Gastrointestinal expression and partial cDNA cloning of murine Muc2

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Van Klicken, B. J. an-Willem, Alexandra W. C. Einerhand, Louise A. Duits, Mireille K. Makkink, Kristien M. A. J. Tytgat, Ingrid B. Renes, Melissa Verburg, Hans A. Boller, and Jan Dekker. 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 extend in the small intestine but was absent in the stomach. In situ hybridization showed that the murine Muc2 mRNA was confined to intestine but was absent in the stomach. 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 immunoprecipitated the metabolically labeled mucins. Second, on the assumption that MCM might be homologous to rat and human MUC2, we sought to isolate murine Muc2, which appears homologous to rat and human MUC2. Because Muc2 is prominently expressed in the colon, it is most likely to be the predominant mucin in the colonic mucus layer.

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), and Sigma (St. Louis, MO). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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 Coomassie 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 1–4 wk at −70°C to Biomax MR films (Kodak). Prestained high-molecular-mass markers were purchased from Bio-Rad (ranging from 49.5 to 205 kDa). For reference to very high-molecular-weight molecules, all metabolically labeled unreduced rat gastric mucin precursors were used (molecular weight of monomer and dimer = 300,000 and 600,000, respectively) (5). The density of CsCl gradient fractions was measured by weighing 1 ml of each fraction using a calibrated pipette. Hexose assay was performed using orcinol (Sigma) according to François et al. (10) with galactose as standard. Monosaccharide analysis was performed according to the method of Savage et al. (22). Amino acid analysis was performed using the o-phthalaldehyde (Pierce) derivative technique and HPLC (35). For some analyses, purified mucins were digested by proteinase K as described previously (28).

Isolation of colonic mucins and preparation of antisera. The mucosa of the entire colon was scraped off of 58 adult healthy mice (27 males). MCM was isolated, and a polyclonal anti-MCM serum was elicited, as described earlier for HCM and RCM (27, 28). Briefly, 2.3 g wet weight of mucosal scrapings were homogenized in 50 ml buffer, pH 7.5, containing 6 M guanidinium·HCl, 0.1 mM dithiothreitol (Sigma) and sulfhydryl groups were carbamylated by iodoacetamide (Sigma). Mucins were purified by equilibrium centrifugation using three consecutive CsCl (Boehringer) density gradients. Mucin-containing fractions from each gradient were pooled and run on the next gradient. In the first and second gradient, CsCl was added to a density of 1.40 g/ml, with a guanidinium·HCl concentration of 4 M. In the last gradient, CsCl was added to a density of 1.50 g/ml, whereas the guanidinium·HCl concentration was reduced to 0.2 M. Isopycnic density gradient centrifugation was performed in a Beckman ultracentrifuge, Ti 60 rotor at 50,000 rpm for 66 h at 4°C. For analysis, the fractions were dialyzed extensively against distilled water at 4°C and stored at −20°C. Purified antigen was mixed with Freund’s complete adjuvant (Difco, Detroit, MI) and injected subcutaneously in a New Zealand White rabbit. After booster injections with Freund’s incomplete adjuvant (Difco), the anti-MCM serum was obtained.

Metabolic labeling of gastrointestinal tissue and immunoprecipitation of mucins. Metabolic labeling of tissue in vitro and immunoprecipitation of mucins were performed as described previously (5, 29, 36). In brief, mucin biosynthesis was studied by metabolic labeling with [35S]methionine/cysteine, Pro-mix (Amersham), to label the polypeptides, or with [35S]sulfate (Amersham) to label mature mucins. Healthy adult female mice (15–20 g) were killed by cervical dislocation. Tissue explants (10 mm²) of stomach, jejunum, or colon were cultured and pulse-labeled with either Pro-mix for 30 min or [35S]sulfate for 60 min, using 100 µCi of each label per 100 µl of medium per tissue explant. In some experiments, chase incubations of 4 h were performed after the pulse-labeling with [35S]sulfate, after which the tissue and the culture medium were collected. After the respective pulse or chase experiments, explants were homogenized in, or culture medium was mixed with, a Tris buffer containing 1% Triton X-100 and 1% SDS and high concentrations of six protease inhibitors. Mucins were immunoprecipitated from the homogenates overnight at 4°C with various antibodies. Immunocomplexes were precipitated using Sepharose CL-4B-coupled protein A (Pharmacia). Immunoprecipitated mucins 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 in paraffin. Small segments of stomach, jejunum, or colon were cultured and pulse-labeled with [35S]sulfate, after which the tissue and the culture medium were collected. After the respective pulse or chase experiments, explants were homogenized in, or culture medium was mixed with, a Tris buffer containing 1% Triton X-100 and 1% SDS and high concentrations of six protease inhibitors. Mucins were immunoprecipitated from the homogenates overnight at 4°C with various antibodies. Immunocomplexes were precipitated using Sepharose CL-4B-coupled protein A (Pharmacia). Immunoprecipitated mucins 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).

