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STRUCTURE AND EXPRESSION OF MURINE INTESTINAL TREFOIL FACTOR: HIGH EVOLUTIONARY CONSERVATION AND POSTNATAL EXPRESSION

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SUMMARY Intestinal Trefoil Factor (ITF) is a member of a family of gastrointestinal tract peptides with region-specific expression which are enhanced at sites of injury and repair. In the present study, the murine homologue gene of ITF was molecularly cloned in order to characterize the structure and expression of this peptide in mice. Murine ITF exhibited 78, 95 and 94% nucleotide homology respectively in exons I, II and III, with overall 90% predicted amino acid identity when compared to the rat ITF. Murine ITF exhibited 70% inferred amino acid identity compared with human ITF. Northern blot analysis of various adult mouse tissues demonstrated that ITF is expressed abundantly in the intestine and colon, and minimally in stomach, but not in brain, lung, spleen, kidney, uterus, pancreas, liver, heart or thymus tissues. Expression of ITF appeared to occur as a post-natal event: antibody specific for ITF stains intensely goblet cells in the intestine and colon of three-day old and older mice, but not in the gastrointestinal tract of younger mice or embryos.

INTRODUCTION - The trefoil peptides comprise a "new" family of gastrointestinal tract peptides with a unique three-loop structure formed by a distinctive motif of cysteine disulfide bonds (1). In humans and rats, three trefoil peptides have been identified: pS2 expressed mainly in the stomach (2,3), SP in the pancreas and stomach (4,5,6), and ITF in the small and large intestine (7,8,9). In contrast to their normal regionally restricted distribution, in inflammatory disease states more than one trefoil peptide may be secreted at sites of mucosal injury and repair, (10,11,12,13). Hyperplastic or metaplastic polyps in the colon also express trefoil peptides (14).

Despite delineation of the pattern of expression of these abundant peptides in the gastrointestinal tract, the functions of the trefoil peptides are incompletely understood. *In vitro* studies in our laboratory suggest that trefoil peptides promote re-establishment of mucosal integrity after injury (15). Addition of trefoil peptides to wounded monolayers of confluent intestinal epithelial cell line resulted in three- to five-fold increase in rate of epithelial migration in wounded monolayers.

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Abbreviations: MITF, RITF, HITF, mouse, rat, and human Intestinal Trefoil Factor; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DAB, draminobenzidine tetrahydrochloride.

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This restitution was further enhanced by addition of mucin glycoproteins purified from colon or small intestine (15).

Further characterization of the functional role of trefoil peptides may be facilitated by comparison of interspecies variation in structure and expression as well as animal species such as mice, in which models of inquiry can be established. As initial study to understand the role of trefoil peptides in mice, we sought to isolate the murine ITF homologue and to define its expression.

MATERIALS AND METHODS

Southern and Northern Blot Analyses - Spleen DNA of 129/J mice was prepared according to Blin and Stafford (16). Whole RNA was prepared from adult and embryo mouse tissues essentially according to Auffray and Rougeon (17). Methods of agarose electrophoresis and transfer onto Hybond -N membrane (Amersham, Arlington Heights, IL) were according to the manufacturer's protocol. The full length rat ITF cDNA (8) was labeled with αP^{32}-GTP using random primers (Strategene, La Jolla, CA). Hybridization was carried out at 65°C overnight (in 5X SSC, 50mM Phosphate Buffer pH7.4, 1% SDS, 100g/ml denatured salmon sperm DNA, $2x10^7$ cpm probe) and washes at 60°C (in 1XSSC 1%SDS for 1 hour, then 0.1X SSC 0.1%SDS for 1 hour).

ITF Gene Isolation and Sequencing - The murine ITF gene was isolated from a partial Sau3A-digested 129 strain liver genomic DNA library (Strategene, La Jolla, CA) using standard screening protocols (18). The gene was subcloned into Bluescript II KS+ phagemid (Strategene, La Jolla, CA), and parts of the gene were sequenced, including its three exons in their entirety, using standard dideoxy (19) and automated sequencing (Taq DyeDeoxy , Applied Biosystems, Foster City, CA) methods. Primers used in sequencing strategy are shown as Figure 2.

