissue-specific factors (present in stomach and intestine but not in other tissues like pancreas) to convey transcriptional activation of TFFs, as has been shown for GATA-1 (Sakai et al., 1998). Along this line may be the earlier observations demonstrating transcriptional stimulation of TFF1 through the phorbol ester TPA in MCF-7 cells, but not in the pancreatic cell line PANC-1 (Beck et al., 1997).

TFF expression is required for normal epithelial restitution and TFF peptides represent healing factor in models of induced lesions and inflammation (Kindon et al., 1995; Alison et al., 1995; Lefebvre et al., 1996; Mashimo et al., 1996; Tran et al., 1999). Thus, in gastrointestinal ulcerative diseases (peptic ulcer, Crohn's disease, ulcerative colitis) the activation of endogenous *TFF* expression may constitute a therapeutic strategy and in this respect the transcriptional activator GATA-6 deserves additional attention. Therapeutic induction of expression *TFFs* may even prevent gastrointestinal cancerogenesis, since mice lacking *TFF1* develop gastric adenocarcinoma. In fact, some 40 % of gastric tumours have lost TFF1 protein expression (Lefebvre et al., 1996). Since mutations of *TFF* genes in tumours have not found so far, it is reasonable to determine if functional loss of the transcriptional activators such as GATA-6 is involved in gastric carcinogenesis.

4.2. The effect of NF-κB

After this study it was interesting to look at another transcription factor which could play a functional role in *TFFs* regulation. Therefore it was shown that TNF-α inhibits the expression of endogenous *TFF* genes as well as reporter genes driven by

approximately 900 bp of the corresponding 5'-flanking region. All three human *TFF* genes were down-regulated in gastric and intestinal cell lines (HT-29 and KATOIII). We further have revealed that this down-regulation is mediated by NF-κB most likely through degradation of IκB, and that some of the multiple putative NF-κB binding sites are functionally important for binding and down-regulation.

TNF-α is 17 -kDa cytokine released by activated macrophages that locally elicits a wide spectrum of metabolic responses and cellular activities (Tracey and Cerami, 1993) and plays an important role during infection and inflammation. Prolonged expression of TNF-α is a signal for chronic inflammation, since TNF-α is up-regulated in inflammatory bowel diseases (IBD) such as Crohn's disease. It is interesting to note that antibodies against TNF-α constitute new promising therapeutic tools for IBD (Asakura and Sugimura, 1999). Although the intracellular signalling of TNF-α is not completely understood, evidence shows TNF-α activates at least two signalling pathways, including several members of the evolutionarily conserved mitogen-activated protein (MAP) kinase family (Raines et al., 1993) and the nuclear transcription factor κB (NF-κB). These pathways may be critical in regulation of cellular proliferation and inflammation respectively.(Traverse et al., 1994; Barnes and Karin, 1997).

Presumably, the prolonged expression of TNF-α leads to down-regulation of the TFF healing peptides, thereby decreasing repair and mucosal protection. This idea is based on many reports showing that oral and systemic application of recombinant TFF peptides promote mucosal defence and heal experimental ulceration in colon and stomach (Playford et al., 1995; Kindon et al., 1995; Tran et al., 1999; Mashimo et al., 1996).

We further have revealed that down-regulation of *TFF* transcription is mediated by NF-κB. Cotransfection of p50 and p65 reduced *TFF* transcription (Fig. 11A,B) and in combination with TNF-α this effect was even more severe (Fig. 11C).
NF-κB is maintained in an inactive state in the cytoplasm by complexing with members of the IκB inhibitory protein family, including IκB-α, (Miyamoto and Verma, 1995; Traenckner et al., 1995) and IκB-β (Thompson et al., 1995). This interaction with IκB-α masks the NF-κB nuclear localisation signal and inhibits the DNA binding

activity of NF-κB (Miyamoto and Verma, 1995). The phosphorylisation of serines 32 and 36 of IκB-α (Traenckner et al., 1995) by the recently cloned IκB-α kinase (Mercurio et al., 1997) stimulates the ubiquitination of IκB-α, followed by degradation of IκB-α by the 26S proteasome complex (Traenckner et al., 1995). NF-κB is then translocated to the nucleus and activates the transcription of a variety of genes. Therefore we tested these effects in two gastrointestinal cell lines. Activation of endogenous NF-κB was mediated via IκB, since IκB cotransfection reversed the effect of NF-κB (Fig.12A) and that of TNF-α (Fig. 12B). This result is in line with other reports demonstrating NF-κB signalling in gastrointestinal epithelia (Jobin and Sartor, 2000; Schmid and Adler, 2000).