RNA isolation. Total RNA was isolated from mucosal scrapeings 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 step (GIBCO BRL) digestion was performed to destroy the RNA. 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'-CACCACTGGGCGTCCAC-3') and P62 (5'-AGGCC-GCTTCTCCAGGT-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 fluorescently labeled nucleotides (Applied Biosystems, Norwalk, CT) and primers P62, P70, and other primers spanning the entire PCR fragment (P71: 5'-GTCACCAACCTGGG-3', P72: 5'-CCCT-CATGTGAAACCAGG-3', P73: 5'-AGTTTGGACAT- CAGAAG-3', P75: 5'-GACTTCGCGAGAACTC-3', P76: 5'-CCCGGTTACACAGGG-3', and P77: 5'-TGAAGTAGT- ATGGTGTCATCC-3'). Sequence reactions were analyzed on...
an Applied Biosystems model 377 sequencer. Sequences were analyzed using Macintosh Sequence Navigator and Auto-
assembler software. Nested primers P61 (5'-TAAGGTCGAG-
CACCATCTACCTCACCC-3') and P63 (5'-GGAATTCTGGAT-
GTTCCAAAATCT-3') were used to amplify and clone a fra-
gment (244 bp) of the 911-bp PCR fragment in the Sal-
I-EcoRI 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 mu-
rine stomach, small intestine, and colon using TRIzol (GIBCO
BRL) following the manufacturer’s protocol. The Northern
blot analysis was essentially carried out as previously de-
scribed (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 Qiabrane
(Merck) was carried out. The blot was hybridized to a
32P-labeled 244-bp
Eco
RI fragment of pBluescript SK (Stratagene, La Jolla, CA).

RESULTS

Identification of MCM as murine Muc2. When ana-
lyzed by SDS-PAGE and PAS staining, MCM, pre-
sented 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
mobilites 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 prote-
ase 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

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

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>MCM</th>
<th>RCM</th>
<th>HCM</th>
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<tr>
<td>Fucose, mol %</td>
<td>5.1</td>
<td>6.2</td>
<td>5.0</td>
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<tr>
<td>Mannose, mol %</td>
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<td>0.1</td>
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<td>GalNAc, mol %</td>
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<td>21.3</td>
<td>12.4</td>
</tr>
<tr>
<td>GlcNAc, mol %</td>
<td>28.2</td>
<td>40.1</td>
<td>44.2</td>
</tr>
<tr>
<td>Sialic acid, mol %</td>
<td>22.9</td>
<td>7.6</td>
<td>19.6</td>
</tr>
</tbody>
</table>

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-acetylgalactosamine. GlcNAc, N-acetylglucosamine.

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 glycans are hallmarks of epithelial
mucins (26).

<table>
<thead>
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<th>PAS</th>
<th>BLOT</th>
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<tr>
<td>HCM</td>
<td>RCM</td>
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<tr>
<td>pk</td>
<td>+</td>
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<tr>
<td>HCM</td>
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Fig. 1. Specificity of the anti-murine colonic mucin (MCM) antiser-
um. 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.
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 mucin 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 monoaonal 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 mucin 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

![Fig. 2. Immunoprecipitation of mucin precursors from metabolically labeled murine colonic tissue. A colonic segment from the middle of the colon was incubated for 30 min with 35S-labeled amino acids and homogenized. A: mucin precursors were immunoprecipitated using anti-MCM, anti-HCM, and anti-RCM antisera and antibodies and the monoclonal anti-MUC2 antibody WE9. Immunodetoxes were analyzed next to the colonic homogenate on SDS-PAGE, followed by fluorography. PAS lane shows the PAS staining of the anti-MCM lane of the adjacent fluorograph. B: mucin precursor was immunoprecipitated using anti-MCM and incubated with or without endoglycosidase H (endo H) as indicated. Arrowheads indicate the borders between the 3% stacking gels and the 4% running gels. At left of A and B, positions of the 300- and 600-kDa markers are indicated.](image)

![Fig. 3. Region-specific expression of murine Muc2 in the gastrointestinal tract by Western blotting. Segments of the stomach, jejunum, and colon were homogenized in the same buffer as used for immunoprecipitation. Protein concentrations were measured and adjusted to allow loading of equal amounts of protein in each lane. Samples of each homogenate were analyzed on SDS-PAGE and stained with PAS (A). In parallel, samples were analyzed on Western blot using 1:500 diluted anti-MCM (B). Arrowheads indicate the positions of the 205-kDa marker are indicated.](image)
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 jejunal and colonic 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 doubly 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 revealed that the amino acid sequences were identical in the NH₂-terminal region of 105 residues.
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. Also, all of the 17 cysteine residues present in murine Muc2 were 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 colonic tissue, we were able to...
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 NH$_2$-terminal sequence of murine Muc2 was determined using RT-PCR on murine colonic RNA. The deduced sequence of the 302 NH$_2$-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 $^3$S-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, 23, 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 displayed a significantly lower mobility on SDS-PAGE than the colonic Muc2. As discussed at length previously (31), the mobilities of mature mucins are generally anomalous and are dependent on the intrinsic negative charge of the mucins, which is imposed by the high sialic acid content. Also, the presence of sulfate esters has been shown to have dramatic effects on 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, differently 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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A preliminary report was in abstract form (38).

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