Histological Staining - Whole mouse embryos of various stages (blastocyst, E8, E12, E16), newborn, 3 day- and 6 day-old, and tissues from adult mice were fixed by immersion in buffered 4% paraformaldehyde in PBS, equilibrated in 20% sucrose in PBS, frozen over dry ice while embedded in OCT Compound (Miles Inc., Elkart, IN), and cut onto glass slides in 12μm sections. These sections were thawed, dried, treated with acetone, taken to water, and stained using primary rabbit antiserum raised to the 14-amino acid carboxy-terminal peptide of ITF which is divergent in structure from the other known trefoil products followed by a biotinylated horse peroxidase-conjugated secondary antibody and 0.1% DAB substrate as suggested by the manufacturer (Vector Laboratories, Burlingame, CA).

RESULTS

Isolation and Sequencing of the Murine ITF Gene - Southern genomic analysis (Figure 1) confirmed the specificity of the rat cDNA ITF probe under high stringency hybridization conditions described above. Single major bands were identified with various restriction enzyme digests, indicative of a single gene. This probe was then used to screen the phage genomic library of 129 strain mouse under the same hybridization and washing conditions. One of the five positive clones contained the entire ITF gene, including 890 base pairs 5' of the transcriptional initiation site, and extending approximately 6.3kb 3' of the stop codon (Figure 2).

Nucleic acid sequencing of subclones revealed an RNA polymerase II promoter site in the 5' flanking region (20). Other salient features in this region include a 220bp AT-rich region, as found in the rat 5' flanking region (21), which contains a Pit-1 (5'-AATTATACAT-3') homeobox domain (22). There is also a 15-fold repeat of dC-dA dinucleotides capable of forming z-DNA sequences

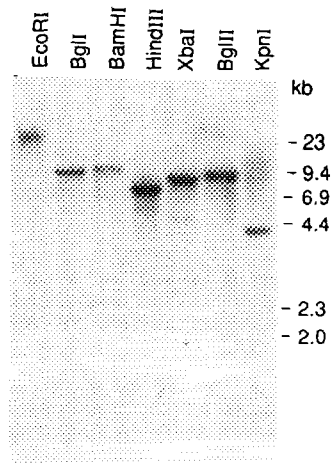

Fig. 1. Genomic Southern Blot Analysis of 129 Mouse DNA Using Rat ITF cDNA Probe. Digested DNA was separated on 0.8% agarose gel. Position of Lambda-Hind III DNA markers as shown.

which are often present in estrogen response elements (23) and which are believed to play a role in evolution of the eukaryote genome by promoting recombination (24). No overt enhancer sequences or known intestine-specific regulatory elements were identified in the regions sequenced.

When compared to the rat ITF gene (21) (Figure 3), the exon borders of murine ITF are conserved, and there are no premature stop codons within these exons. The conservation of DNA sequences is high, particularly in the exons (Table I). The inferred protein structure of ITF reveals absolute conservation of the cysteine residues necessary for the unique disulfide-bonded structure of this family of genes. Moreover, the predominance for silent nucleotide mutations and conserved amino acid changes suggests evolutionary pressure to conserve the ITF structure over the estimated 20-30 x 10^6 years phylogenetic distance between the *Mus* and *Rattus* genera.

The predicted amino acid sequence of murine ITF was compared to those of rat and human trefoil peptides (Table II). The degree of ITF sequence identity is greater between different species (90 and 70% identity) than between the different trefoil homologues within the species (28 and 36% identity), suggesting that these homologues became established before the divergence of rodent and simian mammals.

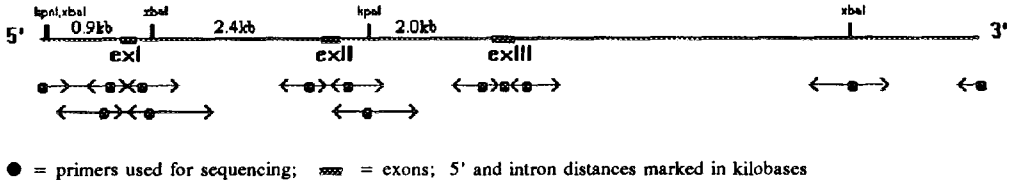

Fig. 2. ITF Gene Sequencing Strategy.

