Int. J. Dev. Biol. 42: 783-789 (1998)

Original Article

Trefoil peptides are early markers of gastrointestinal maturation in the rat

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ABSTRACT Trefoil peptides are members of a unique family of proteins found predominately throughout the gastrointestinal tract, whose proposed functions include mucus stabilization, stimulation and/or differentiation of epithelial cells during wound repair. Recent trefoil knockout studies have reported delays in epithelial cell migration or maturation pathways together with almost a complete lack of mucus. In order to fully explore the role of trefoil peptides in gastrointestinal maturation, these studies were undertaken to accurately characterize the expression of trefoil peptides in the developing rat gut. The results of RPA suggest that trefoil mRNA's are expressed as early as 15 days post coitus (dpc) in the intestine and stomach. Proteins are detected at 17 dpc by radioimmunoassay and immunohistochemical studies, which localize trefoil peptide expression to the lumenal surface of epithelial cells. At 17 dpc the gut is lined by pseudo-stratified, undifferentiated epithelial cells. Polarized, columnar cells are not detected until at least 18 dpc, with sparse mucus staining and parietal cell markers not being detected until 18 and 19 dpc respectively. This data demonstrates that trefoil peptides are early markers of epithelial cell maturation in the developing rat gut. The time course of their expression, well before the mucus cell type is specified, suggests a potential role in epithelial cell differentiation.

KEY WORDS: *trefoil peptides, gut, development*

Introduction

Trefoil peptides are members of a unique family of proteins characterized by containing one or more three-looped structural motifs held together by disulphide bonds between highly conserved cysteine residues. The known members of this family are pS2 (also known as TFF-1), spasmolytic polypeptide (SP, also known as TFF-2) and intestinal trefoil factor (ITF, also known as TFF-3) (Wright *et al.*, 1997). Trefoils are found throughout the adult gastrointestinal (G.I) tract, with pS2 and SP predominately found in the stomach and ITF in the intestine and colon. They are secreted by mucus producing cells of the normal G.I tract and are highly expressed in cells surrounding areas of damage in conditions such as peptic ulceration and inflammatory bowel disease (Rio *et al.*, 1991; Wright *et al.*, 1993; Thim, 1994). The function of trefoil peptides is not fully understood, but it has been proposed that in the normal gut the trefoil peptides may play a role in mucus stabilization. In addition, their up regulation at the site of mucosal injury is consistent with a role for these peptides in migration or differentiation during the epithelial repair process (Sands and Podolosky 1996; Cook *et al.*, 1997).

The results of gene-targeting experiments in which *pS2* (Lefebvre *et al.*, 1996) or *ITF* (Mashimo *et al.*, 1996) genes were made dysfunctional have highlighted the effects of trefoil peptides on migration of epithelial cells. In these studies, the proliferative (stem cell) compartment of the epithelium was greatly expanded in the colon for *ITF* and antral mucosa for *pS2*, strongly implying a defect in cell migration or maturation pathways. The latter was especially evident in *pS2* knockout mice in which the distal stomach was hyperplastic with inappropriate cellular differentiation patterns and almost entirely devoid of mucus. Many studies have demonstrated the motogenic capacity of recombinant trefoil peptides for different epithelial cell lines (Dignass *et al.*, 1994; Playford *et al.*, 1995) and it is possible that this may be an important function for trefoil peptides in fetal gut development.

Very little is known about the molecular mechanisms involved in vertebrate gut development. Reciprocal mesenchymal-epithelial inductive interactions are known to be of great importance in the functional differentiation of distinct regions of the gut (Simon-Assmann and Kedinger, 1993; Yasugi, 1993). Results of recent studies suggest that *Sonic hedgehog-Hox/Bmp* genes may be involved in epithelial-mesenchymal interactions in the earliest

