*Proc. Natl. Acad. Sci. USA*
Vol. 93, pp. 2137–2142, March 1996
Medical Sciences

# Transgenic mice that overexpress the human trefoil peptide pS2 have an increased resistance to intestinal damage

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*Communicated by Paul Nurse, Imperial Cancer Research Fund, London, United Kingdom, November 30, 1995 (received for review June 28, 1995)*

**ABSTRACT** **pS2 is a member of the trefoil peptide family, all of which are overexpressed at sites of gastrointestinal injury. We hypothesized that they are important in stimulating mucosal repair. To test this idea, we have produced a transgenic mice strain that expresses human pS2 (hpS2) specifically within the jejunum and examined the effect of this overexpression on proliferation and susceptibility to indomethacin-induced damage. A transgenic mouse was produced by microinjecting fertilized oocytes with a 1.7-kb construct consisting of rat intestinal fatty acid binding protein promoter (positions −1178 to +28) linked to full-length (490 bp) hpS2 cDNA. Screening for positive animals was by Southern blot analysis. Distribution of hpS2 expression was determined by using Northern and Western blot analyses and immunohistochemical staining. Proliferation of the intestinal mucosa was determined by assessing the crypt cell production rate. Differences in susceptibility to intestinal damage were analyzed in animals that had received indomethacin (85 mg/kg s.c.) 0–30 h previously. Expression of hpS2 was limited to the enterocytes of the villi within the jejunum. In the nondamaged intestine, villus height and crypt cell production rate were similar in transgenic and negative (control) litter mates. However, there was a marked difference in the amount of damage caused by indomethacin in control and transgenic animals in the jejunum (30% reduction in villus height in controls vs. 12% reduction in transgenic animals, $P < 0.01$) but the damage sustained in the non-hpS2-expressing ileal region was similar in control and transgenic animals. These studies support the hypothesis that trefoil peptides are important in stimulating gastrointestinal repair.**

The gastrointestinal tract is constantly subject to trauma from ingested food particles and noxious agents such as alcohol or aspirin. When a mucosal defect occurs, it is vital that a healing response occurs rapidly to prevent additional damage to the submucosa from the luminal acid and proteolytic enzymes. This healing response occurs in two phases: Within the first hour after injury, surviving cells from the edge of the wound begin to migrate over the denuded area to reestablish epithelial continuity, a process called epithelial restitution (1). This is followed by a much slower increase in proliferation and remodeling, which only begins about 24 h later (2).

There is increasing evidence that a family of peptides, called the trefoil peptides because of their unusual "three-leaf" structure (3), are involved in this early phase of the repair process. Three members of this family have been identified in humans: human pS2 (hpS2), intestinal trefoil factor (ITF), and spasmolytic polypeptide (SP). The production of all three trefoil peptides are locally upregulated at sites of injury in conditions such as peptic ulceration and inflammatory bowel disease (4) and in experimental ulcers in rats (5, 6). We have recently shown that systemic administration of recombinant trefoil peptides decreases the amount of damage that occurs in an acute rat model of gastric injury. This protective effect was not mediated through suppression of acid secretion but may have been due to the trefoil peptides stimulating epithelial restitution (5). However, caution has to be shown in interpreting these results as our studies using systemic administration of test peptides did not accurately reflect the situation found *in vivo*, i.e., that of site-specific upregulation. To address this potential problem, we have now produced a transgenic mouse line that overexpresses the human trefoil peptide hpS2 specifically within the villi of the jejunum. We have also performed studies that begin to examine the effects that this local upregulation of trefoil peptide expression has on the susceptibility of the mucosa to injury and to the repair process.

