Gastrointestinal expression and partial cDNA cloning of murine Muc2

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Gastrointestinal expression and partial cDNA cloning of murine Muc2. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G115–G124, 1999.—To help us investigate the role of mucin in the protection of the colonic epithelium in the mouse, we aimed to identify the murine colonic mucin (MCM) and its encoding gene. We isolated MCM, raised an anti-MCM antiserum, and studied the biosynthesis of MCM in the gastrointestinal tract. Isolated MCM resembled other mucins in physicochemical properties. Anti-MCM recognized MCM as well as rat and human MUC2 on Western blots, interacting primarily with peptide epitopes, indicating that MCM was identical to murine Muc2. Using anti-MCM and previously characterized anti-human and anti-rat MUC2 antibodies, we identified a murine Muc2 precursor in the colon of ~600 kDa, which appeared similar in size to rat and human MUC2 precursors. Western blotting, immunoprecipitation of metabolically labeled mucins, and immunohistochemistry showed that murine Muc2 was expressed in the colon and the small intestine but was absent in the stomach. To independently identify murine Muc2, we cloned a cDNA fragment from murine colonic mRNA, encoding the 302 NH₂-terminal amino acids of murine Muc2. The NH₂-terminus of murine Muc2 showed 86 and 75% identity to the corresponding rat and human MUC2 peptide sequences, respectively. Northern blotting with a murine Muc2 cDNA probe showed hybridization to a very large mRNA, which was expressed highly in the colon and to some extent in the small intestine but was absent in the stomach. In situ hybridization showed that the murine Muc2 mRNA was confined to intesti-

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EPITHELIAL MUCINS ARE widely accepted to play cytoprotective roles in many organs (9, 26, 37). Many epithelia express a variety of mucins (37); however, the human colonic epithelium expresses one mucin in very high amounts: MUC2 (28). Importantly, we were able to show that in patients with ulcerative colitis the activity of the mucosal inflammation correlates with a signifi-
cant decrease in MUC2 synthesis (32), implying an important role for MUC2 in colonic cytoprotection. The colon of the mouse constitutes a very feasible organ to test further the cytoprotective nature of mucins under experimental pathophysiological conditions. The colonic epithelium has to confront a particularly hostile environment, naturally necessitating effective protection. Moreover, there are many colitis models in the mouse that are well suited for testing the susceptibility of the colonic epithelium toward luminal substances (reviewed in Ref. 8).

Murine mucins and their encoding genes have not been studied extensively. Four murine mucins were identified so far. Muc1 is a membrane-bound mucin that is very homologous to its human and rat counterparts and is expressed at low levels in many epithelia but shows little tissue-specific expression (25, 41). Murine Muc3 was cloned very recently and was demonstrated to be specifically expressed in intestine (23). Murine Muc SAC was cloned from stomach by Shekels et al. (24) and was, like its human homologue, primarily expressed in stomach and Airways. Recently, a murine mucin was identified (confined to salivary glands) that showed high homology to a rat salivary mucin (7). This mucin shows striking resemblance in overall structure to the human salivary mucin MUC7. However, this mucin still awaits inclusion into the “MUC” nomenclature because it shows no sequence homology to the human salivary mucin MUC7. Thus far, colonic mucins in the mouse were not particularly well studied.

To help us investigate the role of murine colonic mucin (MCM) in the protection of the epithelium of the colon, we aimed to identify the MCM and its encoding gene by two independent approaches. First, we isolated colonic mucin, prepared an antiserum against this, and studied the nature of MCM by immunochemical techniques. Second, on the assumption that MCM might be homologous to rat and human MUC2, we sought to isolate a murine Muc2 cDNA fragment from murine colonic mRNA using RT-PCR and studied the murine Muc2 mRNA expression in the gastrointestinal tract. These approaches led to the same conclusion: murine Muc2 appeared to be expressed in the mouse intestine and was particularly abundant in the colon of the mouse.

MATERIALS AND METHODS

Unless otherwise indicated, chemicals were obtained from the following manufacturers: Amersham (Buckinghamshire, UK), Gibco BRL (Gaithersburg, MD), Merck (Darmstadt, Germany), Sigma (St. Louis, MO), Bio-Rad (Richmond, CA),
Pharmacia (Uppsala, Sweden), BDH (Poole, UK), and Boehringer (Mannheim, Germany).

