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# Osmotic changes and ethanol modify TFF gene expression in gastrointestinal cell lines

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**Abstract The gastrointestinal tract is exposed to environmental insult as a result of food intake or in pathological conditions such as diarrhoea, and is therefore protected by the mucus layer. As part of it, trefoil peptides (TFFs) are able to modify the visco-elastic properties of the mucus, protect against experimental ulceration, and promote repair of the epithelia. We investigated, using transient reporter gene assays and RT-PCR in the gastric carcinoma cell line MKN45 and colon carcinoma cell lines LS174T and HT29, whether ethanol and osmotic changes can modify transcriptional activity of *TFF*s. In a mild hypotonic environment (200 mosmol/l) all three *TFF* genes were up-regulated by at least a factor of 2. In hypertonic medium (400 mosmol/l), *TFF1* and *TFF3* were down-regulated, whereas *TFF2* was up-regulated by elevated concentrations of sodium or chloride in MKN45. Raising the osmolality by ethanol resulted in an up-regulation of *TFF3* in both colon cell lines but not in the gastric cell line. We conclude that alteration in *TFF* gene expression is a response of gut epithelia to deal with osmotic forces and ethanol.**



*Key words:* TFF domain peptide; Trefoil peptide; Osmotic gene regulation; Induction by ethanol

## 1. Introduction

The osmolality of the lumen of the gastrointestinal tract changes considerably due to intake of water and food. The osmolality of drinking water is often below 10 mosmol/l (rain-water). Moreover, in situations where the body is threatened by loss of ions (e.g. diarrhoea), apical water secretion leads to swelling of epithelial cells. On the other hand, digestion or intake of salty food results in high osmolality in the lumen of the intestine. Osmotic stress affects the epithelia of the gastrointestinal tract through changes in cell volume or turgor pressure. Thus, as a first barrier the visco-elastic mucus gel layer covering the gastrointestinal epithelial mucosa provides protection against an osmotically unbalanced environment as well as against bacteria and aggressive chemicals. It is agreed that proton diffusion through the gastric mucus is delayed thereby protecting the epithelia from gastric acid. Recently, it has been demonstrated that the human spasmolytic polypeptide (TFF2) acts synergistically with mucins to decrease proton permeation through gastric mucus in vivo and in vitro [1].

Trefoil factor family (TFF) peptides are expressed in the gastrointestinal mucosa and are highly concentrated in the mucus layer of the gastrointestinal tract [2–4]. The physiological role of these peptides is still a matter of discussion, but evidence has accumulated that one of their functions is to protect epithelia from a variety of insults and to facilitate restitution of wounded epithelia. Orally administered TFF2 and TFF3 protect gastric mucosa from injury due to indomethacin and ethanol in rats [5]. Trefoil peptides are cytoprotective peptides co-secreted with mucins, and both act in a synergistic fashion [6,7]. TFF peptides protect transgenic mice from experimental ulceration [8,9] and stimulate cell migration [10]. These peptides alter the physical properties of the secreted mucins, leading to an increase in the optical density and viscosity of purified mucin preparations possibly by binding to mucus molecules together via oligosaccharide side chains [4,11]. In humans, TFF1 and TFF2 are produced by mucus-secreting cells of the stomach – TFF1 by superficial foveolar cells and TFF2 by gastric mucous neck cells and in the deeper glandular cells – whereas TFF3 is predominantly produced by goblet cells of the small and large intestine [12].

The three human *TFF* genes are clustered within 50 kb in the genome and their expression is coordinated [13]. They are up-regulated around areas of epithelial damage and in meta- and neoplasia [14–16]. Little is known about the physiological signals that govern trefoil expression. TFF3 expression and secretion is up-regulated by neuropeptides (somatostatin, vasoactive intestinal polypeptide) and acetylcholine but not by peptide growth factors and various cytokines [17]. Using human gastrointestinal tumour lines, we showed that the 5′-flanking region of all three *TFF* genes controls cell-specific expression [18,19]. Because they resemble stomach- and intestine-specific *TFF* expression, these tumour cell lines provide a model system to address the question whether transcription is influenced by osmotic changes. Here, reporter gene and endogenous expression studies revealed that hypotonicity and ethanol increase *TFF* activity, whereas hypertonicity decreases *TFF* expression.