## MATERIALS AND METHODS

**Production of Transgenic Mice Using the Intestinal Form of Rat Fatty Acid Binding Protein (I-FABP) Promoter I-FABP$^{-1178\ to\ +28}$-hpS2 Fusion Gene.** Nucleotides −1178 to +28 of the rat I-FABP promoter, inserted into the *Eco*RI–*Sma* I sites of pUC13, was kindly donated by Gordon (7). This promoter sequence, which includes the first 28 bases of the nontranslated region of the FABP, was excised by *Eco*RI and *Bam*HI and ligated to the 5′ end of a full-length (490 bp) hpS2 cDNA. The sequence of hpS2 used corresponds to that described by Jakowlew *et al.* (8), which includes the AUG initiation site starting at position 40 and the putative polyadenylylation signal AUUAAA at position 472. Correct assembly into pGem3Z was confirmed by sequencing the entire hpS2 domain and the final 200 bases of the 3′ end of the I-FABP promoter. The final 1.7-kb cassette was excised with *Eco*RI. This cassette was microinjected into 100 recently fertilized oocytes (strain C57 × CBA). Of 70 live-born mice, Southern blot analysis of DNA from tail snips identified one founder mouse that had incorporated the construct. A line (RPTMhpS2/1) was established from this founder. Southern blot analyses of tail DNA obtained from $F_1$ mice indicated that the transgene copy number was about 100 per haploid genome.

**Ethical Approval of Procedures Involving Animals.** All animal procedures were approved by the local animal ethics committee. All animals were kept on a standard chow diet ad libitum and were sacrificed by cervical dislocation.

**Collection of Samples for Analyses.** The weights and lengths of the small and large intestine of transgenic and negative

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Abbreviations: FABP, fatty acid binding protein; I-FABP, intestinal FABP; hpS2, human pS2; ITF, intestinal trefoil factor; SP, spasmolytic polypeptide; mAb, monoclonal antibody; CCPR, crypt-all production rate; r, recombinant.

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littermates were measured. To maintain consistency between animals, the length of the small intestine and colon were expressed as 100%, and 1-cm samples were taken from the following regions of the small intestine: 5% of small intestine length (defined as duodenum), 30% of small intestine length (defined as jejunum), and 90% of small intestine length (defined as ileum). For assessment of the colon, 10% of colonic length was defined as proximal colon, 50% was defined as middle colon, and 90% was defined as distal colon. Tail snips (for Southern blot analyses) were collected in 700 ml of Tris·HCl (50 mM) (pH 8.0), containing EDTA (100 mM), NaCl (100 mM), and SDS (1%) and incubated in proteinase K (360 μg/ml) at 55°C for 12 h. DNA was then extracted using phenol/chloroform, 1:1 (vol/vol), and stored in Tris (10 mM)/EDTA (1 mM) until analyses. Samples for immunohistochemistry were collected in neutral-buffered formalin and samples for crypt cell production rate (CCPR) and morphometry were collected in Carnoy's solution, left at room temperature for 4 h, and then stored in 70% alcohol until further study.

**RNA Blot Hybridization Studies.** Total cellular RNA was prepared from each intestinal segment by using the guanidinium thiocyanate method of Chomczynski and Sacchi (9). RNA integrity was established by formaldehyde/agarose gel electrophoresis. RNA was transferred to nylon filters by capillary transfer and cross-linked with UV-irradiation (UV Stratalinker 2400, Stratagene). Northern (RNA) blot analyses were performed by using a probe consisting of a *Bam*HI–*Pvu* II (404 bp) fragment of the full-length hpS2, which had been labeled with $^{32}P$ by using a nick-translation kit (GIBCO/BRL). Filters were washed for three 30-min cycles at 65°C (one cycle of 2× SSC, one cycle of 1× SSC, and one cycle of 0.5× SSC; all solutions also contained 0.1% SDS). Equal loading of RNA into the wells was confirmed by stripping the filters and reprobing with a 558-bp $^{32}P$-labeled DNA sequence corresponding to the coding region of 18S rRNA from positions 28 to 586 (kindly donated by A. Gandarillas, Imperial Cancer Research Fund).

**Western Blot Analyses and Immunohistochemistry for hpS2.** Tissue was placed in suspension buffer [100 mM NaCl/10 mM Tris·HCl, pH 7.6/1 mM EDTA, pH 8/aprotinin (1 μg/ml)/phenylmethylsulfonyl fluoride (100 μg/ml)] and dispersed with a sonicator. Protein concentrations were estimated based on their absorption at 280 nm and 10-μg samples were then electrophoresed at 0.5 A for 3 h in precast SDS/18% polyacrylamide gels (NOVEX, San Diego). Protein was then transferred onto nitrocellulose filters by electrophoretic transfer (Transblot cell, Bio-Rad). Protein was fixed onto the filter by washing in 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for 45 min, and nonspecific binding was prevented by washing in PBS containing 5% (vol/vol) goat serum for 1 h at room temperature. The position of hpS2 peptide was visualized by using two anti-hpS2 monoclonal antibodies (mAbs); mAb P2802, previously used by Rio *et al.* (10) and our own mAb GE2. Both of these mAbs were raised against a synthetic peptide containing the C-terminal 28 amino acids of hpS2. The Western blot was then developed by using an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma).