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EXON I
AA:    M  E  T  R  A  L  W  L  M  L  L  V  V  L  V  A
mIFT:  ATGGAGACCAGAGCCCTCTGGCTAATGCTGTTGGTGGTCCTGGTTGCT
rITF:  ATGGAGACCAGAGCCTTCTGGACAACCCTGCTGCTGGTCCTGGTTGCT
AA:                    F     T  T     L  L

AA:    G  S  S  G  I  A  A  D  Y  V  G  L  S
mIFT:  GGGTCCTCTGGGATAGCTGCAGATTACGTTGGCCTGT
rITF:  GGGTCCTCCTGCAAAGCCCAGGAATTTGTTGGCCTAT
AA:          S  C  K  A  Q  E  F        L

EXON II
AA:       P  S  Q  C  M  V  P  A  N  V  R  V  D  C  G  Y
mIFT:  CTCCAAGCCAATGTATGGTGCCGGCAAATGTCAGAGTGGACTGTGGCTAC
rITF:  CTCCAAGCCAATGTATGGTCCCGGCAAATGTCAGGGTGGACTGTGGCTAC
AA:                       V              R

AA:    P  S  V  T  S  E  Q  C  N  N  R  G  C  C  F  D
mIFT:  CCCTCTGTCACATCGGAGCAGTGTAACAACCGTGGTTGCTGCTTTGAC
rITF:  CCCACTGTCACATCAGAGCAGTGTAACAACCGTGGTTGCTGTTTTGAC
AA:       T        S                          C

AA:    S  S  I  P  N  V  P  W  C  F  K  P  L  Q  E  T
mIFT:  TCCAGTATCCCAAATGTGCCCTGGTGCTTCAAACCTCTGCAGGAGA
rITF:  TCCAGCATCCCAAATGTGCCCTGGTGCTTCAAACCTCTGCAAGAGA
AA:       S                                    Q

EXON III
AA:       E  C  T  F  *
mIFT:  CAGAATGCACATTTTGA
rITF:  CAGAATGTACATTTTGA
AA:          C
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Fig. 3. Murine Compared to Rat Intestinal Trefoil Factor Sequence.

Spatial and Temporal Pattern of ITF Expression - Intense staining was found in the goblet cells of intestine and colon in mice at the age of 3 days and beyond (Figures 4,5), but not in embryos or newborns.

This pattern of tissue expression in adult mice parallel the pattern of mRNA detected by Northern blot analysis using the rat ITF cDNA clone as probe (Figure 6). Abundant MITF mRNA was identified in mouse intestine and colon (latter not shown); mRNA was detected in stomach with longer exposure to film. No mRNA could be detected in brain, lung, spleen, kidney, uterus, pancreas, liver, heart or thymus tissues.

TABLE I: Comparison of Murine to Rat ITF Sequences

	NT Homology (%)	AA Homology (%)	Silent Mut'ns (% of changes)
5' Flanking	82		
Exon 1	78	72	33
Intron 1	83		
Exon 2	95	100	100
Intron 2	72		
Exon 3	94	100	100
3' UT	85		

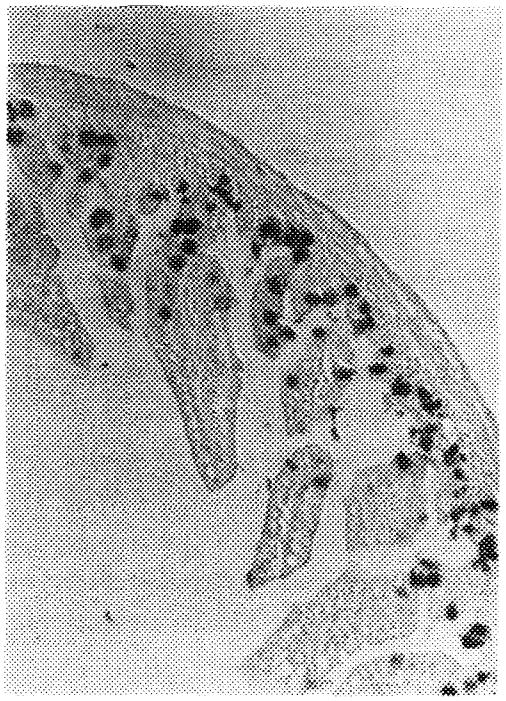
TABLE II:Amino Acid Identity Between Various Trefoil Peptides

