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Molecular Cloning of the Rat Intestinal Trefoil Factor Gene

CHARACTERIZATION OF AN INTESTINAL GOBLET CELL-ASSOCIATED PROMOTER*

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Intestinal trefoil factor (ITF) is a small peptide bearing the unique motif of intrachain disulfide bonds characteristic of the trefoil family. Previous work had localized expression of ITF primarily within goblet cells in the small and large bowel, making it a candidate gene for the study of the molecular basis of intestinal and goblet cell-specific gene expression. In order to study the regulation of ITF expression, we have cloned the rat ITF gene and sequenced 1.7 kilobases of the 5′-flanking region. RNase protection analysis demonstrated a single transcriptional start site. Various lengths of the 5′-flanking region were linked to the reporter gene luciferase and transfected into the colon cancer cell lines LS174T and Caco-2, representing, respectively, cells with and without goblet cell-like phenotype. Expression in the goblet cell-like LS174T colon cancer cell line was nearly 10-fold greater than expression in Caco-2 cells which exhibit columnar enterocyte-like phenotype. The pattern of goblet cell-associated selective transcription required only 153 base pairs of the rat ITF 5′-flanking sequence. Transfection of a construct of human growth hormone under the control of the rat ITF promoter in the N2 subclone of HT-29 cells demonstrated expression of the reporter gene only in those cells exhibiting a goblet cell phenotype as assessed by expression of immunoreactive mucin. These initial studies of the 5′-flanking region of the ITF gene demonstrate the presence of cis-regulatory elements capable of directing goblet cell specific expression.

Goblet cells are abundant constituents of the surface epithelium within the small and large intestine. Although these cells have been long recognized to secrete a complex mixture of mucin glycoprotein onto the cell surface, their functional importance in gastrointestinal tract mucosa has not been well defined. Characterization of the molecular basis of goblet cell differentiation and function has also been quite limited. Although substantial advances have been made in the cDNA cloning of genes encoding the protein backbones of mucin glycoproteins from the gastrointestinal tract and elsewhere, the enormous size and complexity of these genes have made these of limited utility to define those elements responsible for goblet cell specific expression (1–7).

Observations in recent years have led to the recognition of a family of small proteins which are also selectively expressed by mucin-producing goblet cells in gastrointestinal tract mucosa. This family of proteins, designated trefoil peptides, are distinguished by a unique six-cysteine motif (called a "P" domain) which results in the formation of three intrachain loops within these small peptides due to disulfide bond formation in a 1-5, 2-4, 3-6 configuration (8, 9).

Members of the trefoil peptide family appear to be expressed in a region-specific fashion along the length of the gastrointestinal tract. Human spasmolytic polypeptide, or hSP, bears two trefoil motifs and is expressed primarily in the stomach (10), although the porcine homologue was originally isolated from pancreas (11). pS2, bearing a single trefoil motif and initially cloned as the product of an estrogen-responsive gene from a breast cancer cell line (12), is normally expressed only in the gastric antrum in man (13). Cloning of the rodent homologues of pS2 and hSP confirmed that expression of these distinct, but closely related peptides is site-specific along the longitudinal axis of the upper gastrointestinal tract (14, 15).

Intestinal trefoil factor (ITF)[1] is a third member of the trefoil peptide family identified in man (16) and in rat (17) which contains a single P domain. In contrast to pS2 and hSP, ITF is normally selectively expressed in the normal small and large intestinal mucosa complementing the pattern of expression of the other members of the family in the normal gastrointestinal tract (16, 17). More specifically, ITF expression is normally confined to the goblet cell population with the intestinal epithelium (17).

Understanding of the functional role of the trefoil peptides in gastrointestinal tract biology is now emerging. Recent studies using an *in vitro* model have demonstrated that these peptides may play a key role in facilitating healing at sites of mucosal injury by enhancing cell migration from the wound edge (18). This concept is consistent with observed patterns of expression of trefoil peptide in association with injury in gastrointestinal tract disorders. Thus, expression of hSP and pS2, normally confined to the proximal gastrointestinal tract, as well as ITF has been observed adjacent to intestinal ulceration in patients with Crohn's disease and peptic ulcer disease in man (19), whereas ITF is expressed in stomach in conjunction with the other trefoil peptides in an animal model of gastric ulceration (20).

The selective expression of ITF in intestinal goblet cells suggests that characterization of the gene encoding this peptide may provide insight into regulatory elements responsible for goblet cell-associated gene expression. Among the trefoil

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[1] The abbreviations used are: ITF, intestinal trefoil factor; RITF, rat intestinal trefoil factor; EGF, epidermal growth factor; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; EGF, epidermal growth factor.

family members, only the pS2 gene normally expressed in gastric mucosa has been reported (21, 22). Although several genes expressed in the intestine have been cloned and their regulatory elements studied, none are products of goblet cells. Intestinal fatty acid binding protein, cloned in rat and human (23), human villin (24), human and mouse sucrase-isomaltase (25, 26), porcine aminopeptidase N (27), human intestinal alkaline phosphatase (28), and human lactase-phlorizin (29) all represent proteins expressed in the columnar absorptive enterocyte. It is not known whether the regulatory elements which direct intestinal-specific expression of enterocyte products are also important in other intestinal epithelial cell populations. To investigate the mechanisms responsible for goblet cell differentiation within the intestine, and as a basis for further study of the genetic elements responsible for "ectopic" expression of ITF in pathologic conditions, we have cloned the rat ITF gene and its 5'-flanking region. Reporter gene constructs under the control of the rat ITF promoter were able to direct expression in a relatively goblet cell-specific fashion.

MATERIALS AND METHODS

Genomic Library Screening, Mapping, Subcloning, and Sequencing—A rat genomic library constructed by a partial digest of DNA from an adult male Sprague-Dawley rat with *Sau*3A cloned into EMBL3/SP6/T7 λ phage was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). This library contains 2.3×10^6 independent clones with an average insert size of 16 kb (range: 8–20 kb). Phage were plated out on 150-mm plates at a density of 5×10^4 plaque-forming units per plate in a bacterial lawn of competent *Escherichia coli* NM538. After 8 h of growth at 37 °C duplicate nitrocellulose filter lifts were made. After phage denaturation and cross-linking by standard methods, membranes were prehybridized in a solution containing 50% formamide, 5 × SSC (SSC: 0.15 M NaCl, 0.05 M sodium citrate), 20 mM Tris-Cl, pH 7.5, 5 × Denhardt's solution, 0.1% sodium dodecyl sulfate, 100 μg/ml of salmon sperm DNA at 42 °C for 2 h. Membranes were then placed in a solution identical to the one used for prehybridization substituting 10% dextran sulfate for SDS and using 3×10^5 cpm/ml of ^{32}P-labeled T3411 probe. T3411, the rat intestinal trefoil factor cDNA described previously (17), was labeled by random priming using Klenow DNA polymerase (U. S. Biochemical Corp.) (30). Push columns (Primerase columns; Stratagene, La Jolla, CA) were used to remove unincorporated nucleotides. Hybridization was performed for 48 h at 42 °C, followed by three 10-min washes at room temperature in 2 ×SSC, 0.1% SDS, and then two 20-min washes at 37 °C in 0.2 × SSC, 0.1% SDS. Membranes were blotted and exposed overnight to Kodak XAR film with intensifying screens at −70 °C. Positive plaques were picked and phage eluted into λ diluent before secondary screening on 100-mm plates. Conditions for secondary and tertiary screening were performed in a manner identical to primary screening.