Analytic procedures. Rat colonic mucin (RCM) and human colonic mucin (HCM) and antisera against these mucins, anti-RCM and anti-HCM, which recognize rat and human MUC2, respectively, were obtained through earlier work (27, 28). The monoclonal antibody WE9 against human MUC2, which also recognizes rat MUC2, was characterized earlier (29). Protein samples were analyzed on reducing PAGE in the presence of 0.1% SDS. Before SDS-PAGE analyses, samples were boiled for 5 min in buffer containing 1% (vol/vol) 2-mercaptoethanol (Bio-Rad) and 1% (wt/vol) SDS. SDS-PAGE gels were stained with periodic acid-Schiff’s reagent (PAS, Sigma) or with Comassie brilliant blue R-250 (Merck). Western blotting of isolated mucins and gastrointestinal tissue homogenates was described previously (27–29). To visualize radiolabeled bands in protein samples, SDS-PAGE gels were incubated, after fixation in 10% acetic acid-10% methanol, for 10 min with Amplify (Amersham) before drying. Gels were washed, separated by SDS-PAGE using a 3% stacking and 4% running gel, and analyzed by fluorography. In some analyses, immunoprecipitated mucins were digested by endoglycosidase H (endo H) as described previously (28).

Immunohistochemistry. Small segments of stomach, jejunum, and colon of mouse or rat colon were fixed in 4% paraformaldehyde immediately after excision and embedded and prepared for immunohistochemistry as described previously (33). Anti-MCM and anti-RCM were applied at 1:3,000 and 1:500, respectively. Immunoreaction was detected using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA), and staining was developed using diaminobenzidine. To enhance the signal, sections were either boiled in 10 mM citrate buffer (pH 6) for 10 min or treated with 20 µg/ml proteinase K (Boehringer) for 7.5 min in PBS.

RT-PCR and sequence analysis. Total RNA was isolated from mucosal scrapings of murine colon using TRIzol (GIBCO BRL) following the manufacturer’s protocol. One microgram of total RNA was transcribed at 42°C into cDNA using Superscript RT (GIBCO BRL) in a total volume of 20 µl, following the manufacturer’s instructions. The final reaction conditions were as follows: 20 mM Tris (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.01% BSA, 10 mM dithiothreitol, 500 nM random hexamers, 1 µg total RNA, and 500 µM each of dATP, dCTP, dGTP, and dTTP. After a 1-h incubation, an RNase H (GIBCO BRL) digestion was performed for 20 min at 25°C. This was followed by a PCR reaction in a total volume of 20 µl using 1 µl cDNA as template in combination with the primers P70 (5’-CAAGTGATGGGATACGAC-3’) and P62 (5’-AGGCGCTTCCAGGTCAG-3’), which correspond to nucleotides 24–40 and 945–961, respectively, of human MUC2 cDNA (15). These primer sequences are perfectly conserved between rat and human MUC2 (15, 20). Final PCR reaction conditions were as follows: 10 mM Tris (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 0.01% gelatin, 0.2 units Taq polymerase, 200 nM of each primer, cDNA template, and 200 µM each of dATP, dCTP, dGTP, and dTTP. The PCR reaction was carried out as follows: 5 min at 95°C and 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. After the last cycle, a 10-min extension step at 72°C was done. The resulting 911-bp PCR product was isolated after analysis on a 1% agarose gel using the Qiagen gel extraction kit. Subsequently, the purified PCR product was double strandedly sequenced using the Taq dye nucleotide cycle sequencing kit with fluorescein labeled nucleotides (Applied Biosystems, Norwalk, CT) and primers P62, P70, and other primers spanning the entire PCR fragment (P71: 5’-GTCTGAGACCATGCGG-3’, P72: 5’-CCTCATGTGAAACGGG-3’, P73: 5’-AGTTTGGAACATGCAAGA-3’, P75: 5’-GCATGCGGAGAATCTC-3’, P76: 5’-CGCGTTCACATGGG-3’, and P77: 5’-TGAAGTGGATGTTGCATCC-3’). Sequence reactions were analyzed on
an Applied Biosystems model 377 sequencer. Sequences were analyzed using Macintosh Sequence Navigator and Autoassembler software. Nested primers P61 (5'-TAAGGTCA-GACCATCTACCTCACC-3') and P63 (5'-GGAATTCGCA-GTTCCCAAATCTC-3') were used to amplify and clone a fragment (244 bp) of the 911-bp PCR fragment in the Sal I-Eco R I sites of pBluescript SK (Stratagene, La Jolla, CA). This cloned fragment was double strandedly sequenced as described above and used as a probe for Northern blot analysis.

