Delayed Mammary Tumor Progression in Muc-1 Null Mice

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The mucin gene, Muc-1, encodes a high molecular weight integral membrane glycoprotein that is present on the apical surface of most simple secretory epithelial cells. Muc-1 is highly expressed and aberrantly glycosylated by most carcinomas and metastatic lesions. Numerous functions have been proposed for this molecule, including protection of the epithelial cell surface, an involvement in epithelial organogenesis, and a role in tumor progression. Mice deficient in Muc-1 were generated using homologous recombination in embryonic stem cells. These mice appeared to develop normally and were healthy and fertile. However, the growth rate of primary breast tumors induced by polyoma middle T antigen was found to be significantly slower in Muc-1 deficient mice. This suggests that Muc-1 plays an important role in the progression of mammary carcinoma.

The MUC1 protein is a heavily glycosylated cell-associated mucin glycoprotein that is highly expressed and aberrantly glycosylated by the majority of carcinomas and, in particular, by >92% of primary and metastatic breast cancers. This protein was initially identified as a component of the human milk fat globule membrane (1). Subsequently, it was found to be expressed by the majority of simple secretory epithelial cells in addition to being highly expressed by most human carcinomas, including those of the breast, colon, ovary, pancreas, lung, and stomach (2, 3). This widespread expression pattern is in contrast to the other human mucin genes that have been isolated, which appear to have a more restricted spatial expression pattern (reviewed in Ref. 4). MUC1 is also known as PEM, episialin, H23 antigen, DF3 antigen, PUM, and epithelial membrane antigen (or EMA) and by a variety of other names.

Mucins, or mucin-type glycoproteins, can be defined as large extended molecules with a high percentage (50–90%) of their molecular mass made up of carbohydrate that is attached via O-glycosidic linkage through N-acetylgalactosamine to serine and/or threonine. They can be subdivided into the classical secretory or soluble mucins and the membrane-associated mucins. The secretory mucins constitute the viscous mucus of the tracheobronchial, gastrointestinal, and reproductive tracts and typically form extremely large complexed oligomers through linkage of protein monomers via disulfide bonds. These proteins are secreted from the cell and remain at the apical surface in the form of a mucus gel. This secretion serves as a selective physical barrier between the extracellular milieu and the plasma membrane and cell interior. The membrane-associated mucins are intimately associated with the plasma membrane through a hydrophobic transmembrane domain and have not been observed to form oligomeric complexes. However, these proteins can be secreted from the cell surface, most probably through a proteolytic cleavage mechanism (5, 6).

Isolation of cDNA and genomic clones for the human MUC1 gene suggested that the gene coded for a typical type I membrane glycoprotein (7–12). At the amino terminus there was a short signal peptide, and at the carboxyl terminus there was a 28–31-amino acid transmembrane domain and 69–72-amino acid cytoplasmic tail. The majority of the external domain of the protein was made up of multiple copies of a 20-amino acid repeat (up to 125 copies) that was rich in serine, threonine, and proline. Serine and threonine residues act as the attachment sites for O-linked carbohydrate, and it is this region of the protein that is highly O-glycosylated. Isolation of the mouse Muc-1 gene indicated that the most strongly conserved regions corresponded to the serine, threonine, and proline residues within the repeat domain and to the transmembrane and cytoplasmic domains (13, 14). This would suggest that the primary function of the repeat domain is to act as a scaffold for carbohydrate attachment. The sequence conservation of the transmembrane and cytoplasmic domains would suggest an important function for this part of the protein. In this respect, MUC1 has been demonstrated to be associated with elements of the actin cytoskeleton (15), and presumably it is the cytoplasmic domain of MUC1 that is involved in this interaction. An investigation of the expression pattern of Muc-1 during mouse embryogenesis revealed that expression was regulated both spatially and temporally and correlated well with the onset of epithelial differentiation in each organ (16). In addition, the pattern of Muc-1 expression in the adult mouse and in mouse mammary carcinomas correlated well with the pattern of expression observed for the human MUC1 gene (17, 18).