Phage DNA was prepared from plate lysate by a modification of methods described in Maniatis *et al.* (31).[2] Plate lysate was spun at 10,000 × *g* for 10 min at 4 °C and the supernatant collected and subjected to 72,000 × *g* for 3 h at 4 °C. Pellets were resuspended in 500 μl of λ diluent, two samples pooled, spun briefly to pellet contaminants, and the cleared supernatant layered over a CsCl gradient with SM (SM: 100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin) as the diluent, set up as 1 ml with $\rho = 1.7$, 1 ml with $\rho = 1.5$, and 2 ml with $\rho = 1.45$ in a 13 × 51-mm centrifuge tube. Gradients were subjected to 120,000 × *g* at 20 °C for 1 h. Intact bacteriophage was collected from the interface of $\rho = 1.5$ and 1.45 and dialyzed twice for 1 h against 100 mM NaCl, 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$. Phage solution was digested with 50 μg/ml proteinase K at 65 °C for 4–12 h and phage DNA extracted with phenol once, phenol/chloroform once, and chloroform once, then ethanol precipitated and resuspended in 50 μl of TE buffer (10 mM Tris-Cl, pH 8, 1 mM EDTA).

Phage DNA from each plaque-purified cross-hybridizing phage was subjected to restriction digestion with a variety of restriction enzymes and Southern blot was performed with the T3411 probe used to screen the genomic library. Based upon Southern blot, an appropriate restriction fragment containing the entire RITF gene was selected for subcloning into plasmid. The selected restriction fragment was ligated into appropriately cut pKS+ Bluescript plasmid (Stratagene) and used to transform competent XL1-Blue *E. coli* (Stratagene). Large scale preparation of plasmid DNA was performed by CsCl gradient and by affinity resin purification (Magic Maxiprep; Promega, Madison, WI). Exons, large portions of introns, and the entire 5'- and 3'-flanking regions were sequenced using Sequenase (U. S. Biochemical Corp.) and oligonucleotide primers synthesized in a sequential overlapping manner. Final sequence was determined from both strands. Reaction products were resolved on denaturing polyacrylamide gels by standard techniques. Sequence comparisons were made using the BESTFIT and BLAST programs (32). The 5'-flanking region of the RITF gene was searched for potential enhancer, promoter, and repressor elements using Signal Scan v.3.01 (33). The sequences of the 5'-flanking regions of reported genes expressed in intestinal tissue were retrieved from the GenBank® data base and compared with the sequence of the 5'-flanking region of the rat ITF gene using the local and global homology search capabilities of the ALIGN PLUS program (Scientific & Educational Software, State Line, PA).

RNase Protection Assay—The transcriptional start site was established by RNase protection assay based upon the sequence established from the subcloned gene and its 5'-flanking region. A 315-bp fragment straddling the junction of the 5'-flanking region and the first exon was amplified by a polymerase chain reaction primed with synthetic oligonucleotides designed as a sense strand 23-mer starting 230 bp upstream of the translational start site and an antisense 24-mer 61 bp 3' to the translational start site. The RITF gene subcloned into pKS+ plasmid was used as template, and 35 cycles of 94 °C for 1 min, 64 °C for 1.5 min, 72 °C for 2 min were performed, followed by 72 °C for 8 min to complete extension. PCR product was polished with Klenow fragment and subcloned into the *Srf*I site of the plasmid vector PCR Script (Stratagene), and competent XL1-Blue cells were transformed. Plasmid DNA was prepared with plasmid affinity columns (Wizard Miniprep; Promega). Orientation and sequence was confirmed by sequencing with universal primers as described above. The plasmid was linearized by digestion with *Not*I to provide a 5' overhang, and a 315-bp ^{32}P-labeled riboprobe was generated by *in vitro* transcription with priming at the T7 site of the PCR Script vector using the MAXIscript *in vitro* transcription kit (Ambion; Austin, TX). Probe was purified on a 5% acrylamide, 8 M urea gel prior to hybridization with total RNA from rat colon and RNase digestion using the RPA II Kit (Ambion; Austin, TX). Products of digestion were resolved by electrophoresis on a 5% Long Ranger, 8 M urea gel (AT Biochem; Malvern, PA) and exposed to Kodak XAR film at −70 °C between two intensifier screens.

Primer Extension—Confirmation of the predicted transcriptional start site was carried out by primer extension using a primer designated RITF-C (5'-caggaggacgtttcgggtcctt-3' within exon I of the RITF gene (predicted nucleotides +86 to +107). After labeling of the primer 5' end with [α-^{32}P]ATP by standard techniques, primer extension was carried out by the method of Sambrook *et al.* (31) after initial precipitation of the primer with 150 μg of total RNA prepared from rat colonic mucosa in 100 μl by the addition of 3 M sodium acetate (10 μl) and absolute ethanol (250 μl) at −70 °C. Products of primer extension were evaluated on an 8% Long Ranger gel run side by side with a sequence ladder produced with the RITF 20C plasmid, using the RITF-C oligonucleotide as primer (1 pmol/m), ^{35}S-dATP (DuPont NEN and the Sequenase version one sequencing kit (U. S. Biochemical Corp.).

Construction of Luciferase Plasmids—The promoterless luciferase gene construct pXP2 (34) was a gift from Dr. Lee Kaplan. Starting with pRITF20C, an 8.7-kb *Eco*RI fragment of one cross-hybridizing phage containing the entire RITF gene as well as ~1.7 and 1.8 kb of 5'- and 3'-flanking regions, respectively, was subcloned into pKS+ Bluescript and the plasmid cut at the *Nco*I site, a single base pair upstream of the start ATG codon. The 5' overhang was polished by mung bean nuclease digestion followed by ligation with phosphorylated *Xho*I linker using T4 DNA ligase. The plasmid was then digested with *Bam*HI to completion, and the resulting 1.7-kb fragment, containing only the 5'-flanking region, was resolved on a 1% agarose gel and isolated by digestion with GELase (Epicenter Technologies; Madison, WI) according to the manufacturer's instructions and ethanol precipitation. The multiple cloning site of pXP2 was digested with *Bam*HI and *Xho*I to completion and double-cut vector was isolated from a 1% agarose gel with Geneclean (BIO 101; La Jolla, CA) by the manufacturer's instructions. The 1.7-kb *Bam*HI-*Xho*I fragment was ligated into prepared vector to form the construct −1671 RITF-Luc and transformed into competent *E. coli* DH5α cells (Clontech Laboratories; Palo Alto, CA). To confirm correct orientation and preservation of the start codon of luciferase, plasmid DNA was subjected to restriction mapping and sequencing of the insertion junctions.

