Suggestive evidence for two different mucin genes in rat intestine

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In the present report we describe the isolation and sequence of a partial cDNA (M2-798) for a rat intestinal mucin designated M2. A rat intestinal λ ZAP II cDNA library was screened using a polyclonal antiserum which was prepared against deglycosylated high-molecular-mass glycopeptides of the purified mucin. Mucin cDNA clones were found to contain tandem repeats of 18 nt which encoded a threonine- and proline-rich peptide having a consensus sequence of TTPDV. This is the same sequence reported recently by Gum, Hicks, Lagace, Byrd, Toribara, Siddiki, Fearney, Lamport and Kim [(1991) *J. Biol. Chem.* **266**, 22733–22738] for a rat intestinal cDNA called RMUC 176. A novel feature present in the cDNA M2-798 is a 246 nt unique region at the 3' end which encodes a hydrophobic sequence of 82 amino acids. RNA blots probed with M2-798 cDNA produced a single hybridization band between 7.5 and 9.0 kb in rat small intestine and colon. An identical hybridization pattern was obtained with a PCR-generated cDNA probe corresponding

solely to the unique hydrophobic region of M2-798, demonstrating that this region is encoded by the authentic M2 mRNA. Our data suggest that the unique region of M2 has the potential to be either a transmembrane region, or a domain which mediates hydrophobic interactions of the mucin with other molecules. Since we have previously reported another rat intestinal cDNA which encodes the C-terminus of a mucin-like peptide (MLP) [Xu, Wang, Huan, Cutz, Forstner and Forstner (1992) *Biochem. J.* **286**, 335–338], we wished to discover whether M2 was encoded by the same gene. RNA blotting experiments with probes specific for M2 and MLP showed different mRNAs for each. The message for M2 (7.5–8.5 kb) was smaller than that for MLP (> 9.5 kb) and, unlike MLP, gave no signal in human colonic LS174T cells. The results of DNA blots probed with M2-798 and an MLP-probe suggest that M2 and MLP are likely to be single-copy genes. It would appear therefore that normal rat intestine, like human intestine, may express two different mucin genes.

INTRODUCTION

In recent years exciting advances have been made in the field of epithelial mucins to elucidate the structure of their normally heavily glycosylated peptide cores. These advances have been possible largely as a result of improved methods of deglycosylation and successful preparation of antibodies which recognize apomucin fragments in cDNA expression libraries. Although only three full-length mucin cDNAs have been sequenced so far, namely frog skin FIM-A.1 (Hoffmann, 1988), MUC1 for human mammary gland and pancreas (Gendler et al., 1990; Lan et al., 1990) and Muc1, its mouse homologue (Spicer et al., 1991), information derived from these and several other partial cDNAs (Timpote et al., 1988; Gum et al., 1989, 1990, 1991; Crepin et al., 1990; Bhargava et al., 1990; Probst et al., 1990, 1992; Porchet et al., 1991; Eckhardt et al., 1991) suggests that mucin peptides of different organs and species share several characteristics which are relevant to the present study.

(a) The polypeptide backbone contains numerous variable tandem repeats, which comprise as much as 70% of the mucin peptide core and are enriched in hydroxyamino acids and proline. The repeats serve as attachment sites for almost all of the O-linked oligosaccharides of the mucin molecule.

(b) Unique (non-repeating) segments form the C- and N-terminal ends of the peptide. A few N-terminal and many C-terminal segments have now been shown to be enriched in cysteine. In at least one case (human intestinal MUC2), there is another unique segment which contains cysteine, but it is located in the interior of the peptide where it separates different tandem repeat motifs (Toribara et al., 1991).

(c) Consensus sequences for the attachment of N-linked oligosaccharide chains have been found in many mucins cloned to date, indicating that the absence of N-glycans can no longer be considered a hallmark of mucin structure.

(d) In two mucins, MUC1 (Gendler et al., 1990; Lan et al., 1990; Spicer et al., 1991) and rat sialomucin ASGP-2 (Sheng et al., 1992), but not in other mucins reported to date, a putative transmembrane domain has been recognized near the C-terminal end of the peptide.

In previous studies (Fahim et al., 1983, 1987a; Mantle et al., 1984; Robertson et al., 1989) we have shown that, after thiol reduction, purified intestinal mucins separate during gel electrophoresis into a major high-molecular mass component and a 118 kDa minor component, the latter termed a putative 'link' glycopeptide. Recently we have isolated a cDNA for the rat intestinal 118 kDa 'link' glycopeptide (Xu et al., 1992c) and shown that it is not a discrete peptide, but represents the cysteine-rich C-terminal 689 amino acids of a much larger peptide which is expressed in intestinal goblet cells (Xu et al., 1992a) and which we have designated MLP (mucin-like peptide). The mRNA for this larger peptide is approximately 9.5 kb (or greater). The C-terminal 700 amino acids of human intestinal MUC2 show 73% sequence similarity to rat MLP (Gum et al., 1992), although the more central tandem repeat region of MUC2 is not present in rat intestinal mucin (Gum et al., 1991). Moving upstream in an N-terminal direction from the 118 kDa 'link' glycopeptide region of MLP, there is a cysteine-rich area (78 amino acids), an intermediate serine-, threonine- and proline-rich region comprising 4.5 degenerate tandem repeats of 11 or 12 residues each, a 42-amino-acid cysteine-rich segment and a long (258 amino