Traditionally, mucins have been thought of as protective molecules. However, the fact that MUC1 is expressed early during epithelial organogenesis and is highly expressed by tumors and metastatic lesions suggests other functions. In general these functions may be a direct reflection of the large size of the molecule. According to the model of Jantosf (19), the fully glycosylated human MUC1 protein may extend as much as 300–500 nm above the cell surface, far above the glyocalyx. Thus, due to its large extended conformation, one potential function for MUC1 is as an antiadhesive protein, possibly blocking cell-cell and cell-matrix interactions (20–22). Muc-1 in normal polarized epithelia is expressed only on the apical surface. However, in many adenocarcinomas polarization is lost and the protein can be found on basolateral cell surfaces, where it could interfere with cell-cell and cell-substratum adhesion. Alternatively, MUC1 may play an adhesive role by presenting carbohydrates as ligands for selectin-like molecules and thus aiding metastatic dissemination (23–25). This may be particularly relevant as MUC1 has recently been shown to express
sialyl Lewisα and sialyl Lewisβ, ligands for P- and E-selectins (26, 27). Clearly both antiadhesive and proadhesive functions could play significant roles in normal development, tumor progression, and disease. In order to investigate the biological function of the mouse Muc-1 protein, we generated mice deficient in Muc-1 through homologous recombination in mouse embryonic stem (ES) cells. These mice appeared to develop normally and were healthy and fertile. Muc-1-deficient mice exhibited no differences in survival rate and appeared phenotypically normal in all respects. Direct evidence of a role for MUC1 in the development and/or progression of breast cancer has not been demonstrated previously. To investigate the role of Muc-1 in tumor development and/or progression, we compared the growth of mammary tumors in Muc-1−/− and +/+ mice. To generate mammary tumors, we utilized mice transgenic for the polyoma virus middle T antigen (28). In female mice, the middle T antigen is expressed in mammary tumors, we utilized mice transgenic for the polyoma virus middle T antigen (28). In female mice, the middle T antigen is expressed in mammary tumors, and expression is specific for the mammary gland and to a lesser extent the salivary gland. Virgin female mice of this strain have been shown to develop multifocal breast tumors by 2 months of age, and by 4 months of age greater than 50% of these mice will have developed lung metastases. Interestingly, the middle T antigen has been demonstrated to require the presence of the src oncogene for its ability to transform mammary cells (29). Similarly, it has been demonstrated that the neu oncogene, implicated in up to 30% of human breast cancers (30), binds to and activates src tyrosine kinase activity (31). We have employed the middle T oncogene in this study due to its rapid time course of tumor induction, reliable production of spontaneous tumor metastases, and the possible commonality of signal transduction pathways with the neu protooncogene. The results indicate that Muc-1 does indeed play an important role in the progression of mammary carcinoma, as primary tumor growth rate was significantly slower in Muc-1−/− mice as compared with their wild type counterparts.

MATERIALS AND METHODS

Muc-1 Genetargeting Vector Construction—An isogenic Muc-1 genomic clone was obtained through screening a 129SVj mouse cosmid library (Stratagene Cloning Systems, La Jolla, CA) with the Muc-1 cDNA probe, pmuC2TR, previously described (13). Approximately 5 × 105 colonies were plated onto Biodyne nylon membrane (Pall Biodyne, Glen Cove, NY) on LB-agar plates supplemented with ampicillin to 100 µg/ml. Doublelifts were taken from each membrane and screened with pmuC2TR labeled with [α-32P]dCTP (Amersham Corp.) by random priming (32) under conditions recommended by the manufacturer. Positive clones were taken through two further rounds of colony purification to yield pure colonies. An 11-kb EcoRI fragment containing the entire mouse Muc-1 gene (13, 14) was subcloned into pBluescript SKII (Stratagene Cloning Systems, La Jolla, CA) and designated 129Muc-1E2.