A construct consisting of the −1671 RITF promoter driving the

[2] M. Babyatsky, personal communication.

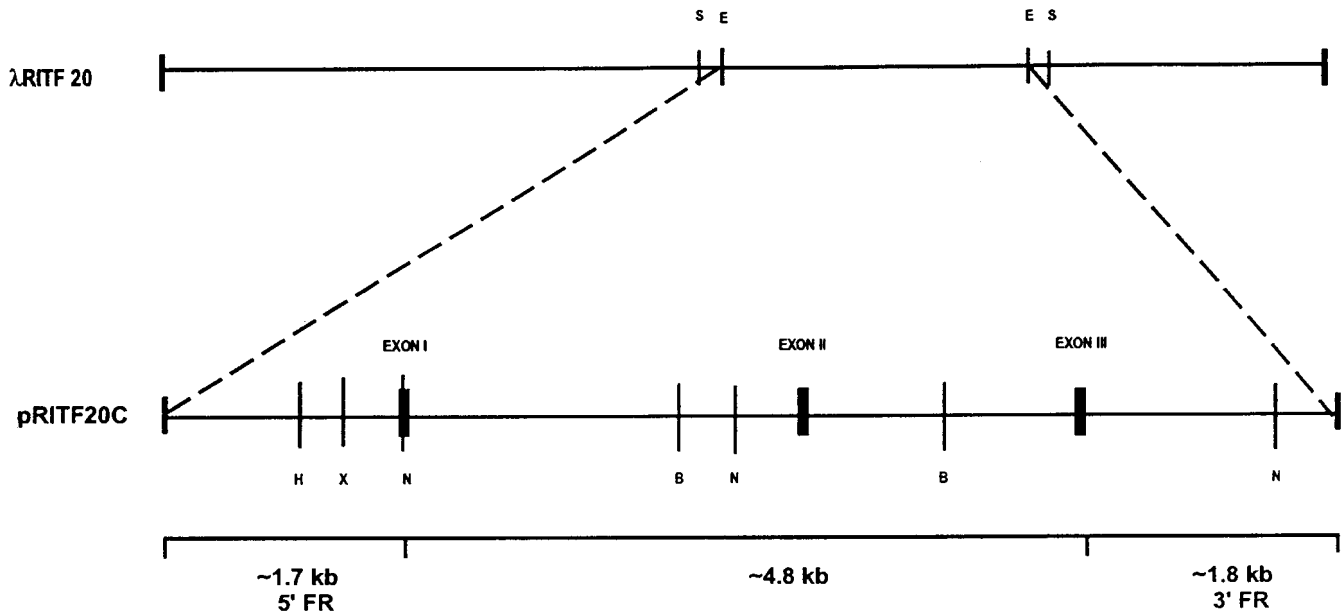

FIG. 1. **Structure of λRITF20 phage clone of the RITF gene.** An 8.3-kb *Eco*RI fragment of the genomic clone was inserted into pBluescript KS+ and designated pRITF20C. The relative locations of each of the three exons are indicated, along with restriction sites utilized for subcloning and creation of deletion constructs. The entire 5′- and 3′-flanking regions (*FR*) and exon-intron borders were sequenced using a strategy of sequential overlapping primers. *S*, *Sfi*I; *E*, *Eco*RI; *H*, *Hin*cII; *X*, *Xba*I; *N*, *Nco*I; *B*, *Bam*HI.

human growth hormone gene was prepared in the following manner: −1671 RITF-Luc was digested to completion with *Bam*HI and *Bgl*II to release the full length of the 5′-flanking region. The ~1.7-kb *Bam*HI-*Bgl*II fragment was isolated electrophoretically on a 1% agarose gel and isolated by GELase as above. pΦGH (Nichols Institute; San Juan Capistrano, CA), a plasmid containing a polycloning site preceding the human growth hormone gene, was digested with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. To increase the efficiency of cloning, *Bam*HI-cut and dephosphorylated pφGH was ligated with T4 DNA ligase, and unligated plasmid was electrophoretically separated from undigested and religated plasmid on a 1% agarose gel and isolated using Geneclean (BIO 101). The *Bam*HI-*Bgl*II fragment of −1671 RITF-Luc and *Bam*HI-cut pΦGH were ligated using T4 DNA ligase, taking advantage of the compatibility of *Bam*HI and *Bgl*II cohesive ends. DH5α *E. coli* were transformed and minipreps of plasmid DNA digested with *Eco*RI to screen for clones with correct insert orientation. The new plasmid, −1671 RITF-hGH, was sequenced at both insert junctions and through the translational start site of the growth hormone gene to confirm the proper sequence of the insert and the initiation site of translation.

Deletion constructs of the 5′-flanking region of the RITF gene driving the luciferase gene were derived from the −1671 RITF-Luc construct taking advantage of convenient restriction sites. For −980 RITF-Luc, the *Hin*cII-*Xho*I fragment of the 5′-flanking region was isolated from −1671 RITF-Luc by electrophoresis on a 1% agarose gel and agarose digestion using GELase followed by ethanol precipitation in the presence of 3 M ammonium hydroxide. Gel-isolated *Hin*cII-*Xho*I fragment was ligated into pXP2 digested with *Sma*I and *Xho*I. Plasmid −704 RITF-Luc was derived by digestion of −1671 RITF-Luc with *Xba*I followed by Klenow treatment of the 5′ overhang to make it blunt. Plasmid was then cut with *Xho*I and the *Xba*I-*Xho*I fragment isolated from an agarose gel as above. The *Xba*I-*Xho*I fragment was ligated into pXP2 digested with *Sma*I and *Xho*I. Further deletion plasmids, −664 RITF-Luc, −331 RITF-Luc, and −153 RITF-Luc, were constructed by treatment of −1671 RITF-Luc with exonuclease III and mung bean nuclease after creation of a 5′ overhang using *Bam*HI digestion to cut the plasmid at the 5′ end of the promoter insert (Erase-a-Base System, Promega). All plasmids were transformed into DH5α *E. coli*, grown in LB media, and plated out. Positive colonies were selected by the method of Grunstein (35) using a 240-bp probe of the 3′-most end of the 5′-flanking region of RITF generated by PCR. Synthetic 19-mer primers for PCR were selected based on the known sequence of the 5′-flanking region of the RITF gene and subjected to denaturing at 95 °C, annealing at 42 °C, and extension at 72 °C for 30 cycles, followed by a terminal extension at 72 °C for 8 min. Correct size of probe was confirmed on agarose gel and identity confirmed by mapping with restriction digestions. Probe was radiolabeled by nick translation with [α-^{32}P]dCTP and [α-^{32}P]dATP. Unincorporated nucleotide was eliminated chromatographically using a Primerase column (Stratagene). All deletion constructs were mapped by restriction digestion and the insert-vector junctions confirmed by sequencing as above. Plasmid for transfections was prepared by alkaline lysis and resin column purification (Qiagen; Chatsworth, CA). Plasmid preparation purity was confirmed by A_{260}/A_{280} of >1.6, and supercoiling of DNA was established by the appearance on agarose gel electrophoresis prior to use in transfection experiments.