NF-κB as a global regulator is essential for the mediating response to infection and play an important role in the inflammatory the intestine (Neurath et al., 1998), but sustained overexpression of NF-κB is associated with chronic inflammation in IBD. A recent report demonstrated increased NF-κB activation in epithelial cells of inflamed mucosa in patients with Crohn´s disease and ulcerative colitis, but not in adjacent uninvolved mucosa (Rogler et al., 1998). Many drugs that inhibit NF-κB such as mesalamine or antioxidants are therapeutic tools in IBD (Jobin and Sartor, 2000; Kaiser et al., 1999). In this respect it is worth noting that these drugs activate *TFF* gene expression in various gastrointestinal cell lines (data not shown). Moreover, IL-1 known to trigger the activation of NF-κB, is capable to down-regulate *TFF3* transcription in HT-29 cells (data not shown).

Many putative binding sites of NF-κB are present in the upstream region of all three human *TFF* genes displaying two or one mismatches to the NF-κB consensus binding site within 900 bp of the 5'-flanking region (Fig. 10). In *TFF2*, one of the putative NF-κB binding sites (at pos. -421) is 90 % identical to consensus sequence. We have shown that mutation of this site abolishes *TFF2* down-regulation after cotransfection of p65 and p50/p65 (Fig. 13). Furthermore specific binding of NF-κB to this promoter element was detected by EMSA.

Although *TFF1* and *TFF3* were shown to be strongly down-regulated by TNF-α and NF-κB (Fig. 9 and Fig. 11), the corresponding 5'-flanking sequences do not display sites with 90 % identity to the consensus NF-κB binding site (Fig. 10). In *TFF3,* two

sites that display two mismatches to the consensus NF-κB binding sites were tested by EMSA, and one of them (pos. –97) showed weak NF-κB binding (data not show). Presumably, also sites with one or two mismatches contribute to inhibition of *TFF* transcription.

This idea also results from the observation that a deletion construct of *TFF*2 in which the two proximal putative binding sites displaying two mismatches were deleted, neither respond to NF-κB nor TNF-α (data not shown).

In line with this observation, it was reported that a NF-κB binding site with two mismatches is functional in down-regulating human papilloma virus HPV16 gene expression, although the affinity of NF-κB for this site is reduced about 250-fold (Fontaine et al., 2000). NF-κB activates a variety of genes involved in early immune response, acute phase and inflammatory responses (Jobin and Sartor, 2000), but it also inhibits transcription of some genes (Fontaine et al., 2000; Wissink et al., 1997; Supakar et al., 1995). We did not elucidate the mechanism of *TFF* repression by NF-κB. One possibility would be that the activity of NF-κB is regulated by interaction with other transcription factors such as AP-1 and C/EBP, the putative binding sites of which are present in the 5'-flanking region of all *TFF* genes.

The two latter proteins belong to the bZIP class of DNA binding proteins characterised by leucine zipper structure and adjacent basic DNA binding domain. The bZIP region has been reported to directly interact with the Rel homology domain of NF-κB. (LeClair et al., 1992; Stein et al., 1993).

The biological implications of *TFF* down-regulation by NF-κB remain unclear, but the perpetuation of the chronic inflammatory state associated with NF-κB overexpression in IBD leads to situations of decreased gastrointestinal mucosal protection and initiation of tumour growth, which may be evoked by the reduced expression of *TFF* healing peptides.

4.3. The effect of USF

We have identified the *TFF2* gene as a target for USF transcription factors. This activation is mediated by an E box DNA sequence element in the promoter of the *TFF2* gene. Despite E box elements in the two other *TFF* genes, *TFF1* and *TFF3*, they are not stimulated by USF. In addition factors of the Myc/Max/Mad network were unable to regulate any of the *TFF* genes. Thus it appears that the *TFF2* promoter is unique among the *TFF* genes in that it supports strong activation by USF.