Northern blot analysis. Total RNA was isolated from murine stomach, small intestine, and colon using TRIzol (GIBCO BRL) following the manufacturer’s protocol. The Northern blot analysis was essentially carried out as previously described (30). Briefly, 10 µg of total RNA derived from each tissue were separated on a 0.8% agarose gel containing 10 mM HEPES (Sigma) (pH 7.5) and 2.2 M formaldehyde (Merck). Integrity of RNA was assessed by analyzing the 28S and 18S ribosomal RNAs after electrophoresis and staining with ethidium bromide. Capillary transfer of RNA to Qiaphane (Qiagen) was carried out. The blot was hybridized to a 32P-labeled 244-bp Sal I-Eco R I fragment (described above). After exposure to Kodak X-Omat AR film, the probe was stripped from the blot. The blot was reprobed with a 32P-labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe as described (33), and all RNA samples were found to contain intact GAPDH mRNA bands of the expected size. The levels of hybridization on the Northern blots to the murine Muc2 probe and the GAPDH probe were measured through autoradiography using a PhosphorImager and ImageQuant software (Molecular Imaging, Sunnyvale, CA), as described previously (33).

In situ hybridization. In situ hybridization was performed by labeling double-stranded cDNA fragments using 32P-labeled dCTP and random priming, as described previously (21). To detect murine Muc2 mRNA, two probes were used: 1) the 244-bp Sal I-Eco R I fragment of the murine Muc2 cDNA, described in RT-PCR and sequence analysis, and 2) a 1.2-kb fragment of the rat Muc2 cDNA sequences that was isolated using RT-PCR on rat colonic RNA, using primers that were based on the published rat Muc2 cDNA sequence (42).

RESULTS

Isolation and characterization of MCM. MCM was isolated by triple-density-gradient centrifugation. Mucins were identified in the fractions of the first gradient with densities of 1.35–1.52 g/ml by orcinol assay after dialysis of aliquots of these fractions (not shown). Analysis of these fractions by SDS-PAGE demonstrated the presence of high-molecular-weight PAS-stainable material, which entered the 4% running gels (not shown). The mucin-containing fractions were rerun on a second- and third-density gradient. All other proteins were removed by this procedure as judged by SDS-PAGE and Coomassie blue staining of dialyzed aliquots of each fraction.

The mean buoyant density of the isolated MCM was 1.46 g/ml. Amino acid analysis revealed a high content of serine and threonine residues, together comprising ~37%, whereas the threonine content exceeded the serine content (threonine-to-serine ratio of 1.69). The monosaccharide analysis revealed a very high content of O-linked oligosaccharides, whereas mannose, an indicator of N-linked glycans, was present in only low amounts (Table 1). In particular, the sialic acid content was very high (22.9%; Table 1). The buoyant density of the isolated MCM, the presence of a high percentage of hydroxylated amino acids, and the presence of a high content of O-linked glycosylation was hallmarks of epithelial mucins (26).

Identification of MCM as murine Muc2. When analyzed by SDS-PAGE and PAS staining, MCM, presented as a single band just entering the 4% running gel, displayed a mobility very similar to RCM and HCM (Fig. 1). Epithelial mucins are known to display a characteristic resistance to enzymatic proteolysis, due to the very high number of O-linked oligosaccharides (26). On exhaustive digestion with proteinase K, the mobilities of MCM, RCM, and HCM slightly increased in a similar manner (Fig. 1), indicating that a large part of the molecules are indeed protected from digestion by proteases.

A polyclonal anti-MCM antiserum was elicited, which was tested for its ability to recognize intact and proteinase K-digested mucins on Western blot (Fig. 1). Purified MCM was well recognized by anti-MCM, but after proteolytic treatment nearly all recognition was lost, implying that anti-MCM recognizes primarily peptide epitopes. Similarly, HCM and RCM were recognized by

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Table 1. Monosaccharide composition of colonic mucins from mice, rats, and humans

Rat colonic mucin (RCM) and human colonic mucin (HCM) data are from Refs. 27 and 28, respectively. MCM, murine colonic mucin. GalNAc, N-acetyl-galactosamine. GlcNAc, N-acetyl-glucosamine.