The cloning strategy is diagrammed in Fig. 1. An Escherichia coli β-galactosidase (LacZ) gene (33) lacking the first 7 codons, including the translation initiation codon, designated LacZ 839 (kindly provided by R. Krumlauf), was subcloned into the BamHI site of pBluescript SKII (Stratagene Cloning Systems, La Jolla, CA) and designated LacZ 839. The cloning step resulted in the loss of the β-galactosidase termination codon and was necessary in order to place the appropriate restriction sites at the 5′ end of the gene to allow an in-frame ligation between Muc-1 and LacZ to be made and to place sites at the 3′ end of the gene to allow further manipulations. A 2-kb Smal fragment of the Muc-1 gene was cloned into the SmaI site created at the 5′ end of the LacZ gene to construct the plasmid pMuc-LacZ. This step created a Muc-1LacZ fusion protein designed to be under the control of the Muc-1 transcription and translational machinery. This plasmid incorporated the promoter and first three codons of the mouse Muc-1 gene ligated in-frame, at the SmaI site in the first exon, to the eighth codon of the LacZ gene. A neomycin (neo) resistance gene under the control of the phosphoglycerate kinase (PGK) promoter andpoly(A) site (34, 35) was released from pcK-1 (kindly provided by M. McBurney) and cloned into the EcoRI-HindIII sites of the plasmid pMuc-LacZ in the same transcriptional orientation as the Muc-1 gene. To allow expression of the LacZ gene, two bases were added to the 3′ end of the LacZ gene and also to place the poly(A) signal of SV40 at the 3′ end of the LacZ gene, a 0.8-kb EcoRI fragment was subcloned from the plasmid pPGK (E/T)LacZ (kindly provided by M. McBurney) into the EcoRI site of the LacZ and Pgkneo genes. This plasmid was designated pMucLacZneo.

The final 129 Muc-1-targeting vector was created as follows: the plasmid 129Muc-1E2 was digested with Smal, and a 10-kb fragment, containing the cloning vector in addition to 7 kb of the Muc-1 gene locus, was gel-purified on DEAE (NA45) paper (Schleicher and Schuell). The 8-kb MucLacZneo cassette was removed from pMucLacZneo by NotI-HindIII fragment. 3′ overhanging ends were filled in using Klenow DNA polymerase, and the blunted fragment was gel-purified. Ligatization of the 10- and 8-kb fragments and selection for plasmids containing the two fragments ligated in the correct orientation resulted in the creation of the final Muc-1-targeting vector, designated 129Muc-1GT.

A total of 5000 colonies were plated onto Biodyne nylon membrane (Amersham), and screened by Southern analysis using the 0.8-kb MucLacZ gene as a probe. Both male and female chimeras were back-crossed with C57BL/6J mice to test for germ line transmission. Agouti F1 progeny were genotyped on the basis of agouti/chinchilla pigmentation in the coat. Both male and female chimeras were back-crossed with C57BL/6J mice to screen for germ line transmission. Agouti F1 progeny were genotyped by PCR analysis of tail DNA utilizing oligonucleotides specific for the Muc-1 gene. 5′-ACCTACACGACGACGCACAG-3′ (corresponding to the Muc-1 gene), 5′-CTGACTACGACGCCG-3′ (corresponding to bp 201–181, antisense strand) (33). Tail DNA was prepared as described (39) and dissolved in water to a final volume of 100 µl. Five µl of each DNA preparation was utilized in a 100-µl PCR reaction under standard buffer conditions with 2% (v/v) deionized formamide, 1 µM each oligonucleotide primer, 200 µM dNTPs, and 2.5 units of Taq polymerase (Boehringer Mannheim). DNA samples were heated for 10 min at 95 °C before the addition of 95 µl of a PCR mix containing all of the necessary components. Amplification proceeded for 40 cycles of 95 °C for 1 min, 62 °C for 30 s, 72 °C for 1 min,
followed by a single final extension of 5 min at 72°C. Amplified products were analyzed by agarose gel electrophoresis. Chimeras demonstrating 100% germ line transmission were further bred with 129S V/J mice in order to establish an inbred line of mutant mice.