## 2. Materials and methods

### 2.1. Human cell lines and reporter plasmids

The poorly differentiated gastric adenocarcinoma cell line MKN45 and the colon adenocarcinoma cell lines LS174T and HT29 were cultivated in RPMI 1640 and DMEM supplemented with 20% FCS or 10% FCS, respectively [19]. Luciferase reporter plasmid pGL3 (Promega) was used to construct *TFF* recombinants using the 5′-flanking region of human *TFF1* (pos. −1100 to +38), *TFF2* (pos. −821 to +61) and *TFF3* (pos. −867 to +63) [18,19].

### 2.2. Transient transfection assays

Cells seeded on 24-well plates were transiently transfected by means of a liposome reagent effectene (Qiagen, Hilden, Germany). Renilla luciferase (pRL-CMV, Promega, WI, USA) and dual luciferase measurements (Promega, #E1910) were used to standardise transfection efficiency and calculate specific transcriptional activity [19]. Seven hours after transfection, medium was exchanged and adjusted to

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200 mosmol/l using medium containing a sodium chloride-reduced medium (Life Technologies, Karlsruhe, Germany) or by dilution (33%) with sterile water. Since the relative TFF1 transcription was high in LS174-T, serum-free medium was used after transfection (Fig. 1B). For hyperosmotic stimulation, media adjusted to 400 mosmol/l were used by addition of 50 mM sodium chloride, 33 mM sodium sulphate, 33 mM calcium chloride, 100 mM D-mannitol (Sigma, #M1902), 100 mM D-raffinose (Sigma, #R0250), or 100 mM ethanol. Forty hours after stimulation cells were harvested. Assays were performed in triplicate or quadruplicate, and the results are expressed as the ratio of firefly luciferase to renilla luciferase compared to the unstimulated control (relative transcriptional activity). The standard deviation was less than 15%. Each experiment was independently performed three times.

*2.3. Cytotoxicity assays*

Cell survival was determined 2 and 5 days after induction using the trypan blue (0.18%, Sigma, #T6146) exclusion method [20].

*2.4. Multiplex RT-PCR*

Total cellular RNA was prepared and reverse-transcribed using oligo-dT primers from cells grown in 6-well plates and stimulated as described before. Twenty-five and 30 cycles of multiplex PCR with TFF1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), TFF2 or TFF1, GAPDH TFF3 primer pools were performed as described. Reverse transcriptase, PCR primers and conditions were used as reported previously [19].

## 3. Results

*3.1. Hypotonic stress activates TFF1, TFF2 and TFF3 transcription*

Since the endogenous expression profiles of *TFF1, TFF2* and *TFF3* are matched by reporter gene expression in eight gastrointestinal cell lines [21], we focused on the stomach-derived cell line MKN45 and the intestinal cell lines LS174T and HT29, and used the transient transfection of luciferase reporter genes to study the influence of osmotic changes on TFF transcription.

To demonstrate the effect of reduced osmolality on TFF transcription cell lines MKN45 and LS174T grown in isotonic medium (300 mosmol/l) were exposed to medium with reduced sodium chloride content resulting in an osmolality of 100 mosmol/l and 200 mosmol/l. At both conditions no cytotoxic effects were observed after 2 and 5 days. A mild hypotonic stress (40 h in 200 mosmol/l) caused a specific increase of about 80% of *TFF1* transcriptional activity revealed by reporter gene assays in MKN45 (Fig. 1A). This increase was equivalent to the results obtained when osmolality was reduced to 200 mosmol/l by 33% medium dilution with sterile water. This dilution of the medium had no effect on cell viability determined by trypan blue exclusion or cell growth. *TFF2* and *TFF3* reporter gene activity increased more than 100% as a consequence of mild hypotonic stress. The same increase in *TFF1* activity was noted when osmolality was reduced to 100 mosmol/l by sodium chloride reduction (data not shown). Activation of all three *TFF* genes was also observed in the intestinal cell line LS174-T (Fig. 1B). Hypotonicity also increased *TFF3* transcription (+95%) in the colon cell line HT29 (data not shown).