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0214-6282/97/$10.00

Printed in Spain

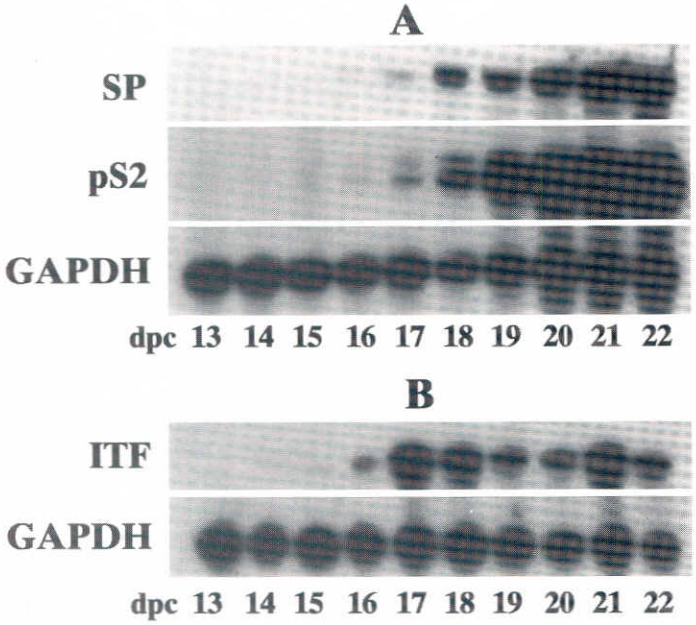

Fig. 1. Trefoil gene expression in the developing gastrointestinal tract. *Tissues were taken from different days of gestation (dpc) from rat embryonic tissues: stomach* **(A)** *or small intestine* **(B)** *or liver (L). Protected fragments of the following genes were detected by RPA in stomach SP, PS2 and GAPDH; and in the small intestine ITF and GAPDH*

stages of chick hindgut (Roberts *et al.*, 1995) and mouse gut (Bitgood and McMahon, 1995) development. In addition, regionalization of the gut tube occurs early in gut development and homeobox-containing genes have been implicated in this process (James *et al.*, 1994; Roberts *et al.*, 1995). The signals that guide stem cell fate decisions and which maintain the differentiated state of epithelial cells remain largely unknown.

Studies in the mouse have shown that in the liver (Gualdi *et al.*, 1996) and pancreas (Gittes and Rutter, 1992), tissue-specific genes are activated prior to morphological signs of differentiation. From the results of these studies, it was concluded that the activation of tissue-specific promoters can be an immediate response to signals that specify cell type, which suggests that tissue-specific genes may play a role in reciprocal interactions between inductive morphogenetic events and events leading to full cytodifferentiation.

There is very little information concerning trefoil peptide expression in the developing rat gut. The results of one study suggested that *ITF* is not expressed in the fetal rat as determined by northern analysis (Mashimo *et al.*, 1995), whereas Jeffrey and colleagues (1994) have shown that SP expression is already well developed by fetal day 20 in the rat, at levels exceeding that of the predominate gastric hormone, gastrin. The studies described in this paper provide evidence that trefoil peptides are expressed in the embryonic rat stomach and intestine, well before morphological signs of stomach/intestine maturation, and the expression is regionalized suggesting a possible role for these peptides in gut epithelial cell differentiation.

Results

The results of RPA are shown in Figure 1. In the stomach (Fig. 1A), mRNA for pS2 and SP are first detected in this assay at 15 and 16 dpc respectively, and continue to increase as the stomach develops, compared to the uniform levels of *GAPDH* gene expression. In the intestine (Fig. 1B), expression of *ITF* is observed starting at 15 dpc.

Protein levels of the trefoil peptides SP and ITF were quantified by RIA and shown in Figure 2. In contrast to the trefoil gene expression seen as early as 15 dpc in the developing rat gut (Fig. 2A), SP is quantifiable at 17 dpc at levels of 5.5 pmol/mg protein, which is low, but detectable. A large increase in SP expression is seen after birth. In an effort to examine different parts of the stomach for SP expression, the neonatal tissue was separated into antrum (Fig. 2A, designated a) and body (Fig. 2A, designated b). No significant difference in levels of SP expression were seen between body and antrum. ITF in the intestine is first detected at 17 dpc at levels of 75 pmol/mg protein and exhibits the same biphasic pattern of expression as seen for the gene expression (see Fig. 1B). There is a substantial increase in ITF after birth.