![Fig. 1. Specificity of the anti-murine colonic mucin (MCM) antiserum. Samples of isolated MCM, human colonic mucin (HCM), and rat colonic mucin (RCM) were analyzed on SDS-PAGE and stained with periodic acid-Schiff's reagent (PAS) (left), before and after digestion with proteinase K (pk). In parallel, these samples were Western blotted using anti-MCM (right). Arrowhead indicates the border between the 3% stacking gel and the 4% running gel. At left, the position of the 205-kDa marker is indicated.](http://ajpgi.physiology.org/ Downloaded from)
anti-MCM before but not after incubation with proteinase K (Fig. 1). We also found that anti-RCM and anti-HCM recognized each of the three mucin preparations, whereas all recognition was lost when digested with proteinase K (not shown). The cross-reactivity of these antisera indicates that homology exists at the polypeptide level among the colonic mucins of these three species. Because we previously identified HCM and RCM as rat and human MUC2, respectively (27, 28), it seems very likely that MCM is identical to murine Muc2.

We took a second approach to try and identify MCM by immunosolating the MCM precursor. Mucin precursors were defined previously as the primary translation products of mucin mRNAs, which are present in the rough endoplasmic reticulum, that do not contain O-glycosylation (26). We used anti-MCM and several established anti-MUC2 antibodies (i.e., anti-HCM, anti-RCM, and WE9) to immunoprecipitate MCM precursors from metabolically 35S-labeled amino acid colonic tissue homogenates (Fig. 2A). Each of these four antibodies precipitated an ~600-kDa protein from the homogenate. A band with a very similar mobility was easily distinguished within the homogenate, strongly suggesting that this was a prominently expressed protein. The polyclonal antisera, anti-MCM, anti-RCM, and anti-HCM, showed precipitation of some additional bands. These bands most likely represent nonspecific products because these were absent from the sample precipitated with the monoclonal WE9. Also, these extra bands were present to variable extents in the immunoprecipitations with the antisera. Compare, e.g., the immunoprecipitation with anti-MCM shown in A and B of Fig. 2: Fig. 2A shows some additional bands, which are absent in the duplicate experiment shown in Fig. 2B. Given the cross-reactivity of the previously characterized anti-MUC2 antisera with the 600-kDa band in the murine colonic homogenates, it is very likely that the 600-kDa band represents the murine Muc2 precursor. PAS staining of the immunoprecipitated mucin also showed the precipitation of the mature mucin, with a mobility slightly higher than that of the Muc2 precursor (Fig. 2A).

The identity of the alleged 600-kDa Muc2 precursor band was further corroborated by digestion with endo H, which hydrolyzes high-mannose N-linked glycans. The presence of these glycans is a hallmark of proteins within the rough endoplasmic reticulum. Endo H digestion of immunoprecipitated Muc2 precursor leads to an increased mobility of the Muc2 precursor band (Fig. 2B), indicating that high-mannose N-linked glycans are present and that this protein indeed resides in the rough endoplasmic reticulum.

Muc2 is a secretory mucin, which is confined to intestinal goblet cells. We studied the expression of Muc2 in murine stomach, jejunum, and colon by Western blotting (Fig. 3). PAS staining of the 4% SDS-PAGE gel revealed very high-molecular-weight glycoproteins in each of these organs, which most likely represent mucins. Western blotting of these samples using anti-MCM revealed extensive staining of a high-molecular-weight product in the colon. The mobility and the appearance of this Muc2 band, stained with anti-MCM, coincided with the PAS-stained band in the corresponding colon homogenate, indicating that Muc2 is highly expressed in the colon.

Staining by anti-MCM was largely absent from the stomach homogenate (Fig. 3). In the jejunal sample, there was only limited staining relative to the intense staining of the colonic sample, of material just entering the running gel. The mobility on SDS-PAGE of this small intestinal Muc2 was lower than the Muc2 in the colon. These findings indicate that the major mucins stained by PAS in the stomach and the jejunum are not identical to Muc2. Because no specific antibodies were
available that recognize murine mucins other than Muc2, we were not able to identify further these gastric and small intestinal bands.