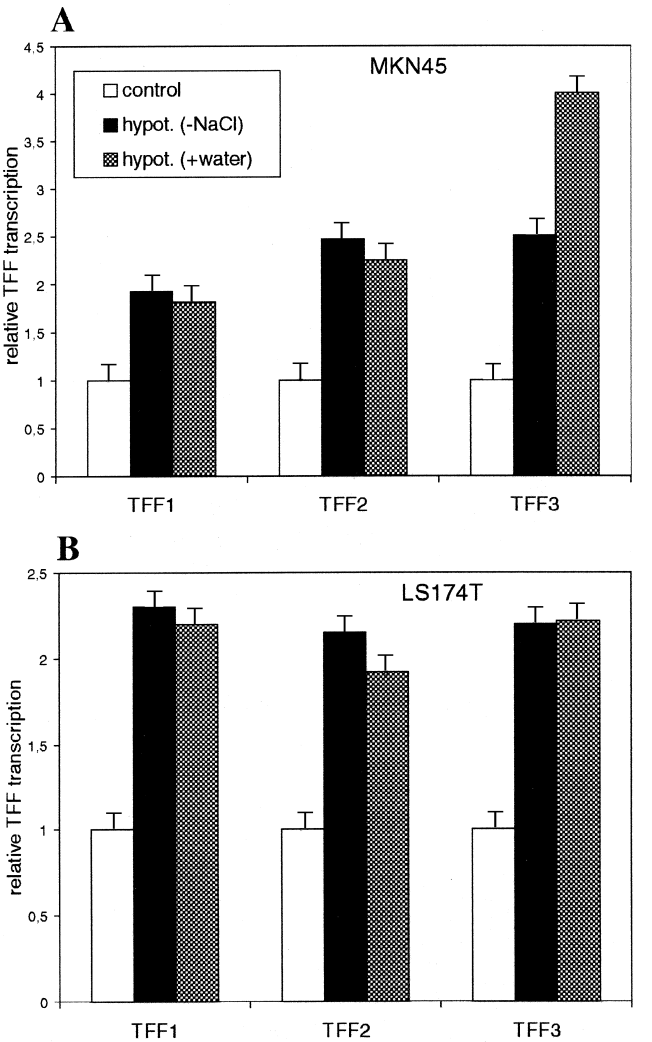


Fig. 1. Effect of hypotonic stress on transcription of *TFF* reporter genes in cell lines MKN45 (A) and LS174T (B). Cells were incubated in isotonic (300 mosmol/l, control) or hypotonic medium (200 mosmol/l) by reduction of 50 mM sodium chloride (−NaCl), and by a 33% dilution of the medium with sterile water (+water). Bars represent mean values ± S.D. of three independent experiments.

*3.2. Hypertonicity causes down-regulation of TFF expression*

To test the impact of high osmolality on *TFF* transcription, cell lines were exposed to medium adjusted to 400 mosmol/l by addition of various substances. The non-metabolisable saccharide derivatives D-mannitol or D-raffinose decreased transcription of all three *TFF* reporter genes by a factor of

Table 1
Effect of ethanol, hypertonicity and hypotonicity on endogenous TFF expression in LS174T

| | Control | Ethanol | NaCl | Raffinose | Water |
|---|---|---|---|---|---|
| TFF1 | 31 | 80 | 16 | 17 | 62 |
| TFF2 | 34 | 101 | 41 | 26 | 72 |
| TFF3 | 52 | 212 | 33 | 12 | 114 |
| GAPDH | 100 | 100 | 100 | 100 | 100 |

Values are displayed in % of GAPDH expression calculated from densities of RT-PCR products after 25 cycles in Fig. 4.