Tissues for immunohistochemical analyses were fixed in neutral-buffered formalin and embedded in paraffin wax. Endogenous peroxidase activity within 5-μm-thick sections was blocked by immersion in 0.3% hydrogen peroxide in methanol for 15 min, and sections were then stained for hpS2 with mAb P2802 (1:200 dilution) or mAb GE2 (1:400 dilution). A brown reaction product was obtained with a peroxidase substrate (diaminobenzidine and PBS in addition to 0.3% hydrogen peroxide).

**CCPR.** The CCPR was used as a robust reproducible index of proliferation (11). Twelve transgenic and control (negative litter mates) mice were injected with vincristine sulfate (1 mg/kg i.p.) and killed by cervical dislocation at intervals from 30 to 180 min later. Samples of fixed jejunal and ileal tissue were hydrated, hydrolyzed, stained with the Feulgen reaction, and transferred to 45% (vol/vol) acetic acid, and the crypts were teased apart under a dissecting microscope. Crypts were then transferred to a glass microscope slide, flattened gently beneath a coverslip, and examined under a compound microscope. The number of blocked mitoses per crypt were counted and the mean and SEM (for 20 crypts) were calculated and plotted against the time since injection.

**Morphometric Analyses.** Samples of the jejunum and ileum were microdissected as for assessment of CCPR by using a stereo dissecting microscope. Villus height, crypt depth, and cross-sectional surface area were assessed by tracing the outline of the crypts and villi by using a precalibrated drawing tube. The tracings were then scanned and analyzed by using a flatbed scanner connected to an Apple Macintosh computer LC475 running the National Institutes of Health public domain program IMAGE. Twenty individual crypts and villi were assessed in each animal at each site and the mean value from these 20 measurements was used in the subsequent ANOVA.

**Indomethacin-Induced Small Intestinal Damage.** The protocol used was a modification of that described by Ettarh and Carr (12). In their paper, two doses of indomethacin (85 mg/kg, s.c.) separated by 24 h were used. However, our pilot studies using an identical protocol gave an unacceptable mortality rate (40%) due to severe gastrointestinal bleeding. We therefore administered a single dose of indomethacin (85 mg/kg, s.c.) to six groups ($n = 7$ per group) of control and hpS2 transgenic animals. Changes in villus architecture (morphology and morphometric assessment of villi and crypts) were assessed at 0, 6, 12, 18, 24, and 30 h after injection of indomethacin. By using this reduced dosage, indomethacin administration causes a marked reduction in villus height but does not affect crypt depth (12).

**Data Analyses.** Data are expressed as the mean ± SEM. For the analyses of CCPR, the slope of the line was fitted by least squares linear regression to give the CCPR for both transgenic and control animals. These slopes were then compared by using the *t* test.

For statistical analyses of the effect of hpS2 expression on indomethacin-induced damage, differences between groups were determined by using two-way analyses of variance with time (since injection of indomethacin) and transgenic positivity as factors. Where a significant effect was seen, individual comparisons between groups were made by using *t* tests based on the mean square error of the residual and degrees of freedom obtained from the ANOVA.

## RESULTS

**Expression of hpS2 in Transgenic Animals.** Northern blot analyses (Fig. 1*B*), Western blot analyses (Fig. 1*A*), and immunohistochemical staining (Fig. 1*C*) showed that expression of hpS2 RNA and protein was restricted to the jejunum. Immunohistochemical analyses of the jejunum of transgenic animals showed that enterocytes along the entire length of the villi expressed hpS2 but the crypts did not (Fig. 1*C*). The equivalent region of negative littermates showed no evidence of hpS2 expression (Fig. 1*D*).