To investigate the cell type-specific expression of murine Muc2, we performed immunohistochemistry using anti-MCM and anti-RCM on sections of the stomach, jejunum, and colon. Anti-MCM and anti-RCM did not stain stomach epithelium (not shown). Immunostaining of jejunal and colonic sections revealed that anti-MCM stained goblet cells in both intestinal segments in a highly specific manner (Fig. 4). Staining was largely confined to the intracellular storage granules (Fig. 4B). However, particularly in the jejunum, thin sheets of extracellular material were also stained by both antisera (Fig. 4). Interestingly, immunostained material, apparently streaming from the goblet cells, was sometimes observed to be continuous with these extracellular sheets (Fig. 4A). The brush-border membranes of the small intestinal enterocytes, which contain high amounts of various glycoconjugates, were free of staining (Fig. 4, A and B). The results obtained when using anti-RCM were very similar to those obtained with anti-MCM (not shown). The goblet cells in the upper part of the colonic crypts stained more strongly with the anti-MCM antiserum than cells in the lower part of the crypts (Fig. 4C), suggesting that more Muc2 is present in the latter cells.

From the immunohistochemical studies, it seemed evident that the murine Muc2 is secreted. To establish this, we performed pulse-chase experiments using [35S]sulfate-labeled segments of jejunum and colon (Fig. 5). Pulse labeling resulted in the accumulation of high-molecular-weight [35S]sulfate-labeled material that was partly secreted into the media of both jejunal and colonic segments during the 4-h chase period. This product could be immunoprecipitated by anti-MCM from both tissue and medium samples of jejunum and colon homogenates and was thus identified as secretory Muc2. The jejunal Muc2 had a lower mobility on SDS-PAGE, as previously noted in Fig. 3, and labeled less intense with [35S]sulfate than the colonic Muc2.

Isolation and sequencing of a partial murine Muc2 cDNA. Part of the murine Muc2 cDNA was cloned by RT-PCR on murine colonic RNA, representing the 5'-region of the murine Muc2 mRNA. A fragment of 911 bp was amplified and double strandedly sequenced, following the strategy shown in Fig. 6A, and was deposited in GenBank under accession number AF016695. It was found to encode a fragment of 302 amino acids of the NH₂ terminus of murine Muc2. The cloned Muc2 sequence was highly conserved among species: compilation of the murine, rat, and human...
MUC2 sequences showed 73% identical amino acids (Fig. 6B). Conservation between rat and murine Muc2 appeared highest with 86% identity. The cloned murine Muc2 sequence contains one putative N-glycosylation site at N159, which is conserved in the rat and human MUC2 sequences. Furthermore, homology (30% identity of amino acids) was observed with the D domain of the human von Willebrand factor, as found earlier for rat and human MUC2 (15, 20).

Murine Muc2 mRNA is highly expressed in the colonic goblet cells. Part of the amplified 911-bp murine Muc2 cDNA sequence was cloned and used as a probe to detect Muc2 mRNA in RNA samples from nine regions of the murine gastrointestinal tract (Fig. 7A). Stomach RNA showed very little hybridization to the Muc2 cDNA probe. However, each RNA sample from small intestine as well as colon showed hybridization to a very high-molecular-weight band. In addition to this band, a smear was noted with an intensity that corresponded to the intensity of the band, suggesting that this smear resulted from degradation of the Muc2 mRNA present in the high-molecular-weight band. It should be noted, in general, that the detection of this type of polydispersed signal for mucin mRNAs on Northern blots is a commonly observed phenomenon (see, e.g., Refs. 11–13). However, the rRNA bands on the gel and the bands detected for GAPDH on the Northern blot showed discrete bands (not shown). The hybridization signal found with the Muc2 probe was quantified relative to the signal observed for GAPDH mRNA in each lane (Fig. 7B). Muc2 mRNA was particularly abundant in proximal and middle colon, indicating that Muc2 mRNA was most abundant in these segments. The signal in the distal colon was relatively low; expression level of Muc2 mRNA was 26% of that found in the other colonic segments.

To localize the Muc2 mRNA in colonic tissue, in situ hybridization was performed. The number and localization of cells with positive signal for Muc2 mRNA were very similar to the number and distribution of the
goblet cells as stained for Muc2 protein in immunohistochemistry (Fig. 4C), indicating that the Muc2 mRNA was confined to goblet cells in the colon (Fig. 8). With the use of a cDNA probe, representing rat Muc2 mRNA, on murine colonic sections, very similar results were obtained (not shown). Also, when the murine Muc2 cDNA probe was used on rat colonic tissue, the signal was confined to rat colonic goblet cells, which could also be stained with anti-rat Muc2 antibody in immunohistochemistry (not shown). It should be noted that goblet cells in the lower part of the colonic crypts hybridize less strongly to the Muc2 cDNA probe than the cells in the upper part of the crypts (Fig. 8), suggesting that less Muc2 mRNA is produced in the goblet cells of the lower crypt region. Because these cells also stain less intensely with the anti-Muc2 antibodies (Fig. 4C), it seems that these goblet cells produce less Muc2 than the goblet cells of the upper crypt region.