Heterozygous agouti offspring were intercrossed to generate mice homozygous for the Muc-1 mutation. Muc-1 +/+ , +/− , and −/− mice were analyzed by PCR analysis using two sets of oligonucleotide primers under the conditions described above. The first set comprised the 5′-Muc-1 primer, previously identified in combination with a 3′-Muc-1 primer, 5′-TCCCCCTGGCCATAGCTG-3′ (corresponding to bp +268 to +284, antisense strand (13)). Together, these primers amplified a 262-bp product from the wild type allele only. The second set of primers was designed to the T portion utilized to screen for the presence of agouti F1 heterozygotes, as described. The validity of the PCR genotyping results was initially confirmed by Southern analysis of EcoRI-digested tail DNA utilizing the 5′-flanking probe described above.

Northern Analysis—Total RNA was isolated from tissues dissected from +/+ , +/− , and −/− littermates, homogenized in guanidinium isothiocyanate/mercaptoethanol solution followed by centrifugation through a CsCl gradient (40). RNA (15 μg) was separated through a 1.2% formaldehyde gel, transferred to Hy-Bond-N nylon membrane (Amersham), and stained with methylene blue solution to detect 18 and 28 S ribosomal RNAs prior to hybridization as described (41). Blots were hybridized overnight, as described (16), with the mouse Muc-1 cDNA probe, pMuc-T2R.

Immunohistostaining—Immunostaining with the polyclonal anti-serum, CT1, raised to a synthetic peptide corresponding to the 17 C-terminal amino acids in the cytoplasmic tail of human MUC1 (17) was performed as described (16, 17). As a negative control, the sections were incubated with CT1 antiserum previously blocked with 5 mg/ml of the synthetic peptide. Immune complexes were detected using fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (Dako Corp, Carpinteria, CA). Photomicrographs were taken on a Nikon FXA photomicroscope with Kodak T-Max 400 film. In addition, sections were routinely stained with hematoxylin and eosin according to standard procedures to investigate morphology. In all cases three +/+, +/− , and −/− animals were analyzed.

Analysis of Mammary Gland Whole Mounts—Intact mammary glands were isolated from twelve week old Muc-1 +/+ , +/− , and −/− virgin littermates, and hematoxylin stained whole-mounts were prepared as described (42).

Gene Expression Analysis—RNA samples isolated from tissues of +/+ , +/− , and −/− littermates were investigated for the expression of a variety of genes utilizing a slot-blot approach. 1 μg of total RNA from +/+, +/− , and −/− tissues was loaded into adjacent wells of a vacuum slot-blot apparatus (Schleicher and Schuell) and vacuum-blotted onto nylon membrane (Hybond-N) under conditions recommended by the manufacturer. Duplicate blots were prepared and hybridized with probes to a large panel of genes (Table II). Genes that were identified as being potentially up-regulated by this approach were further investigated by Northern analysis. Antisense oligonucleotide probes were 5′-labeled by nick-translation utilizing T4 polynucleotide kinase (New England BioLabs, Beverly, MA). All probes were hybridized as described previously for Northern analysis.

Tumor Generation System—To study the role of Muc-1 in tumor formation, FVB mice transgenic for the polyoma virus middle T antigen under the control of the mouse mammary tumor virus promoter (a generous gift of Dr. W. Muller) were crossed with 129SV/J:C57BL/6J animals that have previously lost the Y chromosome (50). For this reason we analyzed the chromosome constitution of the parental cell line, GK129, and Muc-1 targeted clones 56, 31, and 132. Approximately half of the clone 31 metaphase spreads.
Homologous Mutant Mice Do Not Express Muc-1 mRNA and Protein—In order to determine whether the insertion of the LacZ-Pgkneo cassette resulted in the efficient disruption of Muc-1 transcription and subsequent translation, total RNA was prepared from a panel of tissues isolated from +/--, +/+, and --/-- littermates. Approximately equivalent amounts of total RNA were subjected to Northern analysis with the previously characterized Muc-1 cDNA probe, pMuc2TR (13). Expression of Muc-1 was found to be reduced in the tissues of heterozygous mice and undetectable in homozygous mice (Fig. 3). In addition, immunohistochemistry indicated no detectable Muc-1 protein on the apical surface of Muc-1 --/-- secretory epithelial tissues (Fig. 4). Thus, targeted inactivation of the Muc-1 gene by the replacement vector, 129Muc-1GT, resulted in the creation of a null Muc-1 allele.