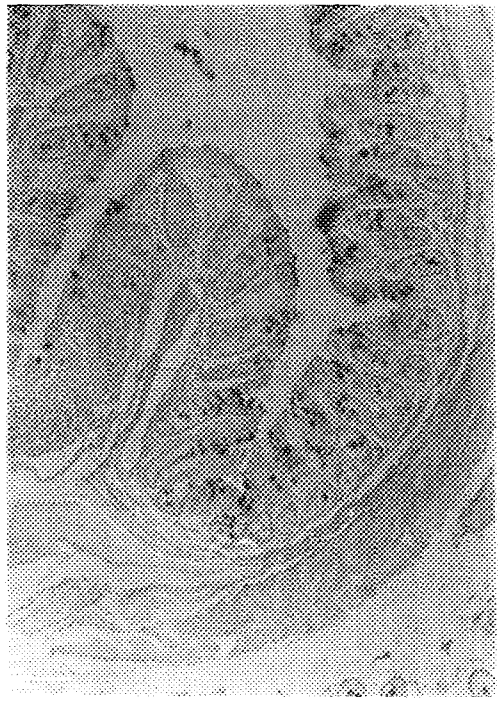
mITF	x	rITF	90%
mITF	x	hITF	73%
hITF	x	hpS2	36%
hITF	x	hSP	28%
mSP	x	rSP	93%
mITF	x	mSP	36%
mITF	x	mpS2	35%

Reference sources for primary sequences: rITF (8,9), hITF (13), hpS2 (25), hSP (26), mSP (27), mpS2 (27); m=mouse, h=human, r=rat.

DISCUSSION

This study establishes: (1) in mice, as in humans and rats, there are at least three homologous and highly conserved trefoil genes, each with distinctive patterns of expression; (2) the degree of ITF sequence homology is greater between different species than between the different trefoil homologues





(4) (5)

Fig. 4. Anti-ITF Staining of 3-day-old mouse Ileum (500X magnification).

Fig. 5. Anti-ITF Staining of 3-day-old Mouse Colon (100X magnification).

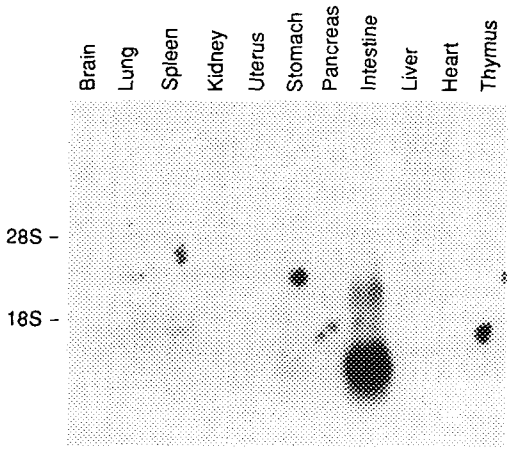


Fig. 6. Northern Blot Analysis of ITF in murine tissues. 20μg of total RNA was loaded per lane and separated on 1% agarose gel.

within the species; and (3) ITF is expressed in the goblet cells of the intestines of three-day old mice or older, but is expressed minimally in younger mice and embryos.

In normal growth and development, small intestinal epithelial cells arise from stem cells in the intestinal crypts and progress up the crypt and villus to be extruded from the villus tip within five days. During this course, latent genes are activated and expressed, and proteins which carry out the functions of mature enterocytes and goblet cells are expressed as the cells undergo differentiation along this crypt-villus axis. As noted, in normal adults the trefoil peptides have regional specific expression in the digestive tract: pS2 in the stomach, SP in the pancreas, and ITF in the intestine and colon. Moreover, all three peptides are expressed at sites of injury and repair. Given such specificity of expression within the digestive tract, these peptides might serve as novel markers for the study of embryological and postnatal development of the gastrointestinal tract. In the mouse, it appears that ITF expression in the intestine occurs only after birth, presumably in response to suckling. Evidence from our laboratory suggests that trefoil peptides play a key role in both sustaining mucosal integrity and facilitating repair after injury. The cloning of the murine ITF has enabled the development of mice unable to produce trefoil peptide through homologous reconstruction of a disrupted gene in pluripotent embryonic stem cells. Characterization of these mice should permit evaluation of the functional role of ITF in the functional maturation of the intestine and maintenance of the mucosal barrier.

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