

# Developmental Expression Of Mucin Genes ASGP (rMuc4) and rMuc5ac by the Rat Ocular Surface Epithelium

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**PURPOSE.** To determine site and time of initiation of expression of the membrane-spanning mucin ASGP (rMuc4) and the goblet cell-specific, gel-forming mucin rMuc5AC by the developing rat ocular surface epithelium.

**METHODS.** Newborn Sprague-Dawley rat pups were killed at 1, 7, and 14 days after birth. Adult rats (weight, 200 g) were used as controls. Reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect ASGP mRNA using  $\beta$ -actin as an internal control. Competitive RT-PCR was performed to quantitate rMuc5AC mRNA using an rMuc5AC-competitive reference standard (CRS) as an internal control. In situ hybridization was performed to localize ASGP and rMuc5AC mRNA. Goblet cells were detected by staining with periodic acid-Schiff (PAS) reagent.

**RESULTS.** ASGP mRNA was detected by RT-PCR at 1 day after birth. Compared with  $\beta$ -actin, the amount of ASGP mRNA showed a progressive increase from 1 to 14 days of postnatal development. By in situ hybridization, the expression of ASGP was first clearly detected at 14 days after birth at the lid margin, where the most stratification of epithelium was seen, and along the adjacent palpebral conjunctiva. This pattern was seen in rat eyelids that were not yet open but appeared about to open. In rat eyelids already open at 14 days after birth, ASGP mRNA was diffusely spread in the apical cell layer of both conjunctival and corneal epithelia. The expression of rMuc5AC was detected by RT-PCR in ocular surface epithelium in rat pups 1 day after birth. Quantitative RT-PCR showed a low level of rMuc5AC RNA expression in conjunctiva of 1-, 7-, and 14-day-old rats followed by a large increase in expression between 14 days and adulthood. The expression of rMuc5AC was first detected by in situ hybridization in a few goblet cells at 7 days after birth. One or two labeled cells were present in the fornix area; some were on the palpebral side of the fornix; others were present on the bulbar side. The distribution and time of appearance of rMuc5AC correlated with that of PAS staining of goblet cells.

**CONCLUSIONS.** The developmental expression of the membrane-spanning mucin ASGP (rMuc4) and the gel-forming mucin rMuc5AC are regionally and temporally separated. Expression of the gel-forming mucin begins at the fornix at 7 days after birth and is correlated with the appearance of goblet cells, whereas, expression of the membrane-spanning mucin begins later at the lid margin at day 14. Expression of the membrane-spanning mucin correlates to eyelid opening. (*Invest Ophthalmol Vis Sci.* 1999;40:1944-1951)

Mucins, the most prominent glycoproteins of the mucus layer, play an essential role on the ocular surface by protecting the epithelium from dryness and by facilitating removal of external pathogens and foreign debris.<sup>1,2</sup> The molecular character of mucins in the tear mucus layer was not well known until the recent isolation of a number of mucin genes<sup>3</sup> enabled the identification of those that are expressed by the ocular surface epithelium. Through the use of northern blot analysis and in situ hybridization, our laboratory demonstrated that, of the nine cloned human mucins, three—MUC1,

MUC4, and MUC5AC—are expressed by the human ocular surface epithelium.<sup>4,5</sup> MUC1 is expressed by both corneal and conjunctival epithelial cells, MUC4 is expressed by both non-goblet and goblet cells of the conjunctival epithelium, and MUC5AC is expressed only by the goblet cells of the conjunctiva.