Figure 3 depicts the different stages of the developing rat stomach. Tissue sections at each time point were examined for immunoreactive SP expression (Fig. 3a-e), mucus as detected by AB/PAS staining (Fig. 3f-j), parietal cells as detected by ATPase proton pump protein antisera (Fig. 3l-o). SP is first detected by specific antisera at 17 dpc and is localized to the apical surface of the epithelial cells of the stomach (Fig. 3b). Note the pseudostratified undifferentiated appearance of the epithelial layer at this stage. As the stomach develops, pit glands are evident at 19 dpc (Fig. 3d) and SP expression is found in the apical region throughout the epithelial layer including the pit cells. The epithelium is now composed of polarized, columnar cells indicative of stomach maturation. At 20 dpc (Fig. 3e) there is extensive pit gland formation and pronounced SP expression. Mucus, first detected at 18 dpc (Fig. 3h,i) is sparse within the epithelial layer at this time and becomes more pronounced around day 20 (Fig. 3j). The parietal cell marker clearly stains cells at 19 dpc within the pit gland (Fig. 3n).

In embryonic intestinal tissue, ITF protein is first detected at 17 dpc dispersed in the apical region of epithelial cells (data not shown). As the tissue differentiates, the protein is later localized to the goblet cells of this developing tissue as seen in Figure 3, panel k, which shows an intestine at 19 dpc.

Discussion

The gut epithelium is a continuous developmental system in which such fundamental processes as cellular proliferation, lineage commitment and differentiation occur rapidly and perpetually in well demarcated anatomic units. However very little is known about these processes neither in the small intestine (Gordon, 1993) nor in the stomach. Therefore, to identify the factors that regulate how an uncommitted crypt or pit epithelial stem cell differentiates into a particular lineage, it is essential to delineate when tissue specific genes are first expressed in the developing gut and to look for factors that regulate this process in the embryo. The studies in this paper show that trefoil gene expression is clearly evident from as early as 15 dpc in the developing rat gut and

become restricted to mucus secreting cells of the stomach or goblet cells of the small intestine by 18 dpc. At 16 dpc, the stomach and intestine are lined by stratified epithelium, and in the latter tissue the lumen and villi are not yet present. At this developmental stage, all the known trefoil genes are activated, well before differentiation of the epithelium (as determined by the presence of polarized columnar epithelial cells) and well before stem cell commitment. At 18 dpc, a monolayer of partially polarized, cuboidal epithelial cells is established, a few secondary lumina are evident with the beginning of granule formation in the apical region of surface cells; and at 19-20 dpc villi and pit glands are beginning to form in the proximal-middle intestine and antral-fundic stomach respectively, with a single layer of polarized epithelial cells facing the lumen marking the differentiated phenotype. Our results are in agreement with previously reported morphological studies of rat fetal gut development (Helander, 1969; Yeomans *et al.*, 1976; Johnson, 1985; Seki *et al.*, 1993).

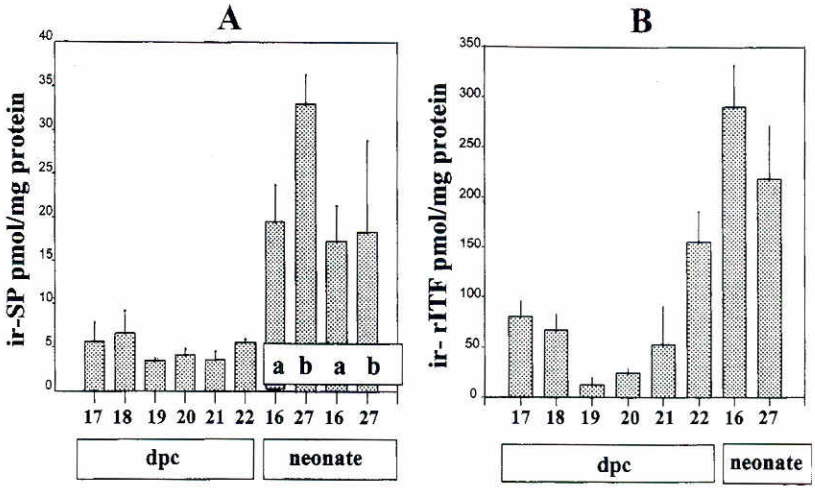

Fig. 2. Developmental expression of trefoil peptides SP and ITF in the gastrointestinal tract. *Levels of trefoil proteins in stomach* **(A)** *and ITF in small intestine* **(B)** *were determined by radioimmunoassay. Tissues were taken from different days of gestation (dpc) or postnatally (neonate). Neonatal tissues were dissected into the antrum (a) and body (b).*

Trefoil proteins are first detectable at 17 dpc, well before the columnar epithelium is evident. In comparison sparse mucus staining is not evident until 18 dpc and morphologically identifiable mucus granules are first detectable around 19 dpc (Yeomans, 1974). Parietal cells can be detected a day later, after the monolayer of cuboidal epithelial cells is well established, and has begun to invaginate the underlying mesenchymal tissue.