Abbreviations used: GPs, high-molecular-mass glycopeptides; TFMS, trifluoromethanesulphonic acid; d-Link, deglycosylated rat mucin 118 kDa glycopeptide; d-HSIM, deglycosylated human small-intestinal mucin; d-GPs, deglycosylated high-molecular-mass mucin glycopeptides; anti-HFB, antiserum to the peptide core of human MUC-2; MLP, mucin-like peptide; NEM, *N*-ethylmaleimide; PMSF, phenylmethanesulphonyl fluoride; 1 × SSC, 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; IPTG, isopropyl β -D-thiogalactopyranoside.

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acids) region enriched in serine, threonine and proline (Xu et al., 1992c; Huan et al., 1992). However, despite a total of 3.5 kb of sequence information, we have not yet identified a typical mucin-like multiple tandem repeat motif.

In an effort to discover whether MLP contains typical tandem repeats, we decided to develop an antibody which could recognize internal regions of the peptide core of rat intestinal mucin (where tandem repeats would be expected), and to use the antibody to screen a rat intestinal cDNA library. As shown here, we did obtain mucin cDNAs encoding tandem repeats, but the repeats appear to belong to an independent mucin gene which we have designated M2. Unlike MLP, M2 does not encode the 'link' region, and it produces a smaller size mRNA than MLP. Tandem repeat sequences of M2 are almost identical with those recently described by Gum et al. (1991) for a rat intestinal mucin partial cDNA called RMUC 176. One of our M2 clones (M2-798) encodes 30 tandem repeats plus a unique hydrophobic sequence of 82 amino acids. The latter differs from any other reported unique sequence in mucins, including the cysteine-rich unique sequence of RMUC 176. Although its function is unknown, the hydrophobic and apolar nature of the M2 unique regions suggest that it has the potential to exist *in vivo* as a transmembrane or membrane-associated region, and/or to form hydrophobic interactions with other molecules.

METHODS

Preparation of rat intestinal mucin and separation of its glycopeptide subfractions

Rat intestinal mucin was purified from homogenates of epithelial scrapings as described previously (Fahim et al., 1987b). The purified mucin was then reduced, alkylated and subjected to density-gradient ultracentrifugation in CsCl and 4M guanidinium chloride [starting density (ρ) 1.4 g/ml] for 48 h at 150 000 *g*; 20 equal fractions were collected, dialysed against water and analysed for carbohydrate (Mantle and Allen, 1978), buoyant density, and electrophoretic mobility by SDS/PAGE (Laemmli, 1970). The high-molecular-mass glycopeptides (GPs), which were rich in carbohydrate and free of the 118 kDa glycopeptide, appeared in fractions 5–9 over a buoyant-density range of 1.38 to 1.45 g/ml. The 118 kDa glycopeptide was harvested from fractions 11–18 (ρ 1.28 to 1.37 g/ml). Both pools were separately concentrated by freeze drying for analyses or further experiments.

Deglycosylation of mucin subfractions

Rat mucin GPs (10 mg as protein) were dried over P₂O₅ for 2 days, and then subjected to two deglycosylation steps using trifluoromethanesulphonic acid (TFMS) for 2 h at 0 °C and 4 h at 0 °C, as described by Edge et al. (1981). Further deglycosylation was achieved by an oxidation–elimination reaction (Gerken et al., 1992), in which oxidation was performed in the dark at 4 °C overnight, and elimination carried out at a constant pH of 10.5 for 1 h at room temperature. The product was incubated for 16 h at 37 °C with O-glycanase (endo- α -N-acetylgalactosaminidase of *Diplococcus pneumoniae*; Genzyme Corp., Boston, MA, U.S.A.) in 10 mM Tris/maleate buffer, pH 6.0. The final mucin product was dialysed against 10 mM NaH₂PO₄/Na₂HPO₄ buffer, pH 7.5, in the presence of 1 mM phenylmethanesulphonyl fluoride (PMSF) in 0.05 % NaN₃. After analyses of the amino acid and amino sugar composition (by the Picotag h.p.l.c. system; Waters Associates, Mississauga, Ontario, Canada) (Cohen and Strydom, 1988), the mucin product was called d-GPs (deglycosylated glycopeptides).

Other mucin preparations used in the present study included deglycosylated rat mucin 118 kDa glycopeptide, called d-link (Xu et al., 1992c), deglycosylated human small-intestinal mucin, d-HSIM (purified as described for rat mucin and deglycosylated as described above for d-GPs, except for the omission of O-glycanase treatment).