The specific activation of the *TFF2* gene by USF must be the result of mechanistic differences that distinguish *TFF2* from the other two *TFF* genes and USF from factors of the Myc/Max/Mad network. One possibility is that differences in DNA binding determine at least in part the responsiveness to factors. Previous studies have demonstrated the importance of E-box flanking sequences for high affinity binding of Myc/Max/Mad network proteins while the flanking sequences seem less critical for USF binding (for review (Lüscher and Larsson, 1999). In agreement the binding of Myc/Max complexes was significantly lower to the E-boxes of the three *TFF* genes than to a high affinity binding site (Fig. 18 and table 6)**.**

In contrast to c-Myc/Max complexes, the differences in USF binding to *TFF* E-boxes and a high affinity site were less pronounced, supporting previous findings that USF proteins have a broader specificity than Myc/Max/Mad complexes (Fig. 18). Thus *in vitro* the differences in binding of the factors analysed to by a methode the 4 E boxes were relatively low.

In vivo DNA binding analyses by ChIP revealed more striking differences. USF binding was predominantly found on *TFF2* while little binding to *TFF1* and *TFF3* was measured (Fig. 19). In addition binding of c-Myc was very low (Fig. 19). Thus DNA binding selectivity is considerably more pronounced *in vivo* than *in vitro* offering a mechanistic basis for the observed specificity. This might reflect distinct accessibility to chromatin embedded binding sites or co-operative DNA binding effects. A different situation has been described for the E box in the *cad* gene.

Both USF and c-Myc bind but only the latter appears to be able to activate transcription, leading to the suggestion that the *cad* promoter is not responsive to USF (Boyd and Farnham, 1997).

Previous studies have shown that USF can activate a number of different promoters in reporter gene assays in many different cell types. In general these activations are rather modest while the activation of the *TFF2* reporter is significantly higher. For example, whereas the promoters of *MAPK phosphatase-1* (Sommer et al., 2000), *BRCA2* (Davis et al., 1999), *CD2* (Outram and Owen, 1994) and *fatty acid synthase* (Wang and Sul, 1997) are activated 3 to 5 fold, the *TFF2* promoter is stimulated at least ten times more efficiently (Fig. 16). We postulate that the regulation of the *TFF2* gene, both the reporter and the chromosomal copy, depends on a factor(s) that cooperates with USF to induce high level, promoter-specific expression. This is also indicated by the narrow tissue specificity of *TFF2* transcription, predominantly in the antral glands and the Brunner's glands of the duodenum (Tomasetto et al., 1990) whereas USF is expressed ubiquitously.

Cooperation of USF with other factors, including Egr-1, STAT1, and cAMP responsive element binding proteins, has been described before (David et al., 1994; Vervoorts and Luscher, 1999; Sommer et al., 2000). Our preliminary analyses suggest that this factor is neither HNF-3, nor C/EBP, nor GATA-6, transcriptional regulators we described previously as activators of *TFF* genes (Beck et al., 1999; Al-azzeh et al., 2000). Further studies are designed to identify the cooperating factor(s).

The *TFF2* gene is activated in response to a number of different insults and to drugs such as 5-amino salicylic acid used for treatment of inflammatory bowel disease or aspirin (EA and PG, unpublished observation). It is largely unclear how these stimuli affect the activity of the *TFF2* promoter. USF might be involved in recognising stress signals (Tsai et al., 1989) and/or cooperate with factors targeted during signalling events (Sommer et al., 2000). Since USF is the major E box binding activity in most cell lysates, USF binding to TFF2 might be a prerequisite for signal stimulated activation.

USF is known to evoke anti-proliferative effects (Qyang et al., 1999). In this respect it is noteworthy that several suggested USF target genes, including BRCA2, p53, and

transforming growth factor ß2, are involved in inhibition of cell proliferation (Qyang et al., 1999).