Cell Culture, Transfections, and Reporter Gene Assays—Caco-2 cells, an intestinal cell line capable of enterocyte-like differentiation obtained from the ATCC, were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/liter D-glucose, 25 mM HEPES, 10% fetal calf serum, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml of streptomycin. Caco-2 cells were grown at confluence for at least 2 weeks prior to use in transfections. LS174T cell line also obtained from the ATCC was maintained in Eagle's minimum essential medium with Eagle's balanced salt solution, non-essential amino acids, sodium pyruvate, L-glutamine, penicillin, and streptomycin. The H2 subclone of the HT-29 cell line obtained as a gift from Dr. Daniel Louvard was grown in either Dulbecco's modified Eagle's medium with glucose (nondifferentiating conditions) or in the presence of galactose as the sole source of carbohydrate to induce goblet cell-like differentiation as described previously (36, 37). All cell lines were grown in 5% CO_2 at 37 °C.

Transfection was accomplished by the calcium phosphate precipitation method. Sixteen hours prior to transfection, 8×10^5 cells were plated out in triplicate in 35-mm wells of a six-well cell culture plate. Complete media was refreshed 2 h prior to transfection. Efficiency of transfection was standardized by co-precipitation of the construct of interest with pTK-GH, consisting of the minimal thymidine kinase promoter driving the human growth hormone gene as a reporter gene (38), and adjusting for the amount of human growth hormone expressed, as determined by a commercially available radioimmunoassay (hGH Allegro Kit; Nichols Institute Diagnostics). Calcium phosphate-precipitated plasmid DNA was added to each well and incubated at constant 5% CO_2 for 4 h before a 2-min exposure to 15% glycerol. Cells were subsequently cultured for 48 h prior to assay for reporter gene expression. For luciferase activity, cells were lysed and assayed immediately for light production in the presence of luciferase substrate using a commercial luciferase assay system (Promega) measured in a luminometer (Analytical Luminescence Laboratory, Monolite 2010). Luciferase activity was expressed as relative light units. All luciferase activity was adjusted for transfection efficiency reflected in the level of growth hormone, expressed as nanograms of hGH/ml of medium. Where noted, promoter activity was expressed as a percentage of the expres-

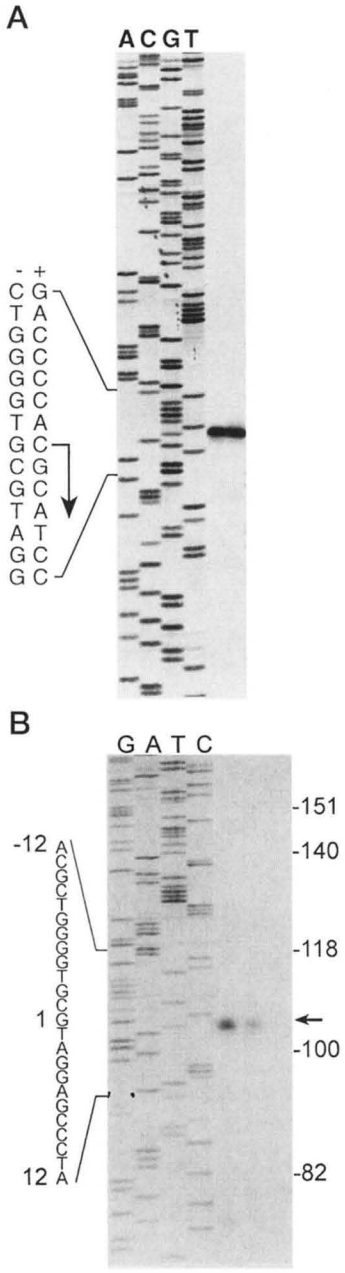

FIG. 2 *A*, RNase protection assay of RITF gene. The transcriptional start site of the RITF gene was first established using an RNase protection assay. A 315-bp fragment spanning −196 to +119 was amplified by polymerase chain reaction, polished with Klenow fragment, subcloned into the *Srf*I site of PCRScript plasmid. Plasmid linearized with *Not*I was primed at the T7 site of the PCR Script vector, and a 315-bp antisense riboprobe was generated using T7 RNA polymerase with incorporation of [α-^{32}P]UTP (800 Ci/mmol; DuPont NEN). Probe purification, hybridization, digestion, and electrophoresis were performed as described under "Materials and Methods." Probe was hybridized with 10 μg of total RNA isolated from rat colon. Sequencing ladders for A, C, G, and T, utilizing the antisense PCR primer used to generate probe and pRITF20C as template, are seen to the *left*. *Right*, a single undigested band is observed, corresponding with the initiation site of transcription denoted by the *arrow* along the sequence displayed at the *far left*. + and − indicate the sense and antisense strands. *B*, Primer extension. RITF transcriptional start site was confirmed by primer extension using a primer oligonucleotide from exon I (putative nucleotides +86 to −107). Primer was precipitated with 150 μg of total RNA isolated from rat colonic mucosa. Hybridization and primer extension were carried out at 30 °C using the method of Maniatis (31). Precipitated products were suspended in 9 μl of TE + 1 μl of 6 × loading buffer, heated at 85 °C for 10 min and then run in duplicate on an 8% polyacrylamide gel (90 watts × 2 h). A sequence ladder using RITF 20C plasmid DNA and ^{35}S-dATP was run simultaneously as displayed in the figure. A single product was observed (*arrow*) corresponding to the predicted start site identified by consensus sequence and RNase protection.

sion of the maximal promoter construct RSV-Luc (a gift from Dr. Loyal Tillotson), consisting of the RSV promoter joined to the luciferase gene.

Co-localization of hGH Expression under Control of RITF 5′-Flanking Region and Expression of Mucin Glycoprotein—The N2 subclone of the HT29 cell line obtained from Dr. Daniel Louvard (36, 37) was grown in differentiating and undifferentiating media (as described for H2 cells) in cell culture flasks with a removable glass slide bottom for subsequent immunostaining (Lab-Tek Chamber Slide System; Nunc, Naperville, IL). N2 cells were seeded as 5×10^5 cells/flask 16 h prior to transfection. Complete medium was replaced with serum-free medium and 2 μg/flask plasmid DNA, either −1671RITF-hGH or pTKGH, was combined with 1.5 μl of cationic liposome (Transfectam; Promega) and added to the cells in culture. Sixteen hours later, complete medium was returned to the cells. After 48 h of further culture, cells were fixed and dried. Slides were blocked with 1.5% normal goat serum for 30 min at 25 °C and then incubated with guinea pig-anti-human growth hormone (Arnel; New York, NY) at 1:20,000 in L4-1-3 supernatant (a mouse monoclonal anti-human mucin previously described; Ref. 39) for 1 h at 25 °C. After washing with PBS, slides were further incubated with rhodamine-conjugated goat-anti-guinea pig IgG (Cappel; Durham, NC) 0.12 mg/ml and fluorescein-conjugated goat-anti-mouse IgG (Cappel) 0.24 mg/ml at 25 °C for 1 h, followed by washing with PBS, drying, and mounting with glycerol:PBS (6:1). Samples were examined using a Bio-Rad MRC 600 scanning confocal imaging system attached to a Zeiss Axiovert 35 inverted microscope (Zeiss; New York). The fluorescence of fluorescein-anti-mouse IgG and rhodamine-anti-guinea pig IgG was excited using the 488- and 568-nm lines, respectively, of the argon-krypton mixed gas laser in the confocal microscope. The confocal parameters of scan rate, aperture, gain, black level, and frames accumulated were the same for all samples.