Preparation and assay of antisera

A polyclonal antiserum to d-GPs was raised in New Zealand White rabbits as described elsewhere (Khatri et al., 1992) using 200 μ g (protein) of antigen for the first, and 100 μ g (protein) for two repeat injections. Preimmune and immune sera at various dilutions were assayed for reactivity with various antigens (1–200 ng of protein) by a slot-blot e.l.i.s.a. (Khatri et al., 1992). For detection, anti-rabbit IgG conjugated to alkaline phosphatase and the Promega protoblot substrate system were employed. Other antibodies used in the present study included an antibody to intact rat intestinal mucin (Forstner et al., 1977) and an antiserum to the peptide core of human MUC2, called anti-HFB (Gum et al., 1989), kindly supplied by Dr. Y. S. Kim, VA Medical Centre, San Francisco, CA, U.S.A.

Electron microscopy and immunogold localization of d-GPs

Electron-microscopic immunolocalization of the antiserum to d-GPs was performed by a slight modification of the method described by Roth (1978). Thin sections (70 nm) of rat small intestine attached on nickel grids were floated for 30 min at room temperature on a drop of 10 mM NaH₂PO₄/Na₂HPO₄/150 mM NaCl buffer, pH 7.5 (PBS), containing 3 % BSA and 0.15 % glycine. After two rinses in PBS (each for 5 min), the sections were floated on a 1:20 dilution of normal goat serum in PBS for 30 min at room temperature. Without rinsing, the grids were then transferred to a drop of antiserum to d-GPs diluted 1:500 in 1 % BSA/PBS and incubated for 2 h at room temperature. After washing with PBS, the sections were floated for 1 h at room temperature on a drop of goat anti-rabbit IgG conjugated to 10 nm particles of colloidal gold (Amersham, Oakville, Ontario, Canada) diluted 1:20 in 1 % BSA/PBS. After washing with distilled water, the sections were air-dried, counterstained with uranyl acetate and lead and examined in a Philips 400 transmission electron microscope at 60 kV.

Isolation and sequencing of cDNAs for rat intestinal mucin

A rat intestinal cDNA library constructed in λ ZAP II by Stratagene has been described earlier (Xu et al., 1992c) and was screened in the present study by the methods of Sambrook et al. (1989a) using a 1:100 (v/v) dilution of antiserum developed against d-GPs of rat mucin. The antiserum was preabsorbed against *Escherichia coli* lysate before use. The amplified library was plated in *E. coli* SURE strain (Stratagene) at a density of 30 000 plaques/150 mm plate, incubated at 42 °C for approx. 3 h, and then overlaid for 4 h with IPTG (isopropyl β -D-thiogalactopyranoside)-saturated nitrocellulose membranes. The membranes were removed and fresh membranes, impregnated with IPTG, were overlaid on the plates for 8 h. Positive plaques were detected with an anti-rabbit IgG–alkaline phosphatase conjugate by the Promega Protoblot system, and then purified to clonality by successive rounds of rescreening. The pBluescript plasmid cDNA inserts were excised *in vivo* from the λ ZAP II vector, and single-stranded DNA was produced with the helper phage R408 (Stratagene protocol). Using universal primers and appropriate internal primers (see the Results section), cDNA

inserts were sequenced by the dideoxynucleotide chain-termination method (Sanger et al., 1977). In a 471 nt region of cDNA insert from M2-798 (nt 328–798), both strands of cDNA were sequenced in their entirety.

Sequences were analysed on a DEC Station 5000/200 computer system using the MBIR software package (Department of Cell Biology, Baylor College of Medicine, Houston, TX, U.S.A.). Comparison with other protein sequences registered in the National Biomedical Research Foundation Protein Identification Resource (PIR release 32.0) and in the GenBank Nucleic Acid Sequence database (release 71.0) were conducted using the FASTA program (Pearson and Lipman, 1988).

Hydropathy plots were generated by the method of Kyte and Doolittle (1982), and secondary-structure predictions were made by the method of Garnier et al. (1978).

Northern- and Southern-blot analyses

Total RNA was prepared from rat intestine, colon, stomach, liver, lung (including bronchi and trachea) and human colonic adenocarcinoma LS174T cells (A.T.C.C.) by the method of Han et al. (1987). For Northern blots, RNA (5 to 20 µg) was separated on 0.8% agarose gels containing 2.2 M formaldehyde, soaked briefly (20 min) in 0.05 M NaOH, and transferred to nylon membranes as outlined by Sambrook et al. (1989b). Southern blots were performed on complete restriction-enzyme digests of genomic DNA from adult male rat liver (Clontech, Palo Alto, CA, U.S.A.) and on *Eco*RI-digested M2-1700, M2-1000 and M2-798. The digests were separated on 0.8–1.0% agarose and transferred to nylon membranes.