The identification of USF as a potent regulator of *TFF2* is in line with a more general role of this transcription factor in negative growth control since TFF peptides have been suggested to possess tumour suppressor activity (see introduction for details). Together the data summarised above suggest that defining the control of *TFF2* expression in more detail will potentially generate opportunities for therapy of gastrointestinal diseases.

5. SUMMARY

5.1.Transcription factor GATA-6 activates expression of gastroprotective trefoil genes *TFF1* and *TFF2*

The up-regulation of TFF peptides is observed in early stages of inflammation and epithelial restitution of the gastrointestinal tract. These secretory peptides belonging to the trefoil factor family (TFF) promote cell migration, protect and heal the mucosa. Their major expression site is the stomach (TFF1, TFF2) and the intestine (TFF3). Several consensus binding sites for members of the GATA transcription factors known to control gut-specific gene expression are located in the 5'-flanking region of the genes. By RT-PCR GATA-6 was shown to be expressed in several tumor cell lines of gastric, intestinal and pancreatic origin. In MKN45, KatoIII and LS174T cotransfection with TFF-reporter genes and GATA-6 expression vectors revealed that GATA-6 activates *TFF1* and *TFF2* 4-6 fold, without an effect on *TFF3*. The functional contribution of GATA binding sequences in the reverse orientation was characterised by reporter gene assays using *TFF2* deletion constructs and gel shift experiments.

5.2.TNF-α and NF-κB inhibit transcription of human *TFF* genes encoding gastrointestinal healing peptides

In HT-29 and KATOIII cell lines the proinflammatory cytokine TNF-α inhibits the expression of endogenous *TFF* as well as reporter genes driven by approximately 900 bp of their corresponding 5'-flanking region. Down-regulation of *TFF* transcription is also observed by cotransfection of NF-κB and is reversed by constitutive form of repressive IκB. By mutational analysis, some of the putative NF-κB binding sites in the 5'-flanking region of *TFF*s were shown to function as silencers, and electrophoretic mobility shift assays indicated binding of NF-κB to these sites.

5.3. Regulation of the gastrointestinal healing peptide *TFF2* gene promoter by USF but not by c-Myc

A consensus E-box element is located at 250bp upstream in the TFF2 promoter, but not in *TFF1* and *TFF3.* Transient transfection studies and mutational analysis demonstrated that the upstream stimulatory factors USF1 and USF2 but not c-Myc, or Myc-Max heterodimers stimulate *TFF2* transcription up to 65-fold in a variety of gastrointestinal cell lines. Ectopically expressed USF1 and USF2 function in a synergistic manner. Different levels of USF activation in these cell lines suggest an

involvement of a coactivator. By *in vivo* chromatin crosslinking and immunoprecipitation the TFF2 E-box was found to be occupied by USF but not by c-Myc. The strong, specific induction of the TFF2 promoter by USF may argue for a specific tumor suppressing or healing function during gut development and repair.

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Diese Arbeit wurde am Institut für Anthropologie und Humangenetik der Universität Tübingen angefertigt. Die wichtigsten Ergebnisse wurden veröffentlicht und sind zur Veröffentlichung eingereicht in:

Ezzal-din Al-azzeh, Petra Fegert, Nikolaus Blin, Peter Gött..(2000). Transcription factor GATA-6 activates the gastroprotective trefoil genes *TFF1* and *TFF2.* Biochem. Biophys. Acta; 1490 (3): 324-332.

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Pia Azarschab, Ezzal-din Al-Azzeh, Wolfgang Kornberger, Peter Gött. (2000). Aspirin promotes TFF2 gene activation in human gastric cancer cell lines. FEBS Lett. (submitted)

Ezzal-din Al-azzeh, Dieter Kube, Nikolaus Blin, Peter Gött. (2000). TNF-α and NF-κB inhibit transcription of human TFF genes encoding gastrointestinal healing peptides. Gastroenterology (submitted)

Ezzal-din Al-azzeh, Oliver Dittrich, Jörg Vervoorts, Nikolaus Blin, Bernhard Lüscher, Peter Gött. (2000). Regulation of the gastrointestinal healing peptide *TFF2* gene promoter by USF but not by c-Myc. EMBO report (submitted)

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Fichtenweg 27/115
72076 Tübingen