RESULTS

Cloning of the Rat ITF Gene—The rat genomic library was screened with the full-length cDNA probe of rat ITF. This probe has been shown previously to specifically hybridize with ITF mRNA in Northern blot analysis and *in situ* hybridization (17). Screening of 500,000 plaques yielded four independent phage picks. Analysis by Southern blot hybridization revealed that all four phage picks contained the entire ITF gene within an 8.3-kilobase *Eco*RI fragment. This fragment was subcloned into the *Eco*RI site of pKS+ Bluescript, and the new plasmid was named pRITF20C. The subcloned fragment contained approximately 1.7 kb of the 5′-flanking region and 1.8 kb of the 3′-flanking region. The gene was observed to be divided into three exons. Approximate exon-intron borders were noted by divergence of the genomic sequence from the cDNA sequence. As depicted in Fig. 1, Exon I extended 119 bp, Exon II extended 144 bp, and Exon III extended 184 bp, whereas Introns A and B were approximately 2.3 and 2.0 kb, respectively.

Identification of Transcriptional Start Site—The transcriptional start site of the ITF gene was first established by ribonuclease protection assay using total RNA isolated from rat colon hybridized to a 315-bp probe spanning an interval from 224 bp 5′ to the translational start site to 91 bp 3′ to the first nucleotide of the start codon. As demonstrated in Fig. 2*A*, optimized digestion with ribonuclease yielded a single protected fragment, corresponding to a transcriptional start site located 34 bp 5′ to the translational start site and 27 bp 3′ to the presumed TATA box. The putative single start site was confirmed by primer extension using RNA obtained from rat colonic mucosa and a complementary primer from nucleotide +86 to +107. As demonstrated in Fig. 2*B*, a single product was identified of the precise predicted size, confirming the putative transcriptional start site.

Analysis of Rat ITF 5′-Flanking Region—The start codon ATG was found to be at +34, embedded within a canonical

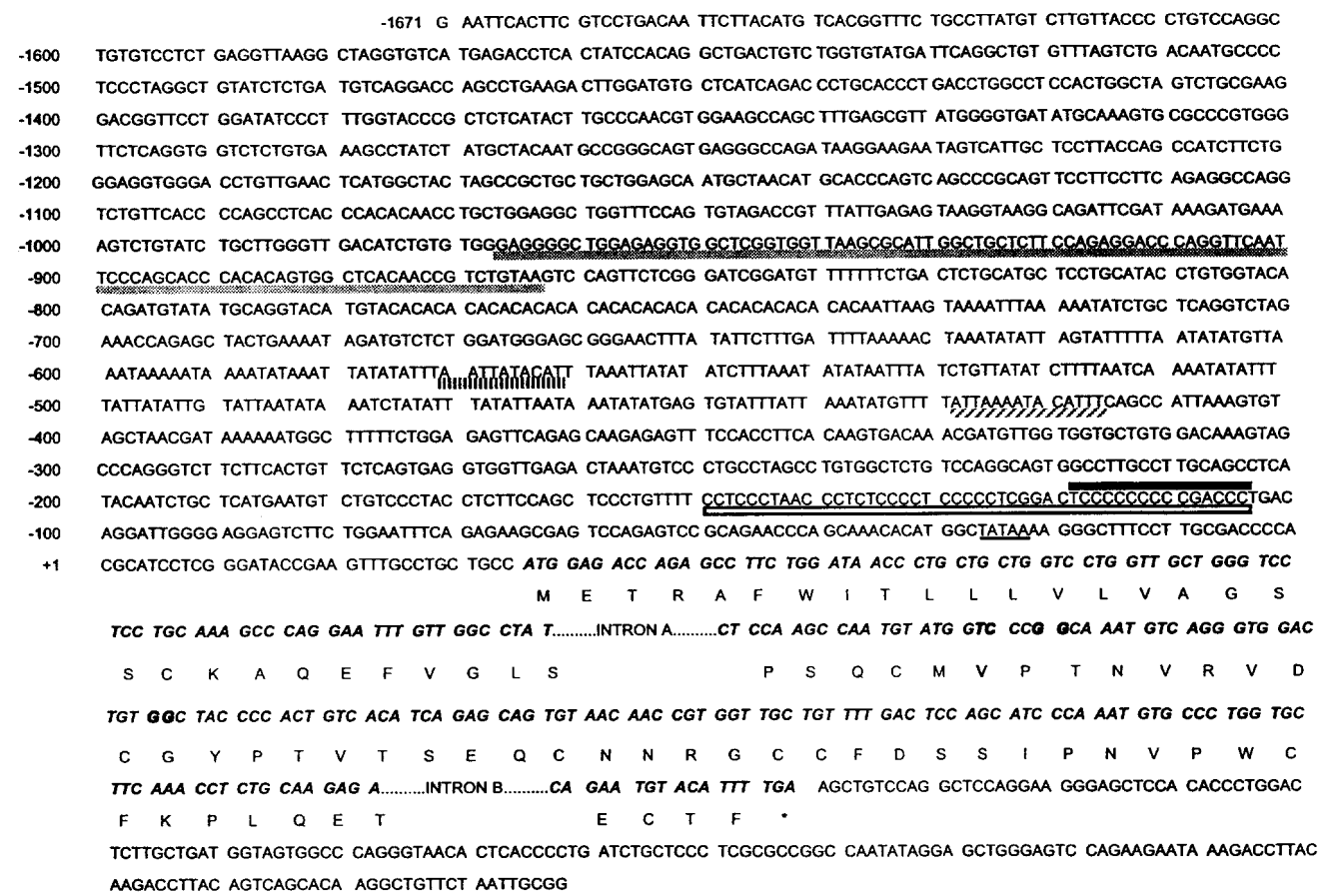

FIG. 3. **Sequence of RITF gene.** The entire 5′-flanking region contained within pRITF20C, as well as the exonic sequences with presumed exon-intron borders, are displayed. Translated regions of exons I, II, and III are indicated by *bold italic letters. Outlined letters* represent corrections of the originally published cDNA sequence of rat ITF. *Stippled underlining* denotes a 100-bp region with 85% identity between the rat ITF and rat intestinal fatty acid-binding protein (rIFABP) genes, with an Alu-like sequence. *Vertical hatch underlining* indicates a Pit-1 homeodomain binding domain consensus sequence, whereas *diagonal hatch underlining* denotes a 14-bp homeodomain-like sequence found also in the 5′-flanking region of the rIFABP gene. An *open box* underlines a region with ~75% GC content, whereas the *solid box* lies over overlapping potential AP-2 sites contained within this region. The TATA box is *underlined*. Also observed are 20 repetitions of the dinucleotide CA between −776 and −737 and a roughly 280-bp span between −657 and −376 with ~90% AT content.