Probes for both Northern blots and Southern blots of rat genomic DNA were made to correspond to selected regions of the cDNAs for the rat intestinal mucins MLP or M2. The probe used for MLP consisted of a *Hind*III cDNA fragment (called MLP-500) corresponding to the 3' half of domain I (Xu et al., 1992c). One of the probes used for M2 was the entire 798 nt cDNA insert of clone M2-798, whereas the second probe consisted of a portion of M2-798 (nts 542 to 798). To obtain the second probe, cDNA was amplified from M2-798 by PCR using *Taq* DNA polymerase (Perkin-Elmer/Cetus) in 100 µl of standard buffer with 0.2 mM each of dATP, dCTP, dGTP and dTTP, and 0.05 µM of each of two opposing primers: 5' CTACGCTATTTCTACCCATC (sense) and 5' CAGCTTG-AAGATGGGGATAC (antisense) corresponding to nt 542–561 and 779–798 respectively, within the non-tandem repeat (unique) region of M2-798. PCR was performed for 30 cycles using a thermal cycler (Ericomp Inc.) for 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. After the last cycle the reaction mixture was incubated at 72 °C for 5 min to complete the extension. The PCR product of 257 nt was excised and amplified by a second round of PCR. The product was separated on 4% NuSieve GTG (FMC Bio Products, Rockland, ME, U.S.A.), eluted and used for radiolabelling as well as nucleotide sequencing. The three probes MLP-500, M2-798 and the 257 nt PCR product of M2-798 were labelled with [α -³²P]dCTP using the T7 Quick Prime kit (Amersham) according to the manufacturer's protocol.

For Southern blots of *Eco*RI-digested cDNAs of rat intestinal mucin M2, inserts from M2-1700 and M2-798 were used as probes. These probes were labelled with digoxigenin using the Non-Radioactive DNA Labelling and Detection Kit (Boehringer-Mannheim G.m.b.H., Mannheim, Germany) according to the manufacturer's protocol.

Hybridizations of both Northern and Southern blots were performed at 42 °C for 18 h with 50% formamide, 4× SSC,

100 µg/ml of sheared salmon sperm DNA, 5× Denhardt's solution, 0.1% SDS, 0.25 M NaH₂PO₄/Na₂HPO₄ buffer, pH 6.5, and approx. 1 × 10⁶ c.p.m./ml of labelled probe, essentially as described by Sambrook et al. (1989c). Partial stripping of the Northern blot was achieved by incubating it for 1 min with shaking in a boiling solution of 0.1× SSC containing 0.1% SDS. Complete stripping of the blot was achieved by one further identical incubation step, and confirmed by re-exposure of the blot to X-ray film for 1 week.

RESULTS

Intestinal mucin was purified from homogenates of rat small-intestinal epithelial scrapings, reduced and alkylated and subjected to density-gradient ultracentrifugation in CsCl and guanidinium chloride (see the Methods section). The high-molecular-mass glycopeptides (GPs) were pooled and deglycosylated by TFMS, oxidation elimination and O-glycanase digestion as outlined in the Methods section. The amino acid and amino sugar compositions of the starting mucin, GPs and d-GPs are shown in Table 1. The lower concentration of Asx and Glx and the higher concentration of Ser, Thr and Pro in the GPs relative to the starting mucin, reflect the removal of the 118 kDa 'link' glycopeptide region of the mucin during density-gradient ultracentrifugation (Fahim et al., 1983; Mantle et al., 1984). After deglycosylation, approx. 70% of the peptide of GPs was recovered. Although the amino acid profile of d-GPs was not completely identical with that of GPs, the concentrations of Ser, Thr and Pro collectively accounted for 63.2% of the peptide, indicating a high recovery of those regions which generally serve as O-glycosylation modules in mucins. The loss of the amino sugars GlcNAc and GalNAc was greater than 99.9%, indicating that the GPs had been effectively stripped of carbohydrate.

An antiserum to the d-GPs was developed in rabbits and tested at several dilutions by slot-blot e.l.i.s.a. against 100 ng each of GPs, d-GP, the 118 kDa 'link' glycopeptide, the d-link and d-

Table 1 Composition of native rat intestinal mucin and its components before and after deglycosylation

Purified mucin was reduced/alkylated, subjected to CsCl-density-gradient ultracentrifugation in the presence of 4M guanidinium chloride, and separated into 20 fractions. The GPs (high-molecular-mass mucin glycoproteins) appeared in fractions 5–9. Amino acids (a) and amino sugars (b) were measured by the PicoTag system after hydrolysis for 24 h (or 7 h for amino sugars) at 110 × C in 5.5 M HCl. d-GPs refers to deglycosylated GPs.

(a)

Amino acid	Composition (mol/100 mol)		
	Native mucin	GPs	d-GPs
Asx + Glx	21.1	11.2	15.1
Ser	10.0	13.6	15.7
Gly	5.7	3.8	5.4
Thr	25.3	33.6	33.4
Pro	11.6	14.2	14.1
Rest	26.3	23.6	16.3

(b)

Amino sugar	Carbohydrate composition (nmol/µg of protein)		
	Native mucin	GPs	d-GPs
GalNAc	3.95	4.20	0.005
GlcNAc	3.60	4.01	0

Figure 1 Subcellular localization of mucin peptide

Gold particles 10 nm in diameter are located predominantly in the rough endoplasmic reticulum of mucus-producing goblet cells, but not in mucus granules or in other cells of the rat small intestine. Magnification: (a) $\times 10530$; (b) $\times 34490$.

HSIM. Only the d-GPs gave a positive reaction with the antiserum (results not shown). Preimmune serum was negative for all antigens.