There are limited studies in the rat in which the developmental expression of stomach and intestinal epithelial-specific genes have been examined. At embryonic day 18, in the intestine several studies have demonstrated that glucagon-like immunoreactive peptides are found in the intestine and duodenum (Larsson, 1977; Brubaker, 1987; Kreymann *et al.*, 1991); and two glucagon mRNA species (one unique to intestine) were detected a day earlier by northern analysis (Hynes and Lund, 1986). Sucrase-isomaltase mRNA has been first detected by northern analysis at 18 days dpc (Leeper and Henning, 1990). Gastrin was detected immunohistochemically in the duodenum at 18 dpc (Larsson, 1977) or 19 dpc (Onolfo and Lehy, 1987). In the stomach, the gestational time of immunohistochemical detection of gastrin cells remains equivocal. For several investigators gastrin cells appear around 19 dpc (Larsson, 1977; Onolfo and Lehy 1987; Seki *et al.*, 1993), although Stein and Morris (1982) observed gastrin cells only after birth, conversely antral tissue gastrin was reported as early as 16 dpc (Braaten *et al.*, 1976). Immunohistochemical detection of insulin and several other peptides including CCK, VIP and secretin were found in the intestine at 18 dpc (Larsson, 1977). In the intestine, the transcripts and protein products of the gene for liver fatty acid binding protein (FABP) are detected between fetal days 17-18 in the jejunum but not until a day later in the ileum; although intestinal-*FABP* gene, mRNA and protein are found a day later (Rubin *et al.*, 1989; Rubin 1992). In the stomach, Iseki and colleagues (1991) localized immunoreactive proteins specific for heart-*FABP* in the parietal cells at 20 dpc, whereas in the surface mucous cells, liver-*FABP* and intestinal-*FABP* were detected at 19 and 22 dpc, respectively. Both lactase activity (reviewed in Henning, 1985) and lactase gene mRNA and protein expression (reviewed in Montgomery *et al.*, 1997; Rings *et al.*, 1992) are found on the 18th day of gestation. Both primitive parietal cells, with typical intracellular microcanaliculi (Helander, 1969), and peptic activity in homogenates of whole stomach have been found as early as 19 dpc (Helander, 1969; Hervatin *et al.*, 1987) but others have shown no basal acid secretion until 20 dpc (Garzon *et al.*, 1981). In the intestine, alkaline phosphatase mRNA was detected as early as day 18 of gestation when columnar epithelium are present in the proximal and middle regions of the intestine (Montgomery *et al.*, 1997) although alkaline phosphatase activity was detected a day earlier in intestinal tissue (Komoda, *et al.*, 1986). Somatostatin-positive cells were only seen in neonate stomachs and at 19 dpc in the duodenum in one study (Onolfo and Lehy, 1987), whereas Larsson (1977) has reported detection of this peptide in the antrum at 19 dpc. Clearly, in the developing rat stomach and intestine, trefoil peptides are expressed well before other markers of epithelial cell differentiation.

Determination and differentiation have been viewed as temporally and functionally distinct processes (Gilbert, 1994). However, recent studies have shown that liver-specific genes are activated prior to morphological signs of liver differentiation (Gualdi *et al.*, 1996) and also pancreatic specific genes are activated prior to detectable morphogenetic events (Gittes and Rutter, 1992). These studies have led to the suggestion that activation of tissue-specific promoters can be an immediate response to signals that specify cell type. In addition, in mouse, pancreas-specific gene expression is localized to the area of the foregut from which the pancreas will eventually be formed, leading Gittes and Rutter (1992) to suggest that there is a "premorphogenetic phase" of organogenesis which may represent the initiation of endocrine lineages, suggesting that the presence of tissue-specific proteins may play a role in reciprocal interactions between morphogenetic events and events leading to full cytodifferentiation. Thus, in the stomach and intestine, similarly in the liver and pancreas, tissue-specific genes are expressed before overt differentiation of epithelial cells. Therefore,