**Effect of hpS2 Expression on Baseline Morphometry and CCPR.** The expression of hpS2 had no significant effect on baseline CCPR (Fig. 2), gut weight, intestinal length, villus height (Fig. 3), or cross-sectional villus area.

**Effect of Indomethacin.** The morphologies of microdissected villi in the jejunum of control or transgenic animals under baseline conditions were identical with long slightly tapering villi (Fig. 4*A*). Villi obtained from control animals that had received indomethacin showed progressive shorten-

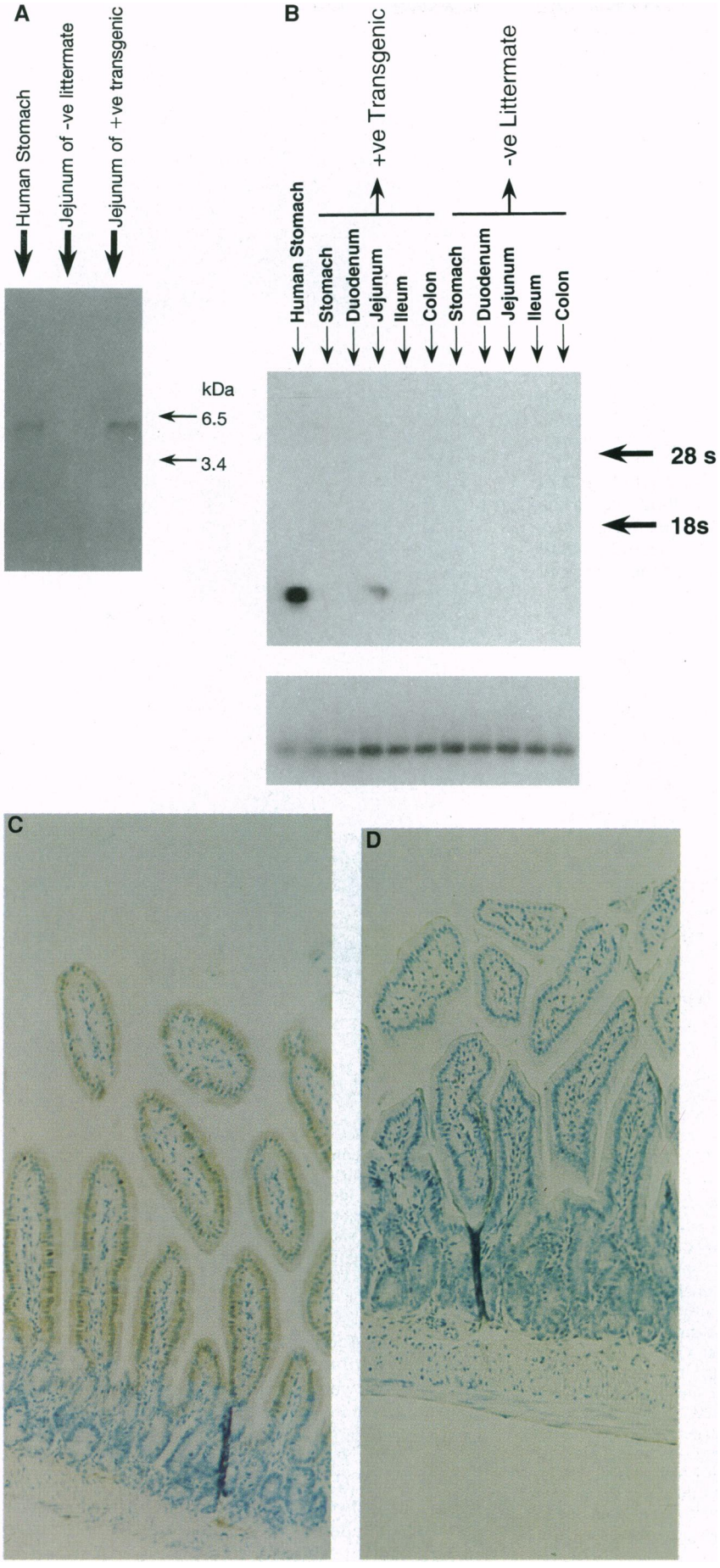


FIG. 1. Distribution of human pS2 in I-FABP$^{-1178\ \text{to}\ +28}$–pS2 transgenic mice. Transgenic mice were identified by Southern blot analysis of tail snips. For the Northern blots, RNA was purified from various regions of the gastrointestinal tract of transgenic positive (+ve) and negative (−ve) littermates. RNA obtained from human stomach was used as positive control. A single band, of the correct size, was seen only in the jejunum of positive animals (*B*). Molecular weight markers for RNA (28 and 18S) refer to *B Upper*. Reprobing of the same filters using a probe for 18S rRNA showed that they were loaded with similar amounts of total RNA (*B Lower*). Western blot analysis gave similar results with a positive band seen in the jejunum of transgenic positive