Electron-microscopic immunolocalization of the antibody to d-GPs was performed by a colloidal gold-second antibody technique on 70 nm thin sections of rat small intestine. As shown in Figures 1(a) and 1(b), gold particles localized over the rough endoplasmic reticulum of goblet cells around the lateral border of the mucin granule mass, with negligible signal over mucin granules or adjacent absorptive cells. Preimmune serum of PBS

controls, used instead of antiserum, gave only background (random) scattering of a few gold particles over the sections (results not shown). Our findings were consistent with the interpretation that the antiserum recognized apomucin in the endoplasmic reticulum but not mature glycosylated mucin in the granules, and would therefore be suitable for the detection of apomucin fragments in a rat intestinal cDNA expression library.

An antibody to the human intestinal apomucin HFB (1:100 dilution of antiserum) reacted positively with d-HSIM, but was negative for all other antigens. This result suggested that the

Figure 2 (a) Sequencing strategy for clone M2-798 and (b) nucleotide sequence of a portion of M2-798 cDNA (i.e. nt 328–798) and the corresponding deduced amino acid sequence (residues 110–266)

(a) The 'ruler' (above) represents the nucleotide numbering in the 5' → 3' direction. Arrows represent the length and direction of individual sequencing reactions. The terminal regions were sequenced using vector-derived universal primers. The open bar indicates the tandem-repeat region and the closed bar the unique region. (b) Twelve 6-amino-acid tandem repeats preceding the unique sequence of the clone are bracketed. The first amino acid of each repeat is preceded with an asterisk. One potential N-glycosylation site was detected at amino acid 125 and is underlined. The predicted secondary structure of the amino acids encoded by the 246 nt unique sequence (nt 552–798) is shown in italics below each triplet codon. Abbreviations: S, β -sheet; H, α -helix; T, β turn; C, random coil. Both strands of DNA were sequenced.

peptide core (random repeat) regions of rat and human intestinal mucins were different immunologically and, presumably, structurally.

Isolation and sequencing of rat mucin cDNAs

With the use of the antiserum to d-GPs, screening of 4×10^5 recombinants from a λ ZAP II rat intestinal cDNA library gave 40 positive clones, 12 of which were purified. The inserts were released by *Eco*RI digestion and separated on 1% agarose. There were three clones, designated M2-1700, M2-1000 and M2-798, containing inserts of approx. 1.7, 1.0 and 0.7 kb respectively. Southern blots of the inserts from each were probed with MLP-500 (Xu et al., 1992a) and showed no hybridization reaction.

Sequencing of inserts from M2-1700 and M2-1000 at both termini revealed a single open reading frame containing a continuous array of 18 nt tandem repeats. Since the tandem repeats of M2-1700 and M2-1000 were highly conserved in their sequence, more internal regions of these clones were technically difficult to sequence. The most common deduced amino acid sequence of the repeats was a six-residue Thr-rich motif TTPDV, suggesting that the repeat regions represent highly O-glycosylated segments of a mucin molecule. Restriction-enzyme analysis revealed that the 18 nt repeating unit contained a site for the restriction enzyme *Mae*III. When the insert from M2-1700 was digested with *Mae*III, the insert was completely digested, leaving only vector-derived fragments, as judged by agarose-gel (1.5%) electrophoresis patterns (results not shown). This finding indicated that the entire cDNA of M2-1700 consisted of repeating units. Southern blots of *Eco*RI-digested M2-1700, M2-1000 and M2-798, probed with digoxigenin labelled inserts from M2-1700 or M2-798 showed cross-hybridization with each other, indicating that the clones likely differed from each other with respect to the number of tandem repeats.

The sequencing strategy for clone M2-798 is presented in Figure 2(a). Sequencing at the 5' end revealed the same 18 nt tandem repeat motif (30 repeats), but the most novel aspect of the cDNA was a 246 nt unique (non-repeat) sequence present at the 3' end (nt 516–798; Figure 2b). A single open reading frame was maintained through the entire repeat region and the unique sequence. Not all of the tandem repeats of M2-798 are presented (in Figure 2b) because they were so similar to the repeating pattern reported by Gum et al. (1991) for a rat intestinal mucin cDNA called RMUC 176. Of the 30 tandem repeats present in clone M2-798, 96.6% contained TTT as the first three amino acids, 76.6% contained a non-polar residue (usually Pro or Ala) at position 4, 73% contained an acidic or basic residue (Asp or Glu) at position 5, and 81.3% contained a hydrophobic residue (Val or Ile at position 6). A similar distribution of residues was calculated from the 59 reported tandem repeats of RMUC 176 (96.6, 81.3, 66 and 83% respectively). One potential N-glycosylation site at residue 125 was present in the M2-798 sequence, whereas in RMUC176 none was detected.

No sequence similarities were noted between the unique region of M2-798 and any other protein or nucleic acid sequence registered in the PIR or GenBank database, including the entire 3.5 kb cDNA we have reported earlier for rat MLP (Xu et al., 1992c; Huan et al., 1992). Compositional analyses based on the deduced amino acid sequence of the unique regions of both M2-798 and RMUC176 are presented in Table 2. The cysteine content of the 82-amino-acid M2-798 region was much lower than that of the 92-amino-acid RMUC176 region (2.4 as against 8.6 mol%) whereas the sum of the other hydrophobic plus apolar residues (Leu, Ile, Val, Phe, Trp, Ala, His, Tyr, Met, Gly, Thr) was much higher in M2-798 (70.2 as against 46.1% in RMUC 176).