An investigation of LacZ expression in Muc-1 +/-- and --/-- mice indicated that the expression of LacZ in the context of the endogenous Muc-1 promoter did not faithfully reflect the previously described expression pattern of Muc-1 in the developing embryo or adult. This was found to be due to a block in transcription of the Muc-1/LacZ fusion gene.2

Muc-1 Null Mice Develop Normally and Are Viable and Fertile—Mice deficient in Muc-1 were obtained at the expected frequency from all crosses. These mice appeared to develop normally and gained weight at the same rate as their heterozygous and wild type littermates (data not shown). Examination of hematoxylin-eosin stained sections prepared from all the major organs revealed no obvious differences between Muc-1 deficient mice and their corresponding littermates (data not shown). Similarly, whole mounts of virgin mammary glands of 12-week old wild type and Muc-1 null animals showed no obvious differences in glandular morphology (data not shown). All possible pairwise crosses of genotypes indicated no differences in the fertility of the parents, subsequent litter size, growth rate, and survival of the litters (data not shown). This would suggest that Muc-1 present in milk is not important for the growth and survival of neonates under pathogen-free conditions.

A Screen for Potential Compensating Genes—We explored the possibility that the up-regulation of expression of one or more mucin-like genes or membrane glycoproteins may have accounted for the apparent lack of a phenotype in Muc-1-deficient mice. Probes were obtained either as antisense oligonucleotides (50 mers) or, alternatively, cloned cDNAs were utilized (Table II). Total RNAs isolated from +/+, +/-, and --/-- littermates were investigated by slot-blot analyses with the various probes. No difference in expression levels was observed for Muc-2 or Muc-4, although high levels of Muc-4 expression were observed in lactating mammary gland, salivary gland, lung, stomach, kidney, and colon (Fig. 5). Similarly, no difference was observable for other mucin-like genes, including ASGP-2, CD34, CD43 (leukosialin), glycoporphin, and MadCAM-1. The expression level of GlyCAM-1 was elevated in several outbred homozygous animals, but this apparent increase in expression was not consistent in inbred homozygotes, nor did it appear to correlate with an increase in GlyCAM-1 protein levels in milk. In addition, no difference was observable in the expression of Thbs-3, although the expression of this gene did appear to be highly variable in the tissues tested (Fig. 5).

Primary Tumor Growth Is Reduced in Muc-1 --/-- Mice—In order to study the effect of the Muc-1 gene on mammary tumor development and progression, 85 female Muc-1 --/-- mice and

2 A. P. Spicer and S. J. Gendler, unpublished data.

Fig. 1. Construction of a replacement targeting vector to inactivate the endogenous Muc-1 gene. Six separate cloning steps, indicated by numbered arrows, were required to construct the final vector, 129Muc-1GT. The cloning strategy is outlined in detail under "Materials and Methods." The LacZ, SV40 pdy(A), and Pgkneo cassettes are represented by the filled arcs, whereas sequence from the mouse Muc-1 locus is represented by the open arcs. In each case, restriction sites utilized in the proceeding ligation are indicated in boldface. Arrows internal of the plasmids indicate the direction of transcription of the respective genes. At step 3, the sequence of the in-frame fusion between the mouse Muc-1 gene and the E. coli LacZ gene is indicated. The initiation methionine is italicized, and the Smal and BamHI sites are underlined.

were found to contain 39 chromosomes (data not shown). In addition, a lower frequency of metaphase spreads containing 39 chromosomes were present in the parental cell line and clones 56 and 132. This would suggest that female germ line chimeras were generated as the result of previous Y chromosome loss during culture.

Approximately 50% of the agouti offspring of the chimeras were heterozygous for the designed mutation. These mice were indistinguishable from their wild type littermates. Heterozygous (+/--) animals were intercrossed to generate animals homozygous for the Muc-1 mutation. Homozygous (--) animals were obtained from clones 56 and 31. Homozygous animals were identified through a PCR-based screening procedure (Fig. 2B), and results were initially confirmed through Southern blotting of EcoRI-digested tail DNA utilizing the 5'-flanking probe (Fig. 2C). In addition, animals homozygous for the disrupted Muc-1 allele (--) were obtained at the expected Mendelian frequency. In addition, inbred 129SV/j heterozygotes were obtained from the original chimeric animals and intercrossed to derive an inbred line homozygous for the Muc-1 mutation. Similarly, inbred C57BL/6j heterozygotes are being derived through a series of back-crosses onto C57BL/6j. These mice are currently at N8 (99% inbred with respect to C57BL/6j ).
35 female Muc-1+/1 mice were utilized. All mice were virgin females positive for the polyoma virus middle T antigen (MTag) transgene. Fifty percent of mice in this study developed palpable lesions of the mammary gland by 68 days of age. There was no significant difference in the rate of appearance of palpable lesions between Muc-1 mutant and wild type mice. Tumors appeared in 100% of wild type mice and in 98% of mutant mice. Tumors in Muc-1 mutant and wild type mice had similar histological appearances and were poorly differentiated adenocarcinomas (Fig. 6A). Pathological analysis showed that the tumors were high grade, based on the high mitotic rate, the solid growth pattern, and the presence of necrosis, and appeared to be typical of tumors generated using the MTag transgene. Immunohistochemical analysis using antiserum directed to the Muc-1 cytoplasmic tail showed that tumors that developed in Muc-1+/1 animals expressed high levels of Muc-1 (Fig. 6B).

Interestingly, tumor growth rate differed significantly between the two groups (Fig. 6C). As early as 104 days of age, Muc-1−/− mice had significantly smaller tumors than did mice with wild type Muc-1 alleles (p < 0.05), and by the 124-day end point, differences in tumor size were highly significant (p < 0.001) (two-sample t test).

Although a lack of Muc-1 protein would be expected to affect the metastatic cascade, no significant differences in rates of metastasis were observed. Overall, 58% of mice developed grossly observable lung metastases, with 53% of Muc-1−/− mice and 67% of Muc-1+/+ mice developing metastases (Fig. 6D). Although this difference suggests a trend toward de-
creased rates of tumor metastasis in Muc-1 −/− mice, it was not statistically significant as assessed by χ-square analysis (p > 0.12). However, based on the sample sizes in this study, the power to statistically detect the observed difference was only 33%. It is possible that with a large sample size, this difference in metastatic rate would be statistically significant. It should be noted that although the rate of metastasis observed in this study was lower than that previously reported in MTag mice (28), this may be a consequence of the alternate type of analysis used to detect metastasis, microscopic examination versus the RNase protection assay.

**DISCUSSION**

**Absence of a Developmental Phenotype in Muc-1 −/− Mice—The ability to create mice that possess deficiencies in specific genes is providing important insights into the physiological role played by specific proteins both during embryonic and postnatal development and during adult life. To investigate the biological function of the Muc-1 membrane glycoprotein, we created a null mutation in the Muc-1 gene using homologous recombination in mouse ES cells. Mice were subsequently derived that were deficient in Muc-1 (Figs. 3 and 4). We and others have postulated that Muc-1 on the apical surface of an aggregation of differentiating epithelial cells may repel adjacent cells or mask adhesive molecules, thus promoting the formation of a lumen (16, 21). However, we were surprised to find that, despite the widespread expression of Muc-1 during epithelial organogenesis, Muc-1-deficient mice were obtained at the expected frequency and appeared normal in all respects. In addition, Muc-1-deficient mice were fertile and produced and weaned litters of average size. Pathological analysis of hematoxylin-eosin-stained sections of all the major organs of inbred 129SvJ and C57BL/6J backgrounds produced mice that appeared normal in all respects.

Other Mucins and Mucin-like Genes May Compensate for Muc-1 Deficiency—We hypothesized that the lack of a specific phenotype in Muc-1-deficient mice might be due to the specific up-regulation of expression of another mucin or mucin-like gene. In humans, eight mucin genes have now been identified (reviewed in Ref. 4), and it is quite likely that there are other mucin genes yet to be discovered. To date, MUC1 is the only human epithelial membrane-spanning mucin gene that has been cloned. Of the human mucin genes that have been identified, only two definite rodent homologues have been isolated, those for MUC1 and MUC2, respectively (13, 14, 51–54). However, a rat intestinal mucin, designated M2 has been isolated (55, 56), which may be the rodent homologue of the human intestinal MUC3 gene, and a mouse gastric mucin gene has recently been reported that may represent the mouse MUC-6 gene. In addition, we have recently isolated genomic clones for the mouse homologue of the human MUC4 gene.3

In an attempt to determine if a mucin-like molecule could potentially compensate for Muc-1 function, we obtained probes for a large panel of mucin and mucin-like genes. Slot-blot analyses of RNA were utilized to screen for their expression in Muc-1 +/+ , +/−, and −/− mice. Muc-2 was strongly expressed in the colon, and Muc-4 appeared to be strongly expressed throughout epithelial tissues (Fig. 5). The structure of the human MUC4 and mouse Muc-4 genes and their encoded proteins has not been fully determined, and it is therefore possible that Muc-4 may be structurally and functionally similar to

3 S. J. Gendler, unpublished data.
Muc-1. Other genes that did not show any consistent alteration in expression included CD34, CD43 (leukosialin), glypican-1, ASGP-2, GlyCAM-1, and MadCAM-1. In addition, the expression levels of genes closely flanking the Muc-1 gene (Thbs-3 and Gene Y) were unaffected by the targeted inactivation of Muc-1 (Fig. 5). We are currently employing the differential display PCR approach (57) on mRNAs isolated from inbred mice in an attempt to identify potential compensating genes.

**TABLE II**

List of genes screened for in Muc-1-deficient mice

| Gene    | Species | Probe name | Type       | Location | Reference |
|---------|---------|------------|------------|----------|-----------|
| Muc-1   | Mouse   | pMuc2TR    | Tandem repeat | bp 468–419 | 13        |
| Muc-2   | Rat     | VR-1A      | 5′ cDNA     | bp 1248–1199 | 53        |
| Muc-4   | Mouse   | pMuc7.18genomic | Footnote | bp 900–851 | 3         |
| GlyCAM-1| Mouse   | Antisense-oligo | bp 1061–1015 | 71        |
| Glypican | Mouse   | Antisense-oligo | bp 1061–1015 | 71        |
| PSGL-1  | Human   | Antisense-oligo | bp 1061–1015 | 71        |
| ASGP-2  | Rat     | Antisense-oligo | bp 2175–2126 | 72        |
| CD34    | Mouse   | Antisense-oligo | bp 1258–1209 | 73        |
| CD43    | Mouse   | Antisense-oligo | bp 1183–1134 | 74        |
| CD44    | Mouse   | Antisense-oligo | bp 1000–951 | 75        |
| CD45    | Mouse   | Antisense-oligo | bp 4250–4201 | 76        |
| Gene Y  | EST01966| Antisense-oligo | 3′ cDNA     | 47        |
| Thbs-3  | Mouse   | mThbs-3-Eco | 2.1-kb EcoRI | 3         |

**FIG. 5.** A screen for genes potentially up-regulated in Muc-1-deficient mice: slot-blot analysis to detect the expression of known genes. Equivalent amounts of total RNA were transferred to a nylon membrane utilizing a slot-blot apparatus. Duplicate membranes were hybridized with radioactively labeled probes for a large panel of mucin and mucin-like genes (Table II). Results are shown for Muc-1, Muc-2, Muc-4, and Thbs-3.

**FIG. 6.** Primary tumor growth rate is reduced in Muc-1 −/− mice. A, hematoxylin-eosin-stained sections of tumors taken at 124 days, showing poorly differentiated adenocarcinomas. Bar (panels A and B) = 100 μm. C, graph showing growth rate of polyoma middle T-induced mammary tumors in Muc-1 −/− (filled square) and Muc-1 +/+ (open circle) mice. At 104 days, Muc-1 −/− mice had significantly smaller tumors than did Muc-1 +/+ mice (p < 0.05). By the 124-day end point, differences in tumor size were highly significant (p < 0.001). Asterisks indicate statistical significance. D, graph showing the percentage of Muc-1 −/− mice with metastases (%).
metastasis (60). However, this is the first study to directly assess the role of the Muc-1 glycoprotein in spontaneous tumor development in the mammary gland. We demonstrated that the presence of the Muc-1 molecule is beneficial to the developing tumor, resulting in a tumor with a significantly faster growth rate than that seen in tumors that do not express the Muc-1 molecule. It is not clear at present how the Muc-1 molecule affects tumor growth rate in the primary tumor. However, it is likely that Muc-1 modulates the immune system in some way. It will be important to determine differences in natural killer and cytotoxic T cell activities at the tumor sites that might underlie the differential tumor growth rates. MUC1 protein can be detected in the circulation of patients with breast and pancreatic carcinomas (61-64), and free MUC1 protein appears to inhibit the cytotoxic T cell lysis of target cells (65) and may be immunosuppressive (66). High levels of circulating Muc-1 might therefore block the specific T cell activity and thus aid the cells in escaping from T cell-mediated lysis. Studies to define the mechanism whereby Muc-1 expression facilitates growth of the primary tumor are currently under way.

The effect on metastasis in the Muc-1−/− mice was less than expected. The trend toward decreased rates of tumor metastasis in Muc-1 null mice suggested that the lack of Muc-1 was showing some effects. However, our sample size was not sufficiently large to reach statistical significance. In addition, the rapid kinetics of the middle T tumor phenotype may have rendered some immune responses impotent. The middle T mice exhibit hyperplastic mammary glands as early as at 3 weeks of age and the rapid production of multifocal mammary adenocarcinomas by 35 days. In many animals tumors developed in every mammary gland. Alternatively, middle T may be modulating the immunogenicity of the tumor cells independently of Muc-1, or up-regulation of other molecules may compensate in the absence of Muc-1. Further analysis of the role of Muc-1 in metastasis may await a tumor model more relevant to human breast cancer. Such a study is in progress, utilizing mice with a lack of Muc-1, mammary tumor growth was significantly less than expected. The trend toward decreased rates of tumor metastasis in Muc-1 deficient mice is expected to be critical in our future understanding of the role of Muc-1 in the development and progression of breast and other cancers. In addition to the work described herein, mucus has been postulated to be involved in adhesion and replication of viruses and bacteria, in receptivity of the uterus to blastocyst implantation, and in diseases such as asthma and cystic fibrosis. Muc1-deficient mice will provide important models in the study of these aspects of mucin biology.

Acknowledgments—We acknowledge the expertise of Suresh Savarirayan in blastocyst microinjection and manipulations, Anita Jennings for expert tissue processing and sectioning, Amy Weaver for statistical assistance, and the expert technical assistance of Melissa Wilson and Steve Ritland. We thank Graham Kay and Sohalia Rastan for the kind gift of the GK129 ES cells, Steve Rosen and Mark Singer for quantitation of GlyCAM-1 in mouse milk, Vania Braga for helpful discussions, Bill Muller for the generous gift of the middle T antigen, Mike McBurney for the kind gift of the pkj-1 and pPGK (E/T)LacZ plasmids, and Robb Krumlauf for the LacZ (number 839) plasmid. We gratefully acknowledge Dr. Robert Cardiff's special expertise in MTag tumor pathology. We acknowledge the graphic expertise of Dawn Taylor and Marvin Ruona.
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J. Biol. Chem. 1995, 270:30093-30101.
doi: 10.1074/jbc.270.50.30093

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