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RESEARCH COMMUNICATION

Localization of intestinal trefoil-factor mRNA in rat stomach and intestine by hybridization *in situ*

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A cDNA encoding rat intestinal trefoil factor (rITF) was prepared by reverse transcription and PCR amplification. The sequence obtained was well conserved with that of other trefoil peptides. An antisense riboprobe produced from the clone was used to localize the sites of ITF expression in the rat gastrointestinal tract using hybridization *in situ*. We found rITF mRNA in goblet cells in the small intestine and colon; a gradient of signal strength greatest near the crypt base was sometimes present. We found no evidence for rITF expression in Brunner's glands, the pancreas, or most regions of the gastric mucosa. Surprisingly, strong signals for rITF mRNA were detected in a region of stomach at the junction of the squamous fore-stomach with the glandular gastric mucosa. This region, which may correspond to the cardiac region, formed part of a larger area of cells staining positive for acid mucins. We hypothesize that concerted expression occurs of particular trefoil peptides with specific mucins, and that this organization reflects a functional relationship between mucins and trefoil peptides.

INTRODUCTION

Trefoil peptides are members of a growing family of stable secreted molecules containing one or more of the unusual 'trefoil' motifs. Trefoil motifs were first named as such because of the 'three-leaved' appearance of the primary structure when drawn to accomodate three pairs of cysteine-based disulphide bonds [1]. Since then, the supersecondary structure of the motifs in porcine pancreatic spasmolytic polypeptide (SP) have been modelled [2], revealing them to have a closely knit and truly novel structure.

Rat intestinal trefoil factor (rITF) is the most recently described trefoil peptide [3]. Northern analyses suggest that rITF mRNA is present in the intestine and kidneys, but absent from the stomach, of the adult rat [3]. Immunohistochemistry, using a polyclonal antiserum raised against a deduced peptide sequence, suggests that expression of rITF protein is confined to goblet cells throughout the small and large intestine, but absent from gastric or pancreatic tissue [3]. However, these two approaches to localizing the sites of expression of rITF have their limitations: (1) Northern analyses suffer from a relative lack of sensitivity in cases where a mRNA is confined to a small tissue compartment; (2) polyclonal antisera to synthetic peptides have the potential to bind not only to the desired peptide but also to others sharing epitopes. Trefoil peptides are difficult in this respect, as shown in Fig. 1, where the immunizing peptide of [3] is aligned with portions of several trefoil peptides [4–7].

In view of the possibilities that a polyclonal antiserum might detect other trefoil peptides in addition to rITF, and that Northern analyses may overlook some sites of expression of the mRNA, we prepared a rITF cDNA clone and used hybridization *in situ* to localize its mRNA in rat stomach, intestine and pancreas.

Peptide	Part	Sequence
rITF[3]	(last 21)	D S S I P N V P W C F K P L Q E T E C T F
xAPEG[7]	(single trefoil)	- • • • • V G • K • • • F • A • L V P S V Y
hpS2	(single trefoil)	- • D T V R G • • • • • Y • N T I D V P P E
mSP[4]	(in trefoil 1)	- • • • • V A G • • • • • H • • P N Q • S E Q -
	(in trefoil 2)	- S N L • F E • • • • • F • Q S V E D • H Y
hSP[4]	(in trefoil 1)	- • • • • V T G • • • • • H • • P K Q • S D Q -
	(in trefoil 2)	- S N F • F E • • • • • F • N S V E D • H Y
pPSP[1]	(in trefoil 1)	- • • Q V • G • • • • • • • • P A Q • S E E -
	(in trefoil 2)	- S D T • • E • • • • • F • M S V E D • H Y
xSPL[6]	(in trefoil 1)	- • • • • • L • T K • • • Y N A T A G P I K K -
	(in trefoil 2)	- • • • • • S G • K • • Y A R T V I T T P A P -
	(in trefoil 3)	- • • T • • E T K • • • Y T E A • A P A R K -
	(in trefoil 4)	- • E C • • D • I • • • E K A V P V V N S

Fig. 1. Comparison of the immunizing peptide of [3] with partial sequences of trefoil peptides

'•' Indicates that the aligned amino acid is identical. Abbreviations: xAPEG, *Xenopus laevis* APEG-rich protein; hpS2, human breast-cancer-associated protein 'pS2'; mSP, mouse spasmolytic polypeptide; hSP, human spasmolytic polypeptide; pPSP, porcine pancreatic spasmolytic polypeptide; xSPL, *Xenopus laevis* spasmolysin.