animals with a molecular mass of about 6 kDa (*A*). Immunohistochemical staining was performed with mouse anti-hpS2 mAbs and a brown reaction product was obtained with a peroxidase substrate (diaminobenzidine and PBS in addition to 0.3% hydrogen peroxide). hpS2 was detected only in the jejunum of transgenic positive animals (*C*) and was not seen elsewhere in the bowel or in the jejunum of transgenic negative littermates (*D*). Note that the I-FABP promoter only induces the enterocytes to produce human pS2 after they have left the proliferative (crypt) zone. (*C* and *D*, ×6.5.)

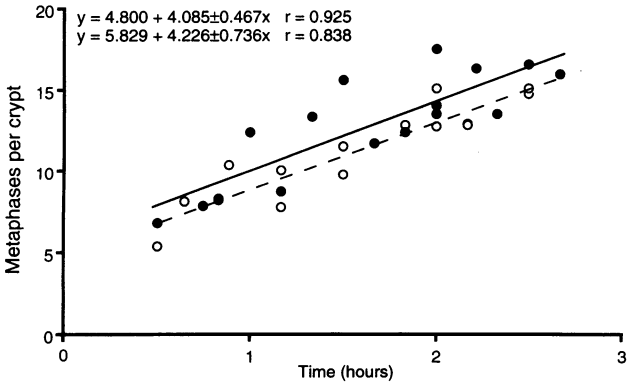


FIG. 2. Effect of expression of hpS2 on the CCPR. Transgenic and control (negative litter mates) mice were injected with vincristine sulfate (1 mg/kg i.p.) and killed at intervals from 30 to 180 min later. The number of metaphases per crypt were then analyzed by using microdissected crypts obtained from the jejunum. There was no significant difference between control (○) and transgenic (●) animals.

ing, reaching a minimum size after 12 h in the jejunum and 24 h in the ileum. Several of the villi also had bulbous expansion of the tips (Fig. 4*B*). These changes in the villus morphology were much less severe in the jejunum of transgenic animals (Fig. 4*C*). Morphometric analyses showed that the degree of