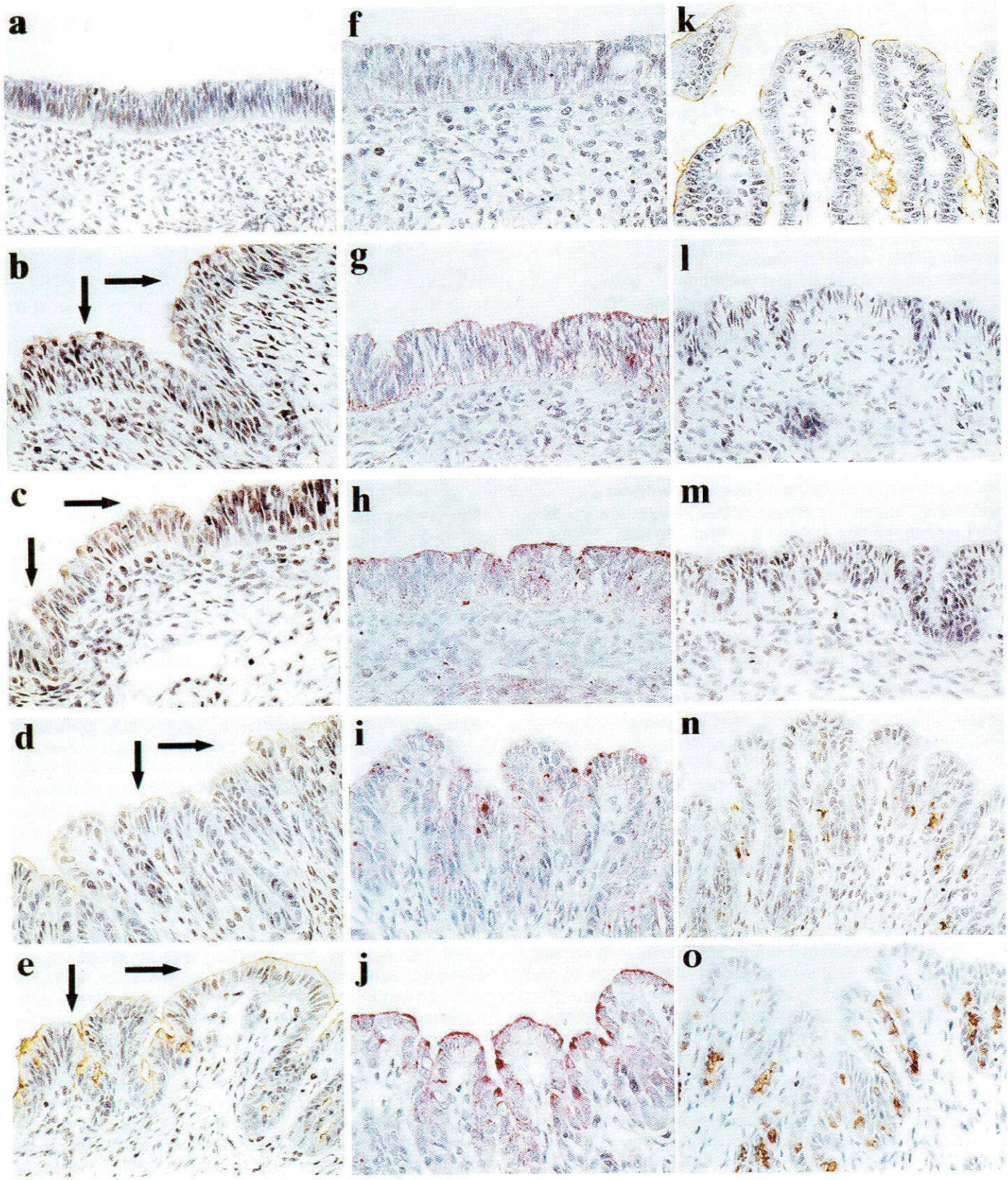

Fig. 3. Localization of trefoil proteins in epithelial cells of the developing stomach and intestine. *Immunohistochemical staining of the stomach tissue from embryonic days 16* **(a,f)**, *17* **(b,g,l)**, *18* **(c,h,m)**, *19* **(d,i,n)** *and 20* **(e,j,o)**. *Tissue sections were treated with antiserum for SP (a-e) and ATPase proton pump protein (l-o). AB/PAS staining is shown in panels f-j. Arrows indicate SP staining in apical surface of epithelial cells (b-e).* **(k)** *A section of small intestine at 19 dpc stained with antiserum for ITF showing localization of trefoil peptide to goblet cells. Magnification 400x.*

it is possible that trefoil peptides may be involved in differentiation of the mucus cell type. This latter possibility is supported by the phenotype of the recent pS2 knockout mice (Lefebvre *et al.*, 1996), in which there is inappropriate cellular differentiation patterns, strongly suggesting that trefoils may be essential for normal differentiation of epithelial cells.

The results of this study indicate that the trefoil proteins are expressed well after their mRNA's are detectable and the genes activated. Others have also reported a lag time between expression of the gene and its corresponding protein. In the pancreas, it was reported that endocrine specific transcripts were detected significantly earlier than the first detection of the corresponding protein by RIA or immunohistochemistry (Gittes and Rutter 1992). These authors have suggested the delay in detection of proteins following detection of transcripts may be due to the sensitivity of RT-PCR which is estimated to be at least 10^8 times more sensitive than protein assays. Alternatively, they have suggested that the early endocrine cell may contain only a constitutive secretory pathway and so the synthesized protein does not accumulate in secretory granules. In agreement, we have shown that trefoil peptides may be secreted either constitutively or in a regulated fashion by cell lines depending on the extent of differentiation (Tran *et al.*, 1998), and this may explain the diffuse localization of the trefoil peptides when first detected at 17 dpc in the apical region of epithelial cells. The lactase protein was not detected on fixed proximal intestinal tissue sections until 2 days after mRNA was first identified in the developing rat intestine at 18 dpc (Rings *et al.*, 1992). However, in a later study, these investigators were able to detect immunoreactive lactase on frozen tissue sections at 18 dpc (Montgomery *et al.*, 1997). We have used fixed tissue sections in our studies and this may account for the delay between message and protein detection. A delay in maturation of the translational machinery is ruled out by the detection of other proteins such as the urea cycle enzymes (Gaasbeek *et al.*, 1988) as early as 14 dpc in the intestinal epithelium.

We have demonstrated that the trefoil peptides are found in pre-mucus epithelial cells of the developing rat gut and that they are expressed prior to the development of the polarized columnar epithelium. This expression occurs several days before expression of other markers of epithelial cell differentiation. Thus it appears that in the rat gut, trefoils are among the first of the known markers of epithelial cells to respond to signals that specify cell type, and may even be involved in the induction of differentiation pathways, or in cell positioning during gut maturation. A motogenic role for trefoil peptides in gut development is suggested by the fact that studies in which the function of the trefoil peptides are ablated have shown that the stem cell proliferation compartment is greatly expanded (Lefebvre *et al.*, 1996; Mashimo *et al.*, 1996) implying a defect in cell migration. In the pS2 knockout mice (Lefebvre *et al.*, 1996), not only was hyperplasia evident in the distal stomach but there was also inappropriate cellular differentiation patterns and almost a complete lack of mucus, strongly suggesting that SP in the stomach may also be involved in epithelial cell differentiation.

Another possible explanation for the early expression of the trefoil peptides in fetal life may lie in the well demonstrated protective and reparative properties of the trefoils, especially in conjunction with the mucus glycoproteins (Kindon *et al.*, 1995; Babyatsky *et al.*, 1996; Playford *et al.*, 1996). There is an important need to establish the protective trefoil-mucus system as part of the formative gastrointestinal mucosal barrier, prior to the development of enzyme and acid secretory capacities. Current studies in our laboratory, using *in vitro* models are underway to examine the effects of recombinant trefoil peptides on epithelial cell differentiation and migration in the developing rat gut.

Materials and Methods

Animals

Sprague-Dawley rat mating pairs were set up. Matings were checked twice daily. Vaginal plug detection was defined as day 0 and assumes mating within a twelve hour period. Rat embryos (8-14 per pregnant female) were obtained from each time point between 13 dpc until just before birth (22 dpc) and pooled.

Tissue extraction

Pooled tissues were placed in either: 10 volumes of Solution D (4M Guanidium thiocyanate, 0.5% sarkosyl, 25 mM NaCitrate, 0.1 M β-mecaptoethanol) for RNA extraction; or 10 volumes of boiling water for 2 min for protein extraction and radioimmunoassay (RIA). Stomach samples used for protein determination by RIA were also taken from rats after birth just prior to weaning (neonate day 16) and after weaning (27days).

Probes

Rat trefoil cDNA's used for generating ITF (423bp/Bluescript) and SP (280bp/pGeml) riboprobes were generous gifts from Professor D.K. Podolsky, Massachusetts General Hospital, Boston, USA and Dr. G. Jeffrey, University of Western Australia, respectively. Rat GAPDH cDNA (300bp/Bluescript) was donated by Dr. P. Fuller, Prince Henry's Institute for Medical Research, Clayton, Australia. Rat pS2 was generated by RT-PCR using the following primers, the restriction sites are underlined: forward primer 5'-CAGCTCGAGTCTTCCCTGGAAGCTGCCAT-3', reverse primer ATGTCT AGACTTGCTGGTTCTCAATGACC. Primer sequences were taken from GenBank entry (accession number D83231) for rat pS2. One μg total adult stomach RNA primed with 0.5 μg oligo-dT and reverse transcribed with 200U MMLV (Promega) in 40 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 40 U RNasin (Promega), 500 μM dNTP's was used as a template for PCR. 10 μl of diluted oligo-dT primed RT reaction was used as template for PCR. Reaction conditions were: 2 mM MgCl, 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton, 200 μM dNTP's, 25 ng of each primer, 2.5U Taq Polymerase (Promega) in total of 50 μl. PCR conditions were as follows: 95°C 3', 1 cycle; 95°C 30s, 50°C 30s, 72°C 60sx30 cycles, 72°C 5' 1 cycle. The XhoI/XbaI RT-PCR-generated 250bp pS2 fragment was subcloned into Bluescript (Stratagene) and sequenced (Omnibase, Promega) for verification.

RNase Protection Assay (RPA)

Total RNA was extracted from embryonic stomach and intestine using the acid phenol method developed by Chomczynski and Sacchi (1987). Antisense ^{32}P-UTP-labeled pS2, SP, ITF and GAPDH riboprobes were incubated with 2 μg of total RNA and hybridized overnight at 42°C essentially as described by Krieg and Melton (1988), except that all hybrids were digested with 20 μg/ml RNase A and 1 μg/ml RNase T1 (Sigma) at 37°C for 30 min. Protected fragments were resolved on 5% acrylamide/8 M urea gels.

Radioimmunoassay

Characterized, specific rat SP and ITF antisera for use in RIA has been previously reported. (Taupin *et al.*, 1995). No antiserum specific for rat pS2 is available and so tissue levels of this peptide were not quantified. Results are expressed as fmol immunoreactivity per mg tissue protein. Protein content was quantified essentially as described by Bradford (1976) except that bovine serum albumin was used as standard. Because of the very small amounts of tissue available, trefoil concentrations could not be accurately quantified in rat gut before 17 dpc.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 2-4 h before embedding in paraffin. Five micron tissue sections were washed in PBS and treated with 3% H_2O_2/methanol for 15 min to quench endogenous perioxidase activity. Sections were preincubated with 5% normal goat serum at room temperature for 30 min followed by incubation with primary antibody to either SP (1:1000 dilution), ITF (1:1000 dilution), 1H9 (antisera against the alpha subunit of the H+K+ ATPase proton pump protein, 1:2000 dilution; a generous gift from Professor T. Masuda, Kyoto University), for 60 min. Sections were subsequently reacted with biotinylated secondary antibody for 30 min before avidin in horseradish peroxidase for 30 min. Product development was observed with the addition of the peroxidase substrate solution diaminobenzidine (DAB). Sections were counterstained with Giemsa and photographed with a Leica MPS 52 camera.

Acknowledgments

The authors wish to thank Dr. D. Newgreen for critical reading of the manuscript. This work in part was supported by NHMRC funding to A.S.G and N.Y.D.

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Received: February 1998
Accepted for publication: April 1998