The common feature of all mucins cloned and sequenced to date is the presence of tandem repeats of amino acids that have a high serine and threonine content, with each mucin having a unique tandem repeat length and sequence.<sup>3</sup> Based on their protein structural features, mucins have been subdivided into three types, membrane-spanning, gel-forming, and soluble.<sup>3</sup> The human ocular surface expresses two membrane-spanning mucins, MUC1 and MUC4, and one large gel-forming mucin MUC5AC. MUC1 is a membrane-spanning mucin found in most epithelial tissues.<sup>3</sup> Although mRNA to MUC1 is expressed by the entire ocular surface epithelium, MUC1 protein is present primarily along the apical cell membrane of cells at the tear interface.<sup>4</sup> A mouse homologue of MUC1, designated

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Supported by Grant R37 EY03306 (IKG) from the National Institutes of Health, and Fight For Sight Fellowship (MT).

Submitted for publication January 11, 1999; accepted May 23, 1999.

Proprietary interest category: N.

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Muc1, has been cloned and sequenced, and the gene has been deleted.<sup>6</sup> Mice with the deletion have no phenotypic alteration other than delayed mammary tumor progression, and extensive examination of the ocular surface of these mice shows no alteration of ocular surface epithelia or tear film.<sup>7</sup> Because of the absence of altered phenotype in a Muc1 null rodent, we have not included MUC1 in our investigation of developmental expression of mucins.

MUC4 appears also to have a wide tissue distribution and is expressed not only by most simple epithelia of wet-surfaced mucosa but also by stratified, squamous, nonkeratinizing epithelium at those sites—that is, ectocervix, vagina, and trachea.<sup>5,8</sup> MUC4 is a relatively uncharacterized mucin, but recent sequencing of the 5' end indicates that the amino acids even outside the tandem repeat are rich in serine and threonine, indicating that it is heavily O-glycosylated.<sup>9,10</sup> The sequence of the 3' end of MUC4 was recently reported to have a membrane-spanning region, and it appears to have high homology (80%) to the rat ASGP mucin.<sup>11</sup> ASGP (ascites sialylglycoprotein) is the product of one gene but is composed of two associated subunits, ASGP-1 and ASGP-2, which together have been termed the sialyl mucin complex.<sup>12,13</sup> The N-terminal region, designated ASGP-1, is a heavily O-glycosylated subunit with tandem repeats, and the C-terminal region, ASGP-2, is an integral membrane glycoprotein that has two EGF-like domains. ASGP-1 is tightly but noncovalently bound to the transmembrane subunit ASGP-2.<sup>14</sup> ASGP-1 does not share sequence homology to human MUC4 (compare GenBank Accession No. AF058803 with M221770 and M15503), but because ASGP-2 does,<sup>11</sup> we make the assumption that ASGP is the rat homologue of MUC4 (which by convention is designated rMuc4). ASGP has been shown to be expressed in a number of wet-surfaced epithelial tissues in adult rats, including small and large intestine, trachea, uterus, lactating mammary gland, and the cornea and conjunctiva of the ocular surface.<sup>15-17</sup> The function of this membrane-spanning mucin at the ocular surface is not known, nor is it known when the mucins appear developmentally.

The third mucin expressed at the ocular surface is MUC5AC. It is a large gel-forming mucin, the expression of which at the ocular surface is limited to the goblet cells of the conjunctiva.<sup>5</sup> The large gel-forming mucin genes are a family of mucins all clustered on chromosome 11p15.<sup>18</sup> These mucins are designated MUC5AC, 5B, 2, and 6, and all have common structural features. Each has cysteine-rich domains at N- and C-terminal regions outside a large central tandem repeat region.<sup>19,20</sup> These cysteine-rich regions contribute to dimerization of individual mucin molecules and are believed to be responsible for gel formation.<sup>21,22</sup> At the ocular surface, MUC5AC may play an important role in forming the gelled mucus layer of the tear film at the air-ocular surface epithelium interface. Its gene represents one of two known goblet cell-specific markers, the other being the cytoskeletal protein keratin 7.<sup>23</sup>

To establish animal models for the study of goblet cell differentiation and the regulation and function of this gel-forming mucin, our laboratory cloned a rat homologue of the human MUC5AC gene.<sup>24</sup> Designated rMuc5AC, a 3783-bp partial sequence of the 3' end of the molecule including a cysteine-rich region believed to be involved in intermolecular disulfide linkage was obtained. In situ hybridization studies

demonstrated that rMuc5AC is expressed in the conjunctival goblet cells of the rat.

Cell kinetic investigations using incorporation of tritiated thymidine into the conjunctival epithelium have shown that the fornical zone is a site enriched by slow-cycling, label-retaining cells in neonatal and adult SENCAR mice,<sup>25</sup> leading Wei et al.<sup>26</sup> to hypothesize that the conjunctival epithelial stem cells are located in the fornical region. Moreover, goblet cells have been reported to derive from the same cells as the stratified cells by analysis of clonal cultures of rabbit conjunctiva grown in athymic mice. These data suggest the existence of a bipotent precursor cell in conjunctival epithelium that can give rise to both goblet and nongoblet cells. Developmental analysis of conjunctival goblet cells using goblet cell-specific markers to determine site of first expression of differentiation markers has not been undertaken.

Thus, the objectives of this study were to use the developing rat and probes to the mucins ASGP (rMuc4) and rMuc5AC to determine site and time of initiation of expression of a membrane-spanning mucin and a gel-forming mucin and to correlate these data to eyelid opening and goblet cell differentiation.

## MATERIALS AND METHODS

### Animals and Tissues

All procedures used in these studies followed the tenets of the Declaration of Helsinki, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

For studies of developing ocular surface, newborn Sprague-Dawley rat pups were killed at 1, 7, and 14 days after birth. Adult rats (weight, 200 g) were used as control subjects. Conjunctiva was immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for RNA isolation with TRIzol reagent (Gibco Life Technologies, Grand Island, NY). Anterior segment (including conjunctiva and lids) was fixed immediately after removal in 4% paraformaldehyde in 0.1 M phosphate buffer and embedded in paraffin for in situ hybridization<sup>5</sup> or histochemical staining with periodic acid-Schiff (PAS) reagent.

### Reverse Transcription-Polymerase Chain Reaction of ASGP

To determine ASGP mRNA expression levels during postnatal development of the rat ocular surface, reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described<sup>9</sup> using  $\beta$ -actin as an internal control. Oligonucleotide primers to the nontandem repeat region of ASGP (Table 1) were designed from published sequence and primers for rat  $\beta$ -actin (Table 1) were purchased from Clontech (Palo Alto, CA). The  $\beta$ -actin primer set spans an intron and, thus, serves as a control for genomic contamination. Total RNA (0.5  $\mu\text{g}/\text{sample}$  for 1-, 7-, and 14-day-old rats, and 0.25  $\mu\text{g}/\text{sample}$  for adult rats) was reverse transcribed using random hexamer primers and reverse transcriptase (Superscript II, Gibco). A larger amount of total RNA was used from 1-, 7-, and 14-day-old tissue to optimize the visualization of the final PCR product. The reverse transcription was performed for 90 minutes at  $42^{\circ}\text{C}$ , followed by incubation with 1 U *Escherichia coli* RNase H (Gibco) for 20 minutes at  $37^{\circ}\text{C}$ . An aliquot of the same RT product from each sample (1/20 of the total volume) was used in the PCR amplification reactions for ASGP and  $\beta$ -actin. All

TABLE 1. PCR Primers and In Situ Hybridization Probes

	Size (bp)	Reference
PCR Primers		
$\beta$ -Actin		
5'-TTGTAACCAACTGGGACGATATGG-3' (1552-1575)	764	Nudel et al. <sup>32</sup>
5'-GATCTTGATCTTCATGGTGCTAGG-3' (2991-2844)		
ASGP		
5'-CGTACTAGAGAACTTGACATGC-3' (4761-4783)	638	Wu et al. <sup>13</sup>
5'-GGTAGGAGAACTTGTCATGG-3' (5398-5378)		
rMuc5AC		
(Primer 1) 5'-TATGAGGTGCGACTGCTTTG-3' (724-743)	470	Inatomi et al. <sup>24</sup>
(Primer 2) 5'-AGAACTCGGGTGCAGTAC-3' (1193-1174)		
(Primer CRS) 5'-AGAACTCGGGTGCAGTACAGATGGCGGGGTCGGGGTGGACAGTG-3' (primer CRS = primer 2 followed by base pairs 1071-1054)		
In Situ Hybridization Probes		
ASGP	638	Wu et al. <sup>13</sup>
rMuc5AC	228	Inatomi et al. <sup>24</sup>

PCR amplifications started with denaturation for 5 minutes at 94°C and ended with termination at 72°C for 7 minutes. The parameters for the 27 cycles of amplification were optimized for each gene using sample tissue RT products and are as follows: ASGP: 94°C for 30 seconds, annealing at 57°C for 1 minute, and extension at 72°C for 1 minute;  $\beta$ -actin: 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute. Fifteen microliters of PCR products were analyzed by ethidium bromide staining of a 1.5% agarose gel.

### Competitive RT-PCR of rMuc5AC

Because preliminary data from tissue taken at 1 day of postnatal development showed very little rMuc5AC mRNA, and because we wanted to correlate rMuc5AC expression to goblet cell development, competitive RT-PCR was performed to determine more precisely when levels of expression of this mucin increased. Three oligonucleotide primers to the nontandem repeat region of rMuc5AC (Table 1) were designed from published sequence.<sup>24</sup> Rat stomach RNA was reverse transcribed as described. The rMuc5AC-competitive reference standard (CRS) was then constructed from the stomach RT product by PCR using the rMuc5AC primer 1 as the sense primer and the CRS primer as the antisense primer.<sup>27</sup> This amplification created a PCR product with a 102-bp deletion from the wild-type rMuc5AC product. The purified cDNA was ligated to a vector (pGEM; Promega, Madison, WI) from which the rMuc5AC-CRS RNA can be transcribed using T7 RNA polymerase (Invitrogen, San Diego, CA). Competitive RT-PCR of rMuc5AC was then performed on conjunctival RNA from 1-, 7-, and 14-day-old rats and adult rats. Decreasing amounts of the rMuc5AC-CRS RNA (3.3 amol to 0.033 amol in a 1:3 dilution scheme for 1, 7, and 14 days after birth; 33 amol to 0.33 amol in a 1:3 dilution scheme for an adult rat) was added to a constant amount of rat conjunctival total RNA (0.25  $\mu$ g). These samples were subjected to reverse transcription using random hexamer primers. PCR amplification with the rMuc5AC primers 1 and 2 (Table 1) was performed on 1/20 of the total volume of the RT product from each sample as follows: denaturation at 94°C for 5 minutes, 40 cycles at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with termination at 72°C for 7 minutes. To determine the equivalent concentration point, the PCR products were analyzed by densitometric

analysis of an ethidium bromide-stained, 1.5% agarose gel using a computing densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA) and software (Image Quant ver. 2.0; Molecular Dynamics). The difference in ethidium bromide incorporation between the wild-type and the smaller CRS product was taken into account.<sup>27</sup> The values were plotted as the logarithm of the wild-type band densities to the corrected CRS RNA band densities (Fig. 1C). The point at which the ratio is equal to 1 is the amount of wild-type RNA present.

### In Situ Hybridization of ASGP and rMuc5AC

To demonstrate when and where ASGP and rMuc5AC expression originates developmentally, in situ hybridization was performed using techniques previously described.<sup>5</sup> A cDNA probe to the 3' end of ASGP was produced by RT-PCR using total RNA from rat cervix and ASGP-specific primers (Table 1) designed from published sequence.<sup>13</sup> The resultant PCR product was subcloned into a vector (pGEM-T; Promega) and sequenced using T7 and SP6 promoters to confirm ASGP sequence and determine the direction of the insert. The cDNA probe to