Kyte and Doolittle (1982) hydropathy plot analysis of the 82-amino-acid unique sequence of M2-798 is shown in Figure 3. Unlike the unique sequence of RMUC176 (result not shown), most of the unique sequence of M2-798 is very hydrophobic. Since the cDNAs of both M2-798 and RMUC176 encode the same basic tandem repeat structure, but have different unique segments, they probably represent different regions of the same mucin core peptide. As yet, however, we cannot map their respective positions on the mucin gene.

Secondary-structure prediction analyses were carried out for the unique region of M2-798 (Garnier et al., 1978) and, as shown in Figure 2(b), the 82-amino-acid region consisted mainly of alternating β -sheet and α -helical segments, each approx. ten

Table 2 Deduced amino acid composition from the unique sequence at 3' end of clones MW-798 and RMUC176 (Gum et al., 1991) for a rat intestinal mucin

Compositions are based on the deduced amino acid residues numbers 185–266 of M2-798 and 356–447 of RMUC176 (calculated from the results of Gum et al., 1991).

Amino acid	Composition (mol/100 ml)	
	Unique region of clone M2-798	Unique region of clone RMUC176
Cys	2.4	8.6
Leu	17.0	4.3
Ile	10.9	1.0
Val	6.1	11.9
Pro	8.5	4.3
Ala	13.4	2.1
Trp	1.2	2.1
Phe	6.1	2.1
Gly	4.8	10.8
Thr	3.6	6.5
Met	4.8	2.1
His	1.2	0.0
Tyr	1.2	3.2
Ser	8.5	9.7
Asp	8.5	4.3
Glu	0.0	7.6
Asn	3.6	6.5
Gln	1.2	5.4
Arg	1.2	4.3
Lys	2.4	2.1

Figure 3 Hydropathy plot of the 246 nt (82 deduced amino acid) unique sequence at the 3' end of clone M2-798 calculated by the method of Kyte and Doolittle (1982) using a window size of 9 amino acids

amino acids in length. The pattern suggests that the unique segment of M2-798 may have a high degree of secondary structure, with sufficient hydrophobicity to predict either a membrane location or at least a capacity for hydrophobic interactions.

Northern blots

RNA blot analyses were carried out using RNA prepared from several rat tissues and probed with the radiolabelled cDNA insert from M2-798 (Figure 4a). Before hybridization, all samples gave sharp 28S and 18S rRNA bands on blots and equivalent

Figure 4 Northern-blot analyses of total RNA probed with M2-798 cDNA (a) or a PCR-generated cDNA probe corresponding to the 3' 246 nts of M2-798 cDNA (b)

Lanes 1 and 2, rat colon (7, 14 μ g); 3 and 4, rat small intestine (7, 14 μ g); 5 and 6, rat stomach (5, 20 μ g); 7 and 8, rat lung (5, 20 μ g); 9 and 10, rat liver (5, 20 μ g); 11 and 12, human LS174T cells (10, 20 μ g). The values on the left represent the positions (kb) of RNA reference markers.

intensities, suggesting equal RNA loading of each tract (results not shown). RNA from human LS174T cells (human colonic carcinoma cell line) was also included in these experiments. A single hybridization band was obtained between 7.5 and 9.5 kb from rat small intestine and colon, but all other samples, including rat lung, liver and stomach, as well as the human cells, were negative. After hybridization with M2-798 cDNA, the blot was completely stripped of the probe (i.e. gave no signal after re-exposure to X-ray film for 1 week). The blot was reprobed with a PCR-generated cDNA corresponding to the 3' 246 nts of clone M2-798 (i.e. the unique region) and, as shown in Figure 4b, gave a pattern identical with that of Figure 4(a). Thus the unique sequence of M2-798 arises from the authentic M2 mRNA and is not an artifact of external DNA recombination. The hybridization signal for both probes appeared within 1 h after exposure of the blots to X-ray film, suggesting that the message was abundant in the small intestine and colon of the rat. Even after 36 h of exposure, however, no signal was detected in other samples. Hybridization patterns identical with those of Figure 4(a) were also obtained when parallel RNA blots were probed with labelled inserts derived from clones M2-1000 and M2-1700 (results not shown).

To compare the mRNA for M2 with that of rat MLP, RNA blots were first hybridized with M2-798 cDNA, the blots were then partially stripped, and reprobed with the MLP-500 cDNA. As shown in Figure 5, each probe gave a different hybridization band and no evidence of polydispersity. In the intestinal tissue of four individual rats (tracts 1–4) the mRNA for M2 was between 7.5 and 9.5 kb and was clearly separated from the mRNA for MLP, which was greater than 9.5 kb. Although both probes had approximately the same specific radioactivity ($\sim 1 \times 10^6$ c.p.m./ml), the MLP signal appeared after a much longer exposure (overnight as against 1 h) of the blot to X-ray film. In LS174T cells the MLP-500 signal was positive (tracts 5, 7 and 8), whereas the M2-798 probe failed to give a signal. The differences between the M2 and MLP blots suggest that the two mRNAs may be encoded by two different genes. Neither probe gave a signal in rat stomach, indicating that there must be yet another gene responsible for gastric mucin.