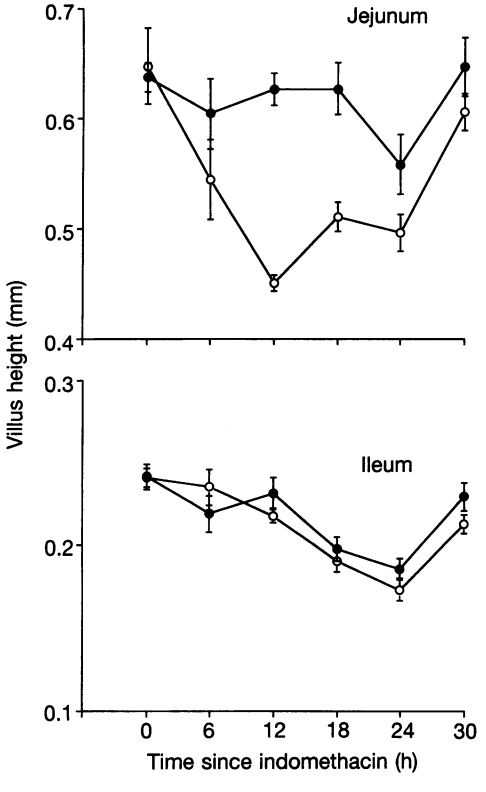


FIG. 3. Effect of indomethacin on the jejunal morphometry of transgenic and control mice. Villus heights were assessed in microdissected villi obtained from control (○) and transgenic (●) animals under baseline conditions (zero hour) and at various times after receiving a single injection of indomethacin (85 mg/kg s.c). $n = 7$ animals per group. Results are expressed as the mean ± SEM. In the jejunum of control animals, indomethacin caused a significant reduction ($P < 0.001$) in villus height during the period 6–24 h after indomethacin administration. In the same region of transgenic animals, the degree of villus shortening was much less severe ($P < 0.001$). In contrast, there was no difference between control and transgenic animals in the amount of shortening that occurred in the ileum due to indomethacin administration, a region that does not express hpS2 in our model.

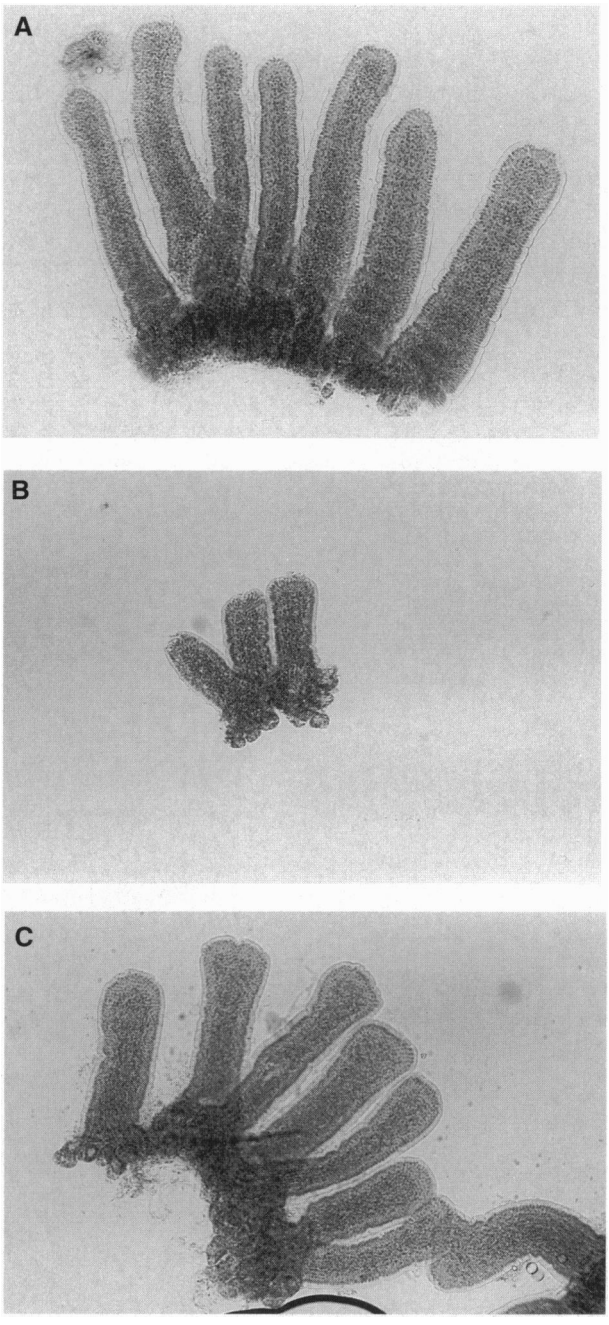


FIG. 4. Effect of indomethacin on the jejunal morphology of transgenic and control mice. The morphology of microdissected villi in the jejunum of control or transgenic animals under baseline conditions was identical with long slightly tapering villi (*A*). Villi obtained from control animals treated with indomethacin 12–24 h previously were shorter and several had bulbous expansion of the tips (*B*). Villus shortening was much less severe in transgenic animals given indomethacin (*C*) (for quantitation of villus height changes, see Fig. 3). (×10.)