Kozak consensus sequence (40). Examination of the roughly 1.7 kb of cloned 5′-flanking region revealed a number of potential transcriptionally active elements (Fig. 3). Over the roughly 280 bp spanning from −657 to −376, an AT-rich region was observed, approaching 90% for these two nucleotides. A smaller region of roughly 75% GC content was observed between −150 and −105. The dinucleotide repeat "AC" was found repeated 20 times from −776 to −737. The sequence of the RITF 5′-flanking region was compared with the reported sequences of 5′-flanking regions of genes expressed in intestinal tissues, including the human and mouse sucrase-isomaltase genes, the rat lactase-phlorizin gene, the human and porcine aminopeptidase genes, and the human and rat intestinal fatty acid binding protein genes (23–29). Using the local and global search functions of the ALIGN program, no areas of high homology were found, except for a 100-bp region of ITF and rat intestinal fatty acid binding protein, which had 85% identity between the two sequences. This corresponds to an Alu-like element of the rat I-FABP gene (23). In addition, a cannonical Pit-1 homeodomain (41) is present at −571 to −561. A 14-bp homeodomain-like element consisting of the nucleotides ATTAAAATACATTT is found at −429, with the identical sequence found at −615 of the rat I-FABP gene.

Comparison with the 5′-flanking region of pS2, the only other trefoil protein for which a genomic clone has been reported (21, 22), revealed no striking homology. Specifically, the 13-base pair imperfect palindrome considered to be the estrogen-responsive element of the pS2 promoter (42) was not found in the 1.7 kb of RITF 5′-flanking region, nor was a canonical estrogen-responsive element discovered. In addition, no strong homology was found with the epidermal growth factor (EGF)-responsive region of pS2 (22) or with other reported EGF-responsive elements (43–45).

Searches for specific known cis-regulatory elements revealed potential overlapping AP-2 binding sites (46) from −119 to −105, within the GC-rich region. A CRE consensus sequence (TGACC) (47) was found from −1431 to −1427. Potential CAAT boxes (either CCAAT or GCAAT) located at −1153 on the sense strand and on the antisense strand starting at −97 and −1225. Four sites, located at −1431, −1185, −908, and −369, also fit the consensus sequence of RG(G/T)TCA which has been reported to promiscuously bind the transcription factors RAR, RXR, HNF-4, and ARP-1 (48).

Expression of the Reporter Gene Luciferase under the Control of Deletions of Rat ITF 5′-Flanking Region in Intestinal Cell Lines—Constructs in which the reporter gene luciferase was ligated to various lengths of the 5′-flanking region of the RITF gene were transfected into LS174T cells, representing a goblet cell-like intestinal cell line, and Caco-2 cells, representing a columnar absorptive-like enterocyte phenotype. The level of expression of the various deletion constructs containing from −1671 to −153 of the 5′-flanking region was between 8 and

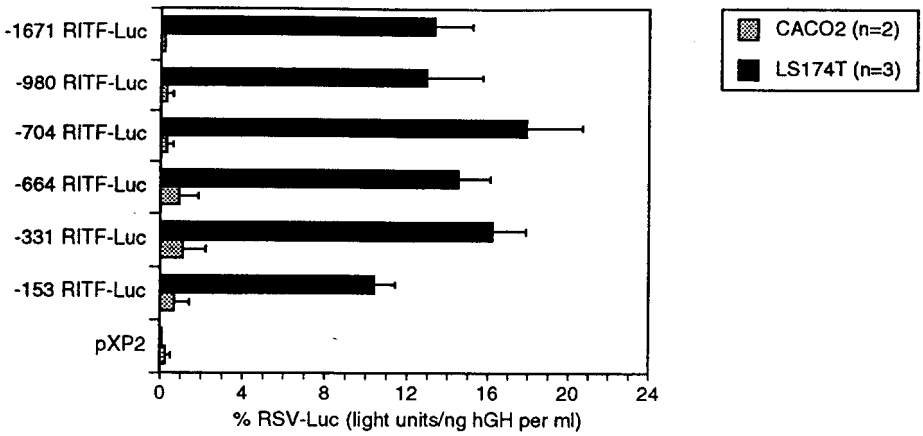

FIG. 4. **Transient expression of RITF promoter-luciferase constructs in intestinal cell lines.** Results are expressed as a percentage of luciferase activity of the RSV promoter driving luciferase gene. Luciferase activity was measured as relative light units adjusted for efficiency of transfection standardized by co-transfection of pTK-GH, the human growth hormone gene under the influence of the thymidine kinase promoter, measured as human growth hormone immunoreactivity in the media of the transfected well using a commercially available radioimmunoassay as described under "Materials and Methods." Deletion constructs are numbered according to the length of the 5′-flanking region relative to the transcriptional start site as determined by RNase protection assay depicted in Fig. 2. *pXP2* is a promoterless luciferase construct. Plasmid constructs were transfected by the calcium phosphate precipitate method as described. *$CACO_2$*, human intestinal cell line; *LS174T*, human intestinal cell line with goblet cell-like phenotype. n = number of independent transfections. Results are expressed as the mean ± S.E.

18% of the expression observed for the maximal promoter-reporter construct, RSV-Luc, in the LS174T cells (Fig. 4). In contrast, the level of expression in Caco-2 cells was consistently less than 2% that of RSV-Luc. The relatively high level of expression in LS174T cells was observed even in cells transfected with the construct containing only 153 bp of RITF 5′-flanking sequence (−153 RITF-Luc).

In an experiment assessing the level of expression of −1671 RITF-Luc in H2 cells grown under differentiating and nondifferentiating conditions, as well as LS174T cells, expression was found to be greatest in the LS174T cell line (Fig. 5). More importantly, H2 cells grown under differentiating conditions were found to express the reporter gene after transfection by the method of calcium phosphate precipitate at levels approximately 2-fold higher than that observed for H2 cells grown in nondifferentiating conditions. Enhanced expression of the RITF-luciferase in the H2 cells in association with differentiating conditions was also observed when cells were transfected by an alternative method using cationic liposome (data not shown). The levels of luciferase activity of these three cell lines were proportional to the concentration of ITF produced in their respective media as assessed by Western blot. Thus, undifferentiated H2 cells produced minimal amounts of ITF, whereas differentiated H2 cells produced greater concentrations and LS174T cells produced nearly 10-fold higher.[3]

Immunofluorescent Localization of hGH Expression Directed by the 5′-Flanking Region of RITF and Co-localization with Mucin Glycoprotein Expression—To further evaluate the ability of the 5′-flanking region of the RITF gene to direct goblet cell-specific expression, we sought to determine the relationship of reporter gene expression directed by the RITF promoter to that of mucin glycoprotein characteristic of goblet cells in the N2 subclone of the HT29 colon cancer cell line. N2 cells grown under differentiating and nondifferentiating conditions were transfected with a construct consisting of the human growth hormone gene under the control of −1671 RITF 5′-flanking region. Double immunofluorescent staining using a commercially available guinea pig anti-human growth hormone antibody and a monoclonal mouse anti-human mucin glycoprotein, previously described, were used as the primary antibodies. Use of rhodamine-labeled anti-guinea pig IgG and fluorescein isothiocyanate-labeled anti-mouse IgG permitted simultaneous co-localization by laser confocal microscopy of expressed human growth hormone in N2 cells transfected with −1671 RITF-hGH by cationic liposome.