Figure 5 Northern-blot analyses of total RNA hybridized first with M2-798, followed by partial stripping of the label and reprobing with the *HindIII* fragment of rat MLP cDNA (MLP-500)

Lanes 1–4 represent RNA from intestinal tissue of four individual rats (10, 10, 10 and 20 μ g of RNA respectively); lanes 5, 7 and 8 refer to human LS174T cells (30, 20 and 10 μ g of RNA respectively); lane 6 refers to rat stomach (10 μ g RNA). The small arrowhead represents hybridization with the M2 probe; the large arrowhead represents hybridization with the MLP probe. The values on the left are positions (kb) of RNA reference markers.

Figure 6 Southern blots of rat genomic DNA probed with MLP-500 (a) and M2-798 (b)

The DNA was digested with the following restriction enzymes: 1, *EcoRI*; 2, *HindIII*; 3, *BamHI*; 4, *PstI*; 5, *XbaI*. Values on the left represent the positions (kb) of DNA reference markers.

Southern blot

Rat genomic DNA was digested with several restriction enzymes and subjected to Southern-blot analyses using the MLP or M2 probes (Figures 6a and 6b). The two probes (500 nt and 798 nt respectively) showed very clear differences in their hybridization patterns. In the case of probe MLP-500, four of the enzymes resulted in the appearance of a single hybridization band. The two bands obtained with *EcoRI* were expected, because MLP-500 cDNA contains an internal *EcoRI* site (Xu et al., 1992c). In the case of probe M2-798, two of the digests gave a single band. Since single hybridization bands from genomic DNA blots are

generally indicative of single-copy genes, M2 and MLP may each represent single-copy genes.

DISCUSSION

In the present study we used an antibody which was expected to recognize the internal core peptide of rat intestinal mucin in order to select positive clones from an intestinal cDNA expression library. From partial sequencing of two M2 clones and complete sequencing of a third, we found tandem repeats of 18 nt which encode a Thr- and Pro-rich protein consensus sequence of TTPDV. One clone (M2-798) also carried a 3' unique (non-repetitive) sequence encoding 82 amino acids. The same repeat sequence, but a different 3' unique sequence, was reported by Gum et al. (1991) for a partial cDNA (called RMUC 176) of rat intestinal mucin. Since both cDNAs have the same tandem repeat and hybridized to an mRNA of approx. 8–9 kb, we conclude that the two cDNAs correspond to different regions of the same mucin gene, and that at least one of the two unique regions must be located in a more internal position than the other, adjacent to a long stretch of tandem repeats. Assuming that the two cDNAs correspond to non-overlapping regions, the mucin peptide can be judged to contain no fewer than 89 tandem repeats.

In previous publications (Xu et al., 1992c; Huan et al., 1992) we have presented 3.5 kb of cDNA sequence corresponding to the C-terminal end of a rat intestinal-mucin-like peptide (MLP). The mucin described in the present paper has been called M2 to distinguish it from MLP. Probes corresponding to the C-terminal region of MLP did not hybridize to the same mRNA as that recognized by probes specific for either the tandem repeats or the unique region of M2-798. Probes corresponding to the two regions of M2 also failed to hybridize to the mRNA for MLP. Unlike the MLP probe, the M2-798 probe gave no signal with human LS174T cells. These findings suggest that rat intestinal tissue may contain two different mucin genes. A parallel exists in human intestine, as there is evidence for different mucin peptides from biochemical studies (Podolsky and Isselbacher, 1984; Wesley et al., 1985) and cDNA cloning (Gum et al., 1989, 1990). Chromosomal-localization studies (Griffiths et al., 1990; Gum et al., 1990) indicate that MUC2 and MUC3 of human intestine encode two distinct mucins. As yet, chromosomal localization for rat M2 and MLP has not been undertaken, and thus unequivocal proof that they represent different genes is lacking. The possibility of M2 and MLP belonging to the same gene seems unlikely, however, not only because of our Northern-blot data, but also because of the general prominence of tandem repeat arrays in most mucins. That is, tandem repeats usually comprise 60–70% of the peptide core of mucins (Strous and Dekker, 1992) and, for M2 and MLP to be part of the same mucin gene, we might expect at least some tandem repeats of M2 to be present in MLP, even if M2 represents an alternative spliced product of MLP. Polydispersity of mucin structure commonly arises from the generation of multiple transcripts which vary on the basis of the number of their tandem repeats. We did not observe mRNA polydispersity for M2 or MLP, however. Splicing variants in which one variant is devoid of the tandem repeats of the other (which would be the case if MLP and M2 are alternate splice variants) have not been observed for mucins to date. Two splice variants of episialin (now called MUC1) have been described (Ligtenberg et al., 1990), but in this case the splice variants each contain the same tandem repeat sequence and differ only with respect to their signal sequence and the region encoding the mature amino terminus. Thus the episialin model is also inappropriate to describe differences between M2 and MLP.