**DISCUSSION**

In this study, we were able to show that the major colonic mucin from the mouse is considered to be identical to murine Muc2, as is evident from the following six considerations.

**Consideration 1.** The physicochemical characteristics of MCM are similar to rat and human MUC2. The threonine plus serine content is around 40% of the amino acids for all three mucins, and characteristically the threonine content exceeds the serine content. The monosaccharide composition is also similar for all three mucins, with low levels of fucose and mannose and particularly high sialic acid contents. The buoyant density of the mucins, which is a measure for the chemical composition of the mucins, is similar, ranging from 1.45 to 1.50 g/ml. Also, the behavior of the isolated mucins on SDS-PAGE is very similar, yielding bands with mobilities corresponding to molecular masses of ~600 kDa. This behavior of these colonic mucins on SDS-PAGE is characteristic yet anomalous with respect to their molecular masses, as will be discussed separately below.

**Consideration 2.** The identity of MCM as murine Muc2 was also corroborated by the cross-reactivities between MCM and previously characterized anti-MUC2 antibodies, which were demonstrated to recognize the polypeptides of rat and human MUC2 (29). Also the cross-reactions of anti-MCM with rat and human MUC2 indicate that homology at the polypeptide level exists among the colonic mucins of these three species. The cross-reactivity between the antibodies and the mucins from these three species was noted in all techniques used: Western blotting of mature mucins, immunoprecipitation of both mature mucins and mucin precursors, and immunohistochemistry. Because these cross-reactivities have been demonstrated to be primarily based on polypeptide recognition, it is very likely that MCM is identical to murine Muc2.

**Consideration 3.** After metabolic labeling of murine colonic explants with radioactive amino acids, an ~600-kDa band was immunoprecipitated using either anti-MCM or a variety of anti-MUC2 antisera, which most likely represent the murine Muc2 precursor. The human MUC2 cDNA was completely sequenced by Gum et al. (11, 14, 15), revealing that the encoded human MUC2 precursor has a molecular mass of ~600 kDa. In line with this expected molecular mass, we identified the human MUC2 precursor as an ~600-kDa band in the human colon and small intestine and a colonic cell line (28, 36, 39). In the rat colon, we were able to

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**Fig. 7. Expression of murine Muc2 mRNA in the gastrointestinal tract.** A: RNA was isolated from the indicated 9 regions of the gastrointestinal tract of the mouse and analyzed by Northern blotting using the 32P-labeled, 244-bp, Sal I-Eco RI Muc2 cDNA fragment. The 18S and 28S rRNA bands and slots (arrowhead) are indicated to the right. B: quantitative analysis of the Northern blot results, in which the signal obtained with the murine Muc2 probe in each lane was expressed relative to the signal obtained with the glyceraldehyde-3-phosphate dehydrogenase probe in the same lane.
identify a very similar 600-kDa band that we could independently identify as the rat Muc2 precursor (27). On the basis of its estimated molecular mass as well as on the cross-reactivity with previously characterized anti-MUC2 antisera, it is very likely that the 600-kDa band represents the murine Muc2 precursor.

Consideration 4. Murine Muc2 mRNA appeared very large, as would be expected when encoding a polypeptide precursor of ~600 kDa. The size of this mRNA, as for other mucins, is very difficult to estimate due to the low resolution of agarose gels for these high-molecular-mass molecules and the lack of appropriate markers. Nevertheless, similar very large mRNAs were detected by Northern blotting for rat and human MUC2 (30, 42), which were consistent with the sizes of the very large MUC2 precursors that these respective mRNAs encode.

Consideration 5. Independently, the NH₂-terminal sequence of murine Muc2 was determined using RT-PCR on murine colonic RNA. The deduced sequence of the 302 NH₂-terminal amino acids was very similar in all aspects to the rat and human MUC2 sequences (15, 20). Particularly, all 17 cysteine residues and the single N-glycosylation site were conserved in all three sequences, indicating that the three-dimensional structure of this part of the polypeptide is likely to be conserved. This high level of similarity, which may also involve other regions of the polypeptide, likely explains the extensive cross-reactivities of the anti-MUC2 antisera with the MUC2 molecules among these three species.