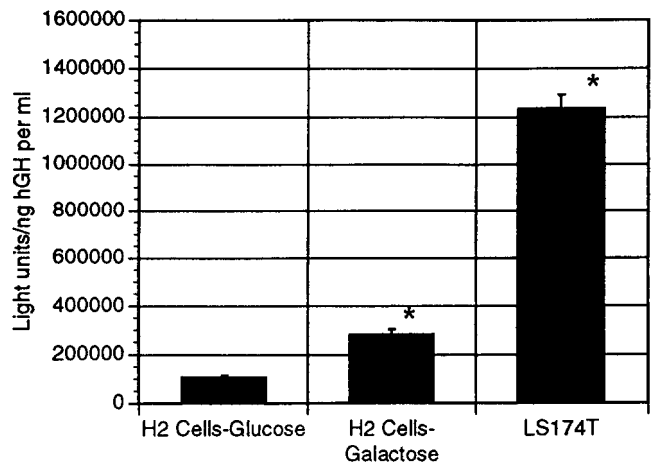

FIG. 5. **Transient expression of RITF-Luc in cell lines with a goblet cell-like phenotype.** Transfections of the full-length construct −1671 RITF-Luc were performed by the calcium phosphate method in the LS174T intestinal cell line and in the H2 subclone of the HT29 intestinal cell line grown in galactose-containing media to induce a goblet cell-like phenotype or in glucose to maintain an undifferentiated phenotype. A representative experiment is shown. Results are displayed as the mean ± S.E. of relative light units standardized for transfection efficiency by co-transfection with pTK-GH and human growth hormone activity as nanograms/ml of medium. *Asterisks* denote $p \leq 0.01$ by a two-sided Student's t test comparing the level of expression to that observed in H2 cells grown in glucose.

Expression of hGH and mucin glycoprotein in differentiated N2 cells transfected with −1671 RITF-hGH is depicted in Fig. 6. Examination of these cells reveals expression of hGH in virtually all cells expressing mucin glycoprotein. No cells were observed to express the reporter hGH in the absence of mucin glycoprotein. In contrast, undifferentiated cells transfected with −1671 RITF-hGH expressed neither mucin glycoprotein nor hGH. In differentiated and undifferentiated N2 cells transfected with pTK-GH, consisting of the human growth hormone gene under control of the thymidine kinase promoter, hGH was

[3] H. Kindon and D. K. Podolsky, unpublished observations.

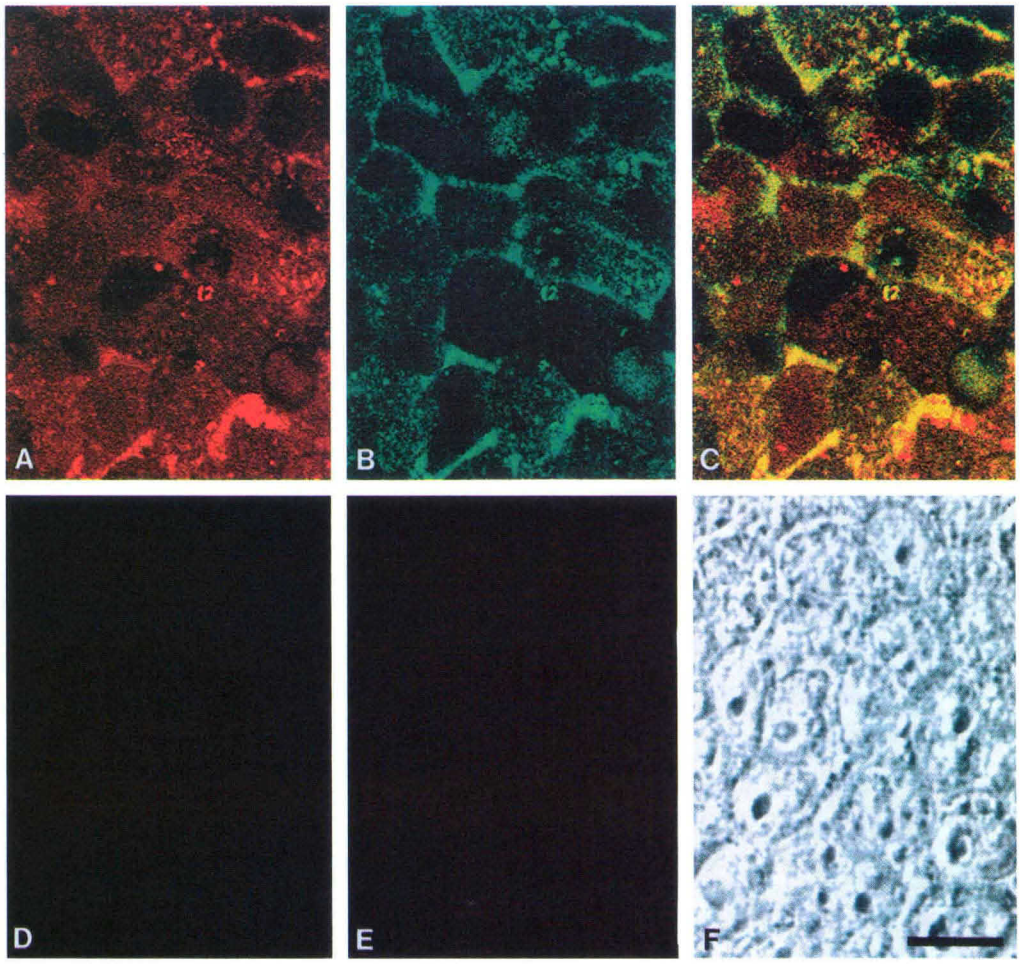

FIG. 6. **Co-localization of the expression of human growth hormone under control of the RITF promoter with native mucin expression in N2 cells.** N2 cells, a subclone of the HT29 intestinal cell line, were grown in galactose-containing media in glass culture dishes to induce goblet cell differentiation before being seeded onto glass slide flasks. The −1671 RITF-hGH construct was transfected by a method of cationic liposome as described under "Methods and Materials." Slides were fixed 62 h after transfection and incubated first with polyclonal guinea pig anti-human growth hormone and monoclonal mouse IgG anti-human mucin glycoprotein as primary antibodies, followed by rhodamine-conjugated goat anti-guinea pig IgG and fluorescein-conjugated goat anti-mouse IgG as developing antibodies. Laser confocal microscopy was performed as described to permit co-localization of growth hormone and mucin. Specificity of fluorescent staining was confirmed by absence of staining with omission of primary antibody (not shown). *A–C* depict representative fields of the goblet cell differentiated cells transfected with −1671 RITF-LGH constituent. *A*, laser-stimulated fluorescence of rhodamine appearing as areas of *red*, indicating expression of ITF-human growth hormone. *B*, laser-stimulated fluorescence of fluorescein appearing as areas of *green*, localizing native mucin expression. *C*, computer-assisted superimposition of images from *A* and *B* display cells co-expressing both human growth hormone and mucin as yellow or orange. Expression of RITF-hGH was not observed in transfected control N2 cells grown in nondifferentiating (glucose-containing) medium (*D*), although nearly all of the nondifferentiated cells did express growth hormone after transfected with a construct (pTK-GH) containing the human growth hormone under the control of the nonspecific thymidine kinase promoter (*E*). The pTK-GH was similarly expressed in nearly all N2 cells maintained in galactose-free media, including those which failed to exhibit goblet cell phenotype. The appearance of N2 cells by conventional phase contrast is depicted in *F*.

expressed in a nonspecific fashion among cells and independent of their acquisition of goblet cell phenotype as assessed by immunostaining for mucin glycoprotein (results not shown).