METHODS

Cloning of rITF

Oligonucleotide primers were designed from the published sequence [3] to allow reverse transcription, then PCR amplication, of the coding region, as shown in Fig. 2. An abundant PCR

Abbreviations used: rITF, rat intestinal trefoil factor; 1 × SSC, 0.15 M-NaCl/0.015 M-sodium citrate; SP, spasmolytic polypeptide.

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBT Nucleotide Sequence Databases.

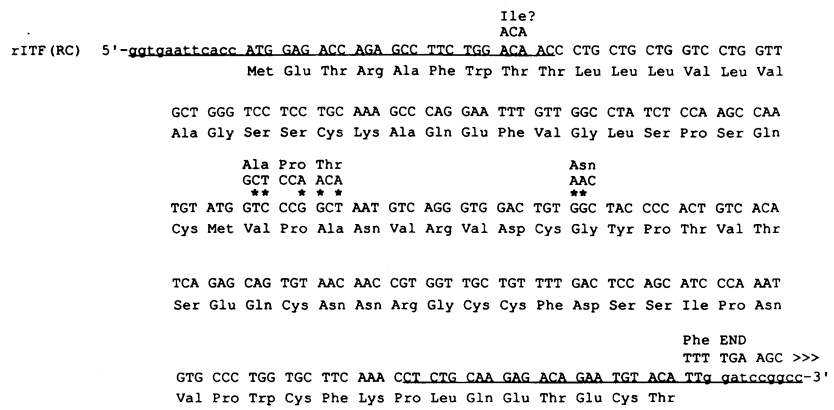

Fig. 2. Priming strategy and sequence of the ITF clone obtained

Primer regions are underlined. Differences with the sequence reported in [3] are given above the main sequence.

product of ~250 bp was obtained by using 5 μg samples of total RNA isolated from the distal small intestine of a male Wistar rat. The product was gel-purified, and an aliquot ligated into the plasmid Bluescript II KS+ (Stratagene) that had been linearized at the *Eco*RV site and T-tailed [8]. Transformants into the HB101 strain of *Escherichia coli* (Promega) were screened, yielding the clone rITF(RC). The sequence, determined on both strands using a Sequenase v2.0 kit (USB), is given in Fig. 2.

Hybridization *in situ*

A single-stranded antisense RNA probe to rITF was transcribed from *Eco*RI-linearized rITF(RC) DNA, using T3 DNA polymerase (Promega), and [^{35}S]UTP (~800 Ci/mmol; Amersham International) as the sole source of UTP. Histological 4 μm sections of rat tissues that had been fixed in neutral buffered formalin and embedded in paraffin wax were treated essentially as described by Senior and colleagues [9]. In brief, 1×10^6 c.p.m. of unhydrolysed probe in 10 μl of buffer [9] was hybridized overnight at 55 °C to sections permeabilized with proteinase K. Post-hybridization steps included several large-volume washes in a 50% formamide buffer at 55 °C to remove unhybridized probe, RNAase A treatment to digest single-stranded and imperfectly hybridized domains, and extensive washing to remove these cleaved fragments. The final washes were in 0.5 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate) at 65 °C for 30 min twice. Slides were dehydrated and processed for autoradiography (Ilford K2) at 4 °C for 7–10 days. Latent images were developed with Kodak D-19, and sections were Giemsa-counterstained.

As controls, parallel sections were hybridized to equal-radioactivity amounts of various other riboprobes, including rat β-actin, rat gastrin and mouse c-MET oncogene (a transmembrane tyrosine kinase; used as an irrelevant control probe), and exposed for at least the same time. Not one of these probes gave the same patterns of hybridization as that seen for the rITF probe.

RESULTS

The sequence of the rITF clone obtained differed in several details from the anticipated sequence [3]. Specifically (Fig. 2) we found two GG-for-AA substitutions, a T-for-A substitution, and a TC-for-CT conflict.