As reported previously (Xu et al., 1992b), a cDNA from human colonic carcinoma cells was found to show sequence similarity to rat MLP in the 3' terminal region, and to hybridize to the same mRNA that was recognized by a probe for the human intestinal mucin, MUC2. The human MLP cDNA mapped to chromosome 11 p 15.5, the known locus of MUC2, which suggested that human MLP and MUC2 represent the same gene. Our data also indicated that rat MLP and human intestinal mucin MUC2 share a common C-terminal amino acid structure. Recent work by Gum et al. (1992) has confirmed that human MLP is the same as MUC2 and that rat MLP and MUC2 show 73% identity at their C-termini. However, the tandem repeats of human MUC2 are not present in rat intestinal mucin (Gum et al., 1991). Similar C-termini with dissimilar tandem repeats are also found in comparisons of frog integumentary mucin (FIM-B.1) and bovine and porcine submaxillary mucins (Eckhardt et al., 1991). Also there is better conservation of C-terminal regions than tandem repeats between human MUC1 and mouse Muc1 mammary-gland mucins (Gendler et al., 1991). Thus C-terminal regions of mucins may be better conserved between species than are tandem repeats, presumably because the C-terminal regions perform a function which will not tolerate many mutationally induced deviations in peptide secondary structure (Pemberton et al., 1992).

Since we now know that rat intestinal MLP is a homologue (at the C-terminus) of human MUC2, it is relevant to ask whether rat M2 could be a similar homologue of human MUC3, the second human intestinal mucin (Gum et al., 1990). As yet there is no evidence to support this speculation, since probes corresponding to the tandem repeat of human MUC3 were not found to hybridize in Northern blots of rat intestinal mRNA (Gum et al., 1991), and, in our own experiments, neither a probe of the tandem repeat segment nor the unique region of M2-798 hybridized to RNA of human LS174T colonic cells (where MUC 3 is known to be expressed). Before ruling out a potential similarity, however, it will be important to extend the sequence of M2 and MUC3 to include their C-terminal domains. If similarities of sequence exist, both rat M2 and MLP genes may serve as targets for studies of mucin-gene expression in rat models of human intestinal disease.

One of the tandem repeats of M2-798 (Figure 2b) contained a consensus sequence for N-glycosylation. This observation adds to the growing list of mucins in which deduced N-glycan attachment sites have been noted (Hoffmann, 1988; Gum et al., 1989; Corfield et al., 1990; Gendler et al., 1990; Lan et al., 1990; Probst et al., 1990; Eckhardt et al., 1991; Xu et al., 1992a). The significance of N-glycosylation in mucin molecules is still unknown, although Dekker and Strous (1990) have suggested that N-glycans are added co-translationally during apomucin synthesis and may stabilize a conformation which facilitates disulphide bond-mediated polymerization and intracellular transport.

The unique region encoded by the cDNA M2-798 was highly enriched in hydrophobic and apolar residues, which distinguish it from the cysteine-enriched unique region encoded by RMUC 176 (Gum et al., 1991). Whether this indicates that M2 has a transmembrane domain is open to question, since there is no independent biochemical or immunological evidence to suggest that the M2 mucin is attached to intracellular or surface membrane structures. A membrane-bound intestinal mucin has been described in COLO 205 human colonic carcinoma cells (Baeckstrom et al., 1991). After deglycosylation, the core peptide of this mucin recognized the monoclonal antibodies SM-3 and HMFG-2, which are directed against the tandem repeats of human MUC1, a mucin known to be membrane-attached. We

have also employed these two antibodies (a gift from Dr. J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London, U.K.) but have detected no reactivity with the deglycosylated glycopeptides of rat intestinal mucin (I. A. Khatri, G. G. Forstner and J. F. Forstner, unpublished work). These findings do not, of course, exclude the possibility that rat intestine may contain a MUC1 homologue with a conserved C-terminus but different tandem repeat sequences.

The 82 amino acids of the M2 unique region constitute a much longer hydrophobic stretch than the putative transmembrane region of either MUC1 (31 amino acids) (Gendler et al., 1990) or the rat mammary carcinoma mucin component ASGP-2 (26 amino acids) (Sheng et al., 1992). Possibly the M2 mucin is not an integral-membrane-bound mucin, but instead contains a hydrophobic domain that serves other important roles. There are many reports of interaction of mucins with hydrophobic probes, lipids, mutagens and non-polar compounds (Sachdev et al., 1979; Smith and LaMont et al., 1984; Slomiany et al., 1988; Kim and Singh, 1990; Shankar et al., 1990; Ng et al., 1992). These findings have led to the general assumption that all mucins contain hydrophobic regions. In many cases the hydrophobic binding sites reside on non-glycosylated ('naked') proteinase-sensitive regions of the mucin peptide. The unique hydrophobic segment of M2 not only appears to lack putative N- and O-glycosylation sites (Aubert et al., 1976; Briand et al., 1981; Timpote et al., 1988) it also contains potential trypsin-sensitive residues. The two cysteine residues in the sequence are also of interest, since theoretically one of these could be a target for palmitic acid acylation (Schmidt, 1989). Thus the unique region of M2 may normally function *in vivo* as a pocket for hydrophobic interactions with apolar compounds in the intestinal environment, including cell membranes.

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