DISCUSSION

Epithelial differentiation in the gastrointestinal tract is a complex and dynamic process. Not only is tissue-specific phenotype maintained along the longitudinal axis from esophagus to large bowel, but in the normal mucosa, vertical differentiation from crypt to villus is perpetuated as well. Moreover, differentiation into several region-specific subpopulations is observed within the epithelium along the length of the gastrointestinal tract. Among the growing list of cloned genes whose products are intestine-specific, ITF represents the first within the gastrointestinal tract exclusively expressed by goblet cells. In this paper we report the cloning of the rat intestinal trefoil protein gene and the characterization of its 5′-flanking region.

ITF is only the second gene from among the members of the family of trefoil proteins to be analyzed. Although the protein products of the ITF and pS2 genes share the characteristic intrachain disulfide bonding constituting the trefoil P domain, the regulatory regions of these otherwise distinct genes appear to share little homology. Strict regulation of the expression of these two trefoil proteins along the longitudinal axis of the gastrointestinal tract, with pS2 normally expressed in the gastric antrum and ITF expressed in increasing amounts in the small and large bowel, may account for the relative lack of shared elements in their promoter region.

The family of trefoil proteins, including ITF, have been implicated in mucosal healing following injury (18). An ulcer-associated cell lineage has been reported to appear adjacent to areas of gastrointestinal ulceration, with cells containing EGF-immunostaining material in the base of the newly budding cell lineage and trefoil protein-producing cells appearing more distally along the developing ductule (49). Recent reports of en-

hancement of healing by trefoil proteins, including ITF, in an *in vitro* model of intestinal epithelial injury (18) and *in vivo* (50) further supports the notion that the trefoil proteins are important to the maintenance of the mucosal barrier of the intestine. Whether the abundantly expressed and luminally secreted trefoil proteins accomplish this by contributing to the physical properties of the mucoviscous layer or by specific ligand-receptor interactions remains unknown. ITF has been observed to be induced in the stomach in animal models of gastric ulceration (20), whereas physiologic expression is normally restricted to the small and large bowel (16, 17) (with the reported exception of the mucous cells of the gastric cardia) (49).

The presence of an EGF-responsive element in the 5′-flanking region of the pS2 gene (22) has led to speculation about the role of EGF in inducing expression of trefoil proteins in a response to mucosal injury (51). Although scrutiny of the 5′-flanking region of the ITF gene demonstrates no significant homology to known EGF response elements, such an element may still be present and could now be localized using the deletion constructs reported here. In addition, the signals which lead to the expression of ITF in response to ulceration, whether a consequence of altered cytoskeletal structure from loss of contact inhibition or from local release of cytokines or other mediators, remain obscure and may be more easily unravelled with isolation of the ITF gene and promoter.

Constructs containing segments of the 5′-flanking region of the ITF gene were found to be capable of directing tissue-specific expression to cells with a goblet cell phenotype, indicating the presence of goblet cell-specific promoter elements or conversely non-goblet cell repressor elements within the length of promoter examined. Indeed, relatively high levels of specific expression with promoters as short as 153 bp of the 5′-flanking region suggests that such an element or elements are present within close proximity to the transcriptional start site. As a marker of intestinal goblet cell differentiation, the relatively short ITF gene may have a distinct advantage over the lengthy mucin genes (1–7) for use in transgenic animal experiments or tissue-directed gene therapy.

A search of the 5′-flanking region of the ITF gene reveals none of the known regulatory elements which have been demonstrated to play a role in intestine-specific expression. Specifically, the SIF-1, −2 and −3 elements of the sucrase-isomaltase gene (26) are lacking in the rat ITF promoter. No areas of significant homology appear to exist between the promoter of the rat ITF gene and the reported 5′-flanking regions of the genes for human intestinal alkaline phosphatase, human intestinal fatty acid-binding protein, porcine aminopeptidase N, human and mouse sucrase-isomaltase, or human lactase-phlorizin hydrolase. Of interest, the 5′-flanking region of rat ITF contains a Pit-1 homeodomain consensus binding site. Although initially described as a cis-regulatory element specifically directing expression in pituitary cells (41), the presence of this element in a rat intestinal gene suggests that it might play a role in tissue-specific gene expression beyond the pituitary, an effect which might be context-specific, depending upon additional as yet undescribed regulatory elements. Although lacking a classic Pit-1 homeodomain, the rat I-FABP gene shares with the rat ITF gene a 14-bp homeodomain-like element (−429 to −416).

Transient expression of deletion constructs containing various lengths of RITF gene 5′-flanking region ligated to a luciferase reporter gene indicate that a length of 5′-flanking region as short as the 153 bp contiguous with the transcriptional start site is capable of preferentially promoting expression in cells with a goblet cell-like phenotype. Reporter gene expression in LS174T cells, a human colon cancer-derived cell line capable of expressing both mucin glycoprotein and native ITF in a fashion typical of goblet cells, was nearly 10-fold higher than the level of expression observed in undifferentiated Caco-2 cells, even when corrected for differences in transfection efficiency.

Transient expression experiments comparing undifferentiated and differentiated H2 cells and LS174T cells demonstrate a gradient of expression which parallels the ability to express native ITF. A small number of H2 cells grown in conditions not conducive to differentiation nevertheless spontaneously acquire a goblet cell-like phenotype and secrete mucin, consistent with the low, but not undetectable, level of luciferase activity in undifferentiated cells transfected with −1671 RITF-Luc. Even in the presence of differentiation promoting culture conditions, acquisition of the goblet cell-like phenotype is not uniform (44), accounting for the level of expression which is nearly 2-fold greater than that observed in undifferentiated H2 cells, but still less than that observed in LS174T cells, which uniformly exhibit a goblet cell-like phenotype. Co-localization of mucin glycoprotein and reporter gene expression under the control of the RITF promoter in H2 cells, which like N2 cells are capable of achieving a goblet cell-like phenotype, lends further support to the goblet cell-specific nature of the RITF promoter. Based on these observations it is likely that the proximal 5′-flanking region of the rat ITF gene contains elements which are capable of directing intestine and goblet cell-specific expression.

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