damage (assessed by villus height and surface area) was only about one-third to one-half as much in the jejunum of transgenic animals when compared to the same region of negative control littermates (Fig. 3). ANOVA for jejunal villus height gave a significant effect for the presence of transgenic DNA ($F_{1,72} = 33.6$; $P = 0.000$) and time since administration of indomethacin ($F_{5,72} = 6.8$; $P = 0.000$), with a significant interaction between transgenic status and time since administration of indomethacin ($F_{5,72} = 3.73$; $P = 0.005$). This showed that the effect of the presence of transgenic DNA

varied according to the time since administration of indomethacin: No significant difference between the villus height of transgenic and control animals was seen under baseline circumstances (zero hour) or in the final recovery phase (30 h) (Fig. 3). However, in the intermediate time points (6–24 h), the amount of damage seen was much less severe in the transgenic animals ($P < 0.01$ at each time point compared with the equivalent control animal group). In contrast, examination of the ileum of these animals (a region that did not express hpS2 in our transgenic animals) showed no significant difference between transgenic animals and negative littermates in the amount of damage that had been sustained in this region (Fig. 3); ANOVA for ileal villus height gave a significant effect due to time since administration of indomethacin ($F_{5,72} = 18.08$; $P = 0.000$), but no effect due to the presence of transgenic DNA ($F_{1,72} = 1.65$; $P = 0.12$), with no interaction ($F_{5,72} = 18.41$; $P = 0.30$). This showed that indomethacin caused a similar amount of villus shortening in transgenic and control animals at all the time points studied. There was no significant effect on the crypt depths due to the presence of transgenic DNA or indomethacin administration.

## DISCUSSION

We have produced a transgenic mouse strain that expresses hpS2, a trefoil peptide, specifically within the jejunum by linking it to an I-FABP promoter. Expression of hpS2 within the jejunum of mice appeared not to have any effect on morphology or morphometry under baseline conditions but markedly reduced the susceptibility of the jejunum to indomethacin-induced damage. To our knowledge, this is the first demonstration of a biological action for hpS2.

Trefoil peptides form a family of molecules that share a motif of six cysteine residues, termed a trefoil or P domain, which is distinct from those found in other peptide families (such as the epidermal growth factor family). The term trefoil was developed by Thim (3) who postulated that the six cysteine residues could contribute to the formation of three intrachain loops. Three members of this family have been identified in humans consisting of one (pS2 and ITF) or two (SP) trefoil domains. pS2 was originally identified through its apparent ectopic expression in human breast carcinomas (10), although it has subsequently been shown that the normal breast also produces pS2 (13). Trefoil peptides are also expressed in the normal gastrointestinal tract in a site specific fashion; pS2 and SP are expressed predominantly in the stomach mucosa, whereas ITF is produced by the goblet cells of the colon. Interest in this family of peptides has been further heightened by the findings that expression of all three of the human trefoil peptides are markedly upregulated at sites of gastrointestinal injury in conditions such as peptic ulceration and inflammatory bowel disease (14, 15).

Gastrointestinal mucosal integrity depends on a balance between aggressive factors and mucosal defense mechanisms. When a mucosal lesion occurs, it is vital that a rapid healing response occurs to prevent extension of the damage due to luminal acid and/or proteolytic enzymes. One of the earliest responses is a stimulation of cell migration over the denuded area to reestablish epithelial continuity, a process called epithelial restitution. We have recently shown that systemic, but not intraluminal, administration of recombinant (r) human SP or rat ITF decreases indomethacin-induced gastric damage (5, 16). This effect may have been due to stimulating epithelial restitution as human SP, human ITF, and rat ITF stimulate cell migration in an *in vitro* wounding model of restitution (5, 16, 17). The findings that the trefoil peptides are expressed together at sites of injury and that they all stimulate cell migration suggest that there is likely to be some degree of overlap in their functions *in vivo*. The amino acid sequences of the trefoil peptides are highly conserved with human and murine pS2 sharing about 70% homology (18). Importantly, human and mouse pS2 are identical in their sequence of externally exposed hydrophobic residues, which may be involved in receptor binding (19). Until now, the lack of availability of mouse or human rpS2 has prevented any detailed functional studies. However, we (15, 16) and other workers (17) have shown that rat ITF, human ITF, and human SP possess biological activity when used in studies with rat or human cells lines (5, 16, 17). This conservation of biological activity between human and rodent systems is also supported by our present finding that expression of hpS2 decreases indomethacin-induced damage in the mouse intestine.