Hybridization signals for rITF were detected readily over a high percentage of goblet cells in the duodenum, small intestine and colon (Fig. 3). When the plane of sectioning was favourable, the distribution of silver grains over a goblet cell was in a cresent form, corresponding with the distribution of cytoplasm beneath the theca. A gradient in intensity of signal was present sometimes, with stronger signals over goblet cells near the crypt base, although there were regions, in the colon especially, where this gradient appeared reversed. No signals were detected over any cells within the Brunner's-gland acini; thus no cross-hybridization was occurring between our rITF probe and rat SP mRNA, which is abundant in that location (R. Poulsom, L. A. Rogers, J. M. Longcroft, A. M. Hanby, G. Jeffery, P. Oates & N. A. Wright, unpublished work). Similarly, no signals for rITF mRNA were found over cells in the pancreas, although probes for rat β-actin and rat SP gave distinct patterns of hybridization. We were unable to detect hybridization of our rITF probe to sections of human colon.

Surprisingly, we found signals for rITF mRNA in the stomach localized to an area of mucosa at the junction with the squamous fore-stomach (Fig. 3). The intensity of signal in this region was at least as great as that found in sections of intestine hybridized simultaneously.

DISCUSSION

Given the fact that *Taq* polymerase can introduce errors in cloning [10], we cannot state categorically that the sequence we found derived faithfully from an allele in the Wistar-rat RNA; however, we note that the peptide sequence deduced from our clone is conserved more closely than that reported by Suemori and colleagues ([3], with some ambiguities) to relevant regions of other trefoil peptides (Fig. 4).

Our *in situ*-hybridization results support the claim [3] that rITF is expressed in goblet cells and further show that Brunner's-gland acini and cells of the pancreas had undetectable levels of rITF mRNA. We consider that rITF, although a single trefoil peptide, is most unlikely to be the rat counterpart of the human single trefoil peptide pS2 [5], which is oestrogen- and epidermal-growth-factor/urogastrone-inducible, because we find pS2 to be expressed in goblet cells only in extreme circumstances, for example, in regions adjacent to chronic ulceration and next to the ulceration-associated cell lineage (UACL) [11–13].

We are not certain at present if the rITF hybridization signals we find in rat stomach are present in 'cardiac' gastric mucosa or in an area of intestinal metaplasia, which in man we find to affect

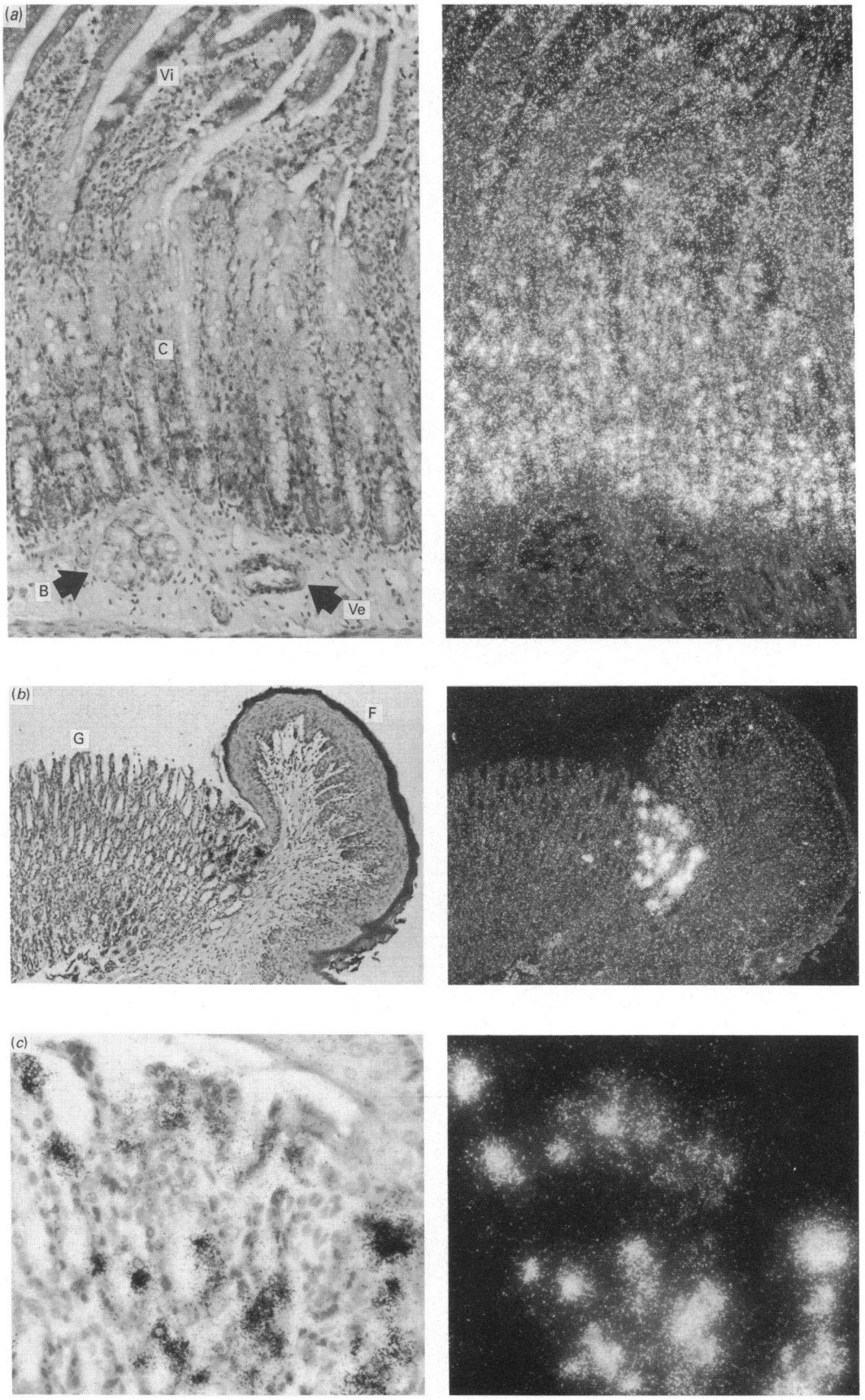

Fig. 3. Conventional (left-hand side) and dark field (right-hand side) micrographs of histological sections of rat tissues probed for rITF mRNA

(*a*) Low-power view of a region of the duodenum showing hybridization signals over goblet cells in the crypts (C) and on the villous surface, but not in Brunner's gland (B), villous core (Vi) or blood vessels (Ve). (*b*) Very-low-power view of the junction between the glandular gastric mucosa (G) and the keratinized squamous fore-stomach (F), showing intense hybridization signals over a restricted population of cells; the rest of the glandular gastric mucosa had no detectable signals. (*c*) High-power view of the junctional region.

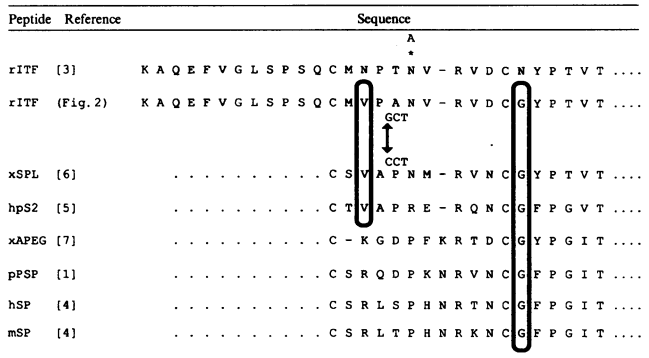

Fig. 4. Comparison of partial deduced sequences of rITF with parts of other trefoil peptides

*, These amino acids are A or N in Figs. 1 and 2 of [3]. The GCT codon is only one base different from hpS2, whereas the equivalent codon of [3] is two bases different. Abbreviations are as in Fig. 1.

the expression of both pS2 and human SP [14]. We speculate that the reported absence [3] from rat stomach of ITF mRNA and protein may be due to the relative insensitivity of Northern analysis compared with *in situ* hybridization and the examination by immunohistochemistry of only a limited region of the stomach. We hypothesize that the expression of trefoil peptides usually occurs only within areas of cells expressing a limited range of mucins of the MUC family: ITF with the intestinal mucins MUC2 and MUC3, for example [15], is perhaps even co-expressed in individual cells. Our rITF clone and its human counterpart will be of value in testing this unifying hypothesis.

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