Consideration 6. The tissue and cell type-specific expression of MCM, as shown by Western blotting, immunohistochemistry, Northern blot, and in situ hybridization is similar to rat and human MUC2, since MCM expression is 1) high in the colon, 2) low in the small intestine, 3) confined to intestinal goblet cells, but 4) undetectable in the stomach. Similar observations were made for human MUC2 expression (1-4, 11, 14, 15, 36) as well as for the expression of rat Muc2 (12, 18, 20, 42, 43).

We set out to identify the mucins in the murine colon that are involved in cytoprotection through the mucus layer. The colonic mucus is copious and could consist of a mixture of mucins. Our strategy for the identification of the MCMs would enable us to isolate a potential mixture of mucins, because our isolation was based on a buoyant density around 1.4 g/ml, a ubiquitous characteristic of secretory mucins (9, 26). As indicated above, it seems very likely that MCM is identical to murine Muc2. Nevertheless, we cannot exclude the possibility that other mucins are present in small amounts in the

Fig. 8. Cellular expression of the murine Muc2 mRNA. In situ hybridization was performed on a section of the middle of the colon using a 35S-labeled, 244-bp fragment of the murine cDNA, as described in MATERIALS AND METHODS. L, lumen of the colon; S, serosal side of the tissue.
colon of the mouse, which remain as yet undetected in our studies.

That the interactions of anti-MCM are primarily limited to polypeptide recognition was substantiated by three observations. 1) All epitopes of MCM that are recognized by anti-MCM were protease sensitive, whereas protease treatment left intact the major part of the molecule, carrying virtually all glycosylation. This implies that anti-MCM recognizes primarily peptide epitopes, as was previously found for a large number of anti-mucin antisera, which were prepared following an identical protocol (29). 2) Anti-MCM was able to recognize and immunoprecipitate the murine Muc2 precursor, which very likely contains no O-glycosylation. Therefore, it is very likely that the anti-serum is primarily directed against peptide epitopes. 3) Recognition of mucins at the histological level was limited to intracellular granules of intestinal goblet cells and extracellular material. In contrast, the brush border and the Golgi apparatus of enterocytes, which lies characteristically in a supranuclear position in enterocytes, are completely devoid of any staining. Because both of these cellular structures contain high amounts of very diverse glycoconjugates, it seems very unlikely that anti-MCM would recognize carbohydrate structures.

On SDS-PAGE, homogenates of the stomach, jejunum, and colon showed similar amounts of PAS-stainable, high-molecular-weight mucin. With Western blotting of these samples, staining by anti-MCM was absent from the stomach, whereas staining was low in jejunum relative to the very intense staining of mucin in the colon. Moreover, the mobility of the Muc2 in jejunum samples, as detected by anti-MCM, was dissimilar from the mobility of the PAS-stainable mucin band. These findings therefore indicate that the major mucins stained by PAS in the stomach and the small intestine are very likely not identical to Muc2. The major murine stomach mucin has been identified as murine MucSAC, explaining why anti-MCM fails to recognize the murine stomach mucin. In line with this, neither anti-MCM nor anti-RCM stained gastric epithelium immunohistochemically, and also the Muc2 mRNA was virtually absent from stomach RNA using Northern blot. In the small intestine of rat, human, and mouse, a second major mucin has been identified: Muc3 (13, 18, 19, 36, 40). Therefore, the PAS-stained band, which was not stained using anti-MCM, most likely represents mature murine Muc3. Because no antibodies are available that recognize mature murine Muc3, we were unable to identify further this small intestinal mucin band.

The mature Muc2 that was detected, by Western blotting and immunoprecipitation, in the small intestine are very likely not identical to Muc2. The mobility of some mucins on gel (6, 34); therefore, potential differences in sulfation may also contribute to the observed differences in mobility between the small intestinal and colonic MUC2. Thus the Muc2 in the small and large intestine of the mouse may differ in their glycan structure and composition. Similarly, different glycosylated forms of rat and human MUC2 were detected in various parts of their respective gastrointestinal tracts (17, 36).

Taking all data together, it is evident that Muc2 is expressed throughout the mouse intestine and that Muc2 is very likely the prominent colonic mucin in the mouse. Studies are now underway to quantify the Muc2 synthesis in various colitis models in mice, to evaluate the role of Muc2 in the cytoprotection of the colonic epithelium against luminal threats.

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