Previous studies examining the possible functions of trefoil peptides have utilized cell culture systems and/or systemic administration of recombinant peptides. Although these approaches provide supportive evidence for a role of trefoil peptides in the repair process, they can all be criticized on the grounds that they do not accurately reflect the situation that occurs *in vivo*, i.e., local upregulation of the expression of trefoil peptides. To address this problem, we therefore developed a transgenic model that would allow us to target expression of the hpS2 to the small intestine by linking it to rat I-FABP$^{-1178\ \text{to}\ +28}$. This promoter sequence has previously been used to target expression of human growth hormone to the mouse intestine (20). The distribution of expression of hpS2 produced by this promoter in our model (restricted to the jejunum) appears to differ from the distribution of the human growth hormone reported by Cohn *et al.* (20) who found expression in the ileum as well as the jejunum. This apparent discrepancy is probably due to Cohn *et al.* (20) collecting and using the entire terminal one-third of the small intestine as the "ileal segment," whereas our "ileal segment" consisted of only the very terminal region of the small bowel (at the position of 90% of total length). In the present study, transgenic animals had an increased resistance to damage only in the region that expressed hpS2 (i.e., the jejunum) with no protective effect seen in the nonexpressing ileal segment. This suggests that the protective action of hpS2 expression was mediated by local, rather than distant, effects.

Indomethacin causes damage to the gastrointestinal tract by several mechanism including reduction in mucosal prostaglandin levels, reduction of mucosal blood flow, and actions on neutrophil function (21). The detailed mechanism by which jejunal expression of hpS2 reduced the degree of damage is unclear although our previous findings that hSP and ITF stimulate cell migration (5, 16, 17), a key component of the early repair process (22), provides one possible explanation. In contrast to most other peptides thought to be important in mucosal repair (such as epidermal growth factor), most studies have reported that trefoil peptides have little or no proliferative activity (5, 16, 17), although one group found that administration of porcine SP induced a proliferative response in HCT116 cells (23). No effect on proliferation was seen in the jejunum of our transgenic animals; however, it is important to note that the I-FABP promoter used only induced the production of hpS2 in the enterocytes after they have left the proliferative (crypt) region. Although expression of hpS2 had no effect on gut morphology under baseline (nondamaged) conditions, it did result in a marked reduction in the degree of damage that occurred in response to indomethacin. These findings are important in interpreting the previous studies of Tomasetto *et al.* (24) who developed a transgenic model in which hpS2 was expressed in the mouse breast (by linking it to an acid-whey protein promoter). They found that the breast architecture appeared normal and that the offspring of these mice had a normal gut when suckled on hpS2-containing milk (24). Their findings are therefore entirely consistent with our results as they did not examine the effect of hpS2 expression in the damaged breast nor in the damaged bowel of the offspring fed hpS2-containing milk.

Little is known about the trefoil peptide receptor(s). Immunoprecipitation and cross-linking experiments have recently identified a 45-kDa protein complex from rat intestinal membranes which binds $^{125}$I-labeled rITF (25). Binding of labeled rITF to this complex could be displaced by unlabeled rITF or human SP and binding of unlabeled rITF resulted in phosphorylation of a tyrosine residue within the complex. The presence of trefoil binding sites on rat enterocytes is also supported by the *in vivo* studies of Rasmussen *et al.* (26) who showed that radiolabeled porcine SP binds to rat enterocytes after intravenous administration. A detailed examination of the distribution of trefoil binding sites on enterocytes has not been performed. However, our previous studies (5) showing that human rSP stimulates repair when given via the systemic circulation but not when given orally (5) suggests that the putative receptor(s) for the trefoil peptides are located on the basolateral, but not the apical, membranes of the enterocytes. A similar distribution has been reported for the epidermal growth factor receptor in rats and humans (27, 28).

Further studies are clearly required to fully understand the function of trefoil peptides *in vivo*. However, initial studies using our transgenic mice that overexpress hpS2 within the jejunum strongly support the hypothesis that trefoil peptides are important in stimulating gastrointestinal repair.

We thank the transgenic animal unit of the Imperial Cancer Research Fund for assistance, Miss N. Mandir for assistance with the assessment of CCPR, the Medical Research Council for funding of R.J.P. and T.M. and the Wellcome Trust for funding R.A.C.

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