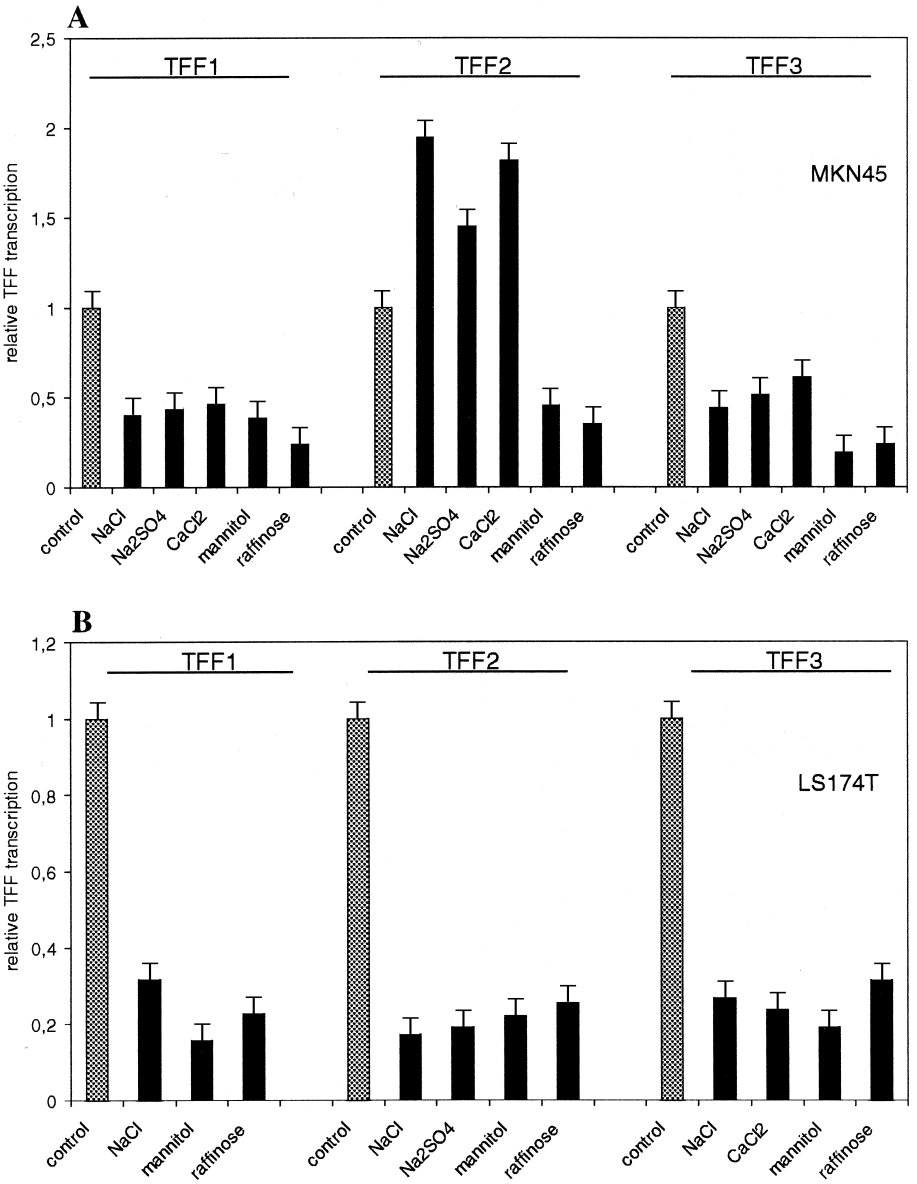


Fig. 2. Effect of hypertonic stress on transcription of *TFF* reporter genes in cell lines MKN45 (A) and LS174T (B). Cells were incubated in isotonic (300 mosmol/l, control) or hypertonic medium (400 mosmol/l) by addition of 50 mM NaCl, 33 mM $Na_2SO_4$, 33 mM $Ca_2Cl$, 100 mM D-mannitol or 100 mM D-raffinose. Bars represent mean values ± S.D. of three independent experiments.

at least 2. This was observed for both cell lines MKN45 (Fig. 2A) and LS174T (Fig. 2B). Addition of sodium chloride (50 mM), sodium sulphate (33 mM) or calcium chloride (33 mM) also decreased *TFF* expression, except for *TFF2* in MKN45. Here an up-regulation of up to two-fold was observed, which was not observed in LS174T. No effects on cell viability and growth occurred in hyperosmotic medium due to addition of sodium chloride, sodium sulphate, mannitol or raffinose.

### 3.3. Ethanol activates TFF3 expression in intestinal cell lines

To test whether a reduced TFF activity is the consequence of a reduction in cell volume we increased osmolality by addition of ethanol, since ethanol is freely permeable and does not evoke an osmotic force on cells. Moreover, ethanol is a drug to which gastrointestinal cells are frequently exposed. Addition of 50 mM or 100 mM ethanol did not alter *TFF3* gene expression in MKN45 (Fig. 3). However, in the intestinal cell lines LS174T and HT29 ethanol leads to a dose-dependent up-regulation of TFF3 transcription. There was no significant cytotoxicity of cells exposed to ethanol up to 250 mM.

### 3.4. Osmotic changes and ethanol evoke comparable effects on endogenous TFF expression

To test whether osmotic changes and ethanol evoke comparable effects on endogenous *TFF* gene expression, cells were cultivated and stimulated under identical conditions as for reporter gene analysis and specific mRNA levels of TFF1, TFF2, TFF3 and GAPDH were determined after semiquantitative multiplex RT-PCR. Fig. 4 and Table 1 show the results for the cell line LS174T. Hypertonicity (addition of sodium chloride or raffinose) led to reduced *TFF* gene expression, and on the other hand, hypotonicity and ethanol activated expression of all three *TFF* genes.

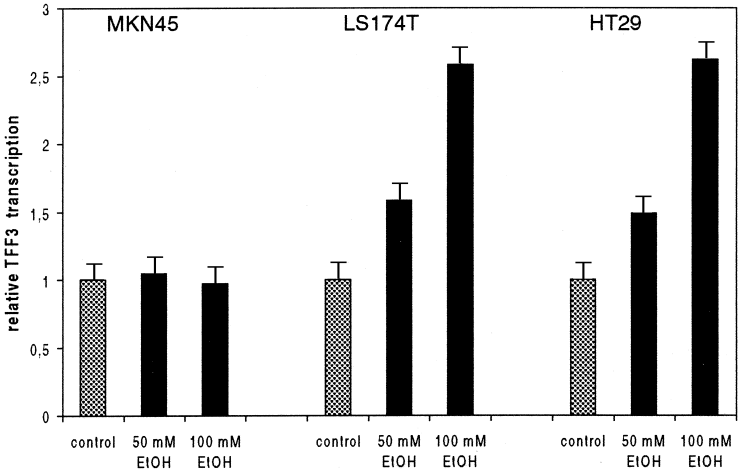


Fig. 3. Effect of ethanol on transcription of *TFF3* reporter genes in the gastric cell line MKN45, and the intestinal cell lines LS174T and HT29. Bars represent mean values ± S.D. of three independent experiments.

## 4. Discussion

Here we show that all three human TFFs are transcriptionally regulated in response to osmotic environment and ethanol in gastric and intestinal cell lines. This regulation may have important consequences for the physiology of the corresponding tissues. The role of mammalian TFF peptides in mucosal protection and healing of the gastrointestinal epithelia is becoming increasingly clear [5,8,9,22,23]. However, little is known about the physiological factors that regulate TFF expression. Expression and secretion of TFF3 is up-regulated by neuropeptides and acetylcholine in the intestinal cell line HT29, indicating that regulation is integrated into the enteric neuroendocrine system [17]. *TFF* genes are up-regulated after experimental injury and ulcerative lesions [14–16]. Recently we found one of the molecular events underlying *TFF1* activation, which is the recruitment of the transcription factor HNF-3 (Beck and Gött, unpublished).

Here we have exposed gastric and intestinal cell lines to a mild hypotonic stress and found an increase in the expression of all three *TFF*s. This up-regulation may also be part of a protection mechanism of the gut in vivo. Osmotic swelling of epithelial cells can threaten the organism in situations that may arise after intake of hypotonic drinking water, in periods of food shortages or under pathological situations (e.g. diarrhoea).

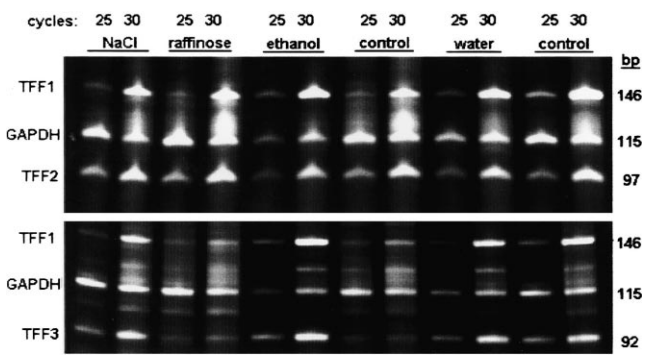


Fig. 4. Osmotic changes alter the endogenous transcription of *TFF* genes in LS174T. Cells were harvested 40 h after induction with 50 mM NaCl, 100 mM D-raffinose, 100 mM ethanol, or a 33% dilution of the medium with water. Twenty-five and 30 cycles of multiplex RT-PCR were performed and visualised by ethidium bromide staining on 6% acrylamide-TBE gels.

On the other hand, under mild hypertonic conditions, we found that transcription of all *TFF*s is down-regulated. If transferred to the in vivo situation, reducing the TFF content of the mucus layer will likely result in an increased absorption rate of intestinal epithelium, which may provide an evolutionary benefit in periods of abundant food supply.

However, uptake of food and beverages may not always be beneficial. In this respect, we found that increasing the osmolality by ethanol lead to a three-fold up-regulation of *TFF3* in two intestinal cell lines but not the gastric cell line. It should be noted that treatment with 100 mM ethanol (= 0.46%) did not alter cell viability but this concentration is reported to be created in the intestine by bacterial ethanol fermentation. In addition, ethanol concentrations up to 6% can be found in the intestine of alcoholics, which causes adaptive cytoprotection [24,25]. It has been shown that orally administered TFF-peptides protect against ethanol-induced gastric injury in rats [5], which argues for a beneficial function of up-regulation of TFFs by ethanol. Moreover, transcriptional up-regulation of tracheobronchial mucin and mucin genes *MUC1* and *MUC2* (intestinal mucin) as a result of treatment with ethanol has recently been reported [25], supporting a synergistic protective function of TFFs and mucins [6,7].

Generally, a hypertonic stress resulted in down-regulation of all *TFF*s. However, there was one exception. In the gastric cell line MKN45, hypertonic stress caused by sodium chloride, sodium sulphate or calcium chloride resulted in up-regulation of *TFF2* whereas raffinose and mannitol caused down-regulation. The mechanism of this up-regulation is difficult to analyse, because in response to non-isotonic conditions cells accumulate or dump many osmotically active ions or organic solutes, which beside other effects can alter gene regulation. Presumably, increased extracellular sodium or chloride concentrations up-regulate specifically the expression of *TFF2* in MKN45. It may be interesting to note that MKN45 is an adenocarcinoma cell line showing properties of gastric parietal cells [26]. In vivo, expression of TFF2 is restricted to the glandular and ductal cells of the antrum, where secretion of hydrochloric acid occurs. Moreover, TFF2 can protect cells

from hydrochloric acid, since it decreases proton permeation through gastric mucus in vivo and in vitro [22]. Gastric acid secretion may therefore be part of a regulatory network modulating *TFF2* expression.

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## References

[1] Tanaka, S., Podolsky, D.K., Engel, E., Guth, P.H. and Kaunitz, J.D. (1997) Am. J. Physiol. Gastrointest. Liver Physiol. 272, G1473–G1480.
[2] Rio, M.C., Bellocq, J.P., Daniel, J.Y., Tomasetto, C., Lathe, R., Chenard, M.P., Batzenschlager, A. and Chambon, P. (1988) Science 241, 705–708.
[3] Thim, L. (1989) FEBS Lett. 250, 85–90.
[4] Thim, L. (1997) Cell. Mol. Life Sci. 53, 888–903.
[5] Babyatsky, M.W., DeBeaumont, M., Thim, L. and Podolsky, D.K. (1996) Gastroenterology 110, 489–497.
[6] Playford, R.J., Marchbank, T., Chinery, R., Evison, R., Pignatelli, M., Boulton, R.A., Thim, L. and Hanby, A.M. (1995) Gastroenterology 108, 108–116.
[7] Kindon, H., Pothoulakis, C., Thim, L., Lynch Devaney, K. and Podolsky, D.K. (1995) Gastroenterology 109, 516–523.
[8] Playford, R.J., Marchbank, T., Goodlad, R.A., Chinery, R.A., Poulsom, R., Hanby, A.M. and Wright, N.A. (1996) Proc. Natl. Acad. Sci. USA 93, 2137–2142.
[9] Mashimo, H., Wu, D.C., Podolsky, D.K. and Fishman, M.C. (1996) Science 274, 262–265.
[10] Williams, R., Stamp, G.W.H., Lalani, E.-N. and Pignatelli, M. (1996) J. Cell Sci. 109, 63–71.
[11] Williams, G.R. and Wright, N.A. (1997) Virchows Arch. 431, 299–304.
[12] Sands, B.E. and Podolsky, D.K. (1996) Annu. Rev. Physiol. 58, 253–273.
[13] Gött, P., Beck, S., Machado, J.C., Carneiro, F., Schmitt, H. and Blin, N. (1996) Eur. J. Hum. Genet. 4, 308–315.
[14] Alison, M.R., Chinery, R., Poulsom, R., Ashwood, P., Longcroft, J.M. and Wright, N.A. (1995) J. Pathol. 175, 405–414.
[15] Welter, C., Theisinger, B., Seitz, G., Tomasetto, C., Rio, M.C., Chambon, P. and Blin, N. (1992) Lab. Invest. 66, 187–192.
[16] Nogueira, A.M.M.F., Machado, J.C., Carneiro, F., Reis, C.A., Gött, P. and Sobrinho-Simoes, M. (1998) J. Pathol. (in press).
[17] Ogata, H. and Podolsky, D.K. (1997) Am. J. Physiol. Gastrointest. Liver Physiol. 273, G348–G354.
[18] Beck, S., Fegert, P. and Gött, P. (1997) Int. J. Oncol. 10, 1051–1055.
[19] Beck, S., Sommer, P., Blin, N. and Gött, P. (1998) Int. J. Mol. Med. 2, 353–361.
[20] Verma, M., Sanadi, A. and Davidson, E.A. (1994) Glycobiology 4, 825–836.
[21] Thompson, A.M., Steel, C.M., Foster, M.E., Kerr, D., Paterson, D., Deane, D., Hawkins, R.A., Carter, D.C. and Evans, H.J. (1990) Br. J. Cancer 62, 78–84.
[22] Li, D.C., Wang, D.M., Majumdar, S., Jany, B., Durham, S.R., Cottrell, J., Caplen, N., Geddes, D.M., Alton, E.W.F.W. and Jeffery, P.K. (1997) J. Pathol. 181, 305–310.
[23] Lefebvre, O., Chenard, M.P., Masson, R., Linares, J., Dierich, A., LeMeur, M., Wendling, C., Tomasetto, C., Chambon, P. and Rio, M.C. (1996) Science 274, 259–262.
[24] Dinda, P.K., Wasan, S., Beck, I.T. and Kossev, P. (1996) Can. J. Pysiol. Pharmacol. 74, 598–602.
[25] Verma, M. and Davidson, E.A. (1997) Gene 189, 9–12.
[26] Yoshida, T., Sato, R., Mahmood, S., Kawasaki, S., Futai, M. and Maeda, M. (1997) FEBS Lett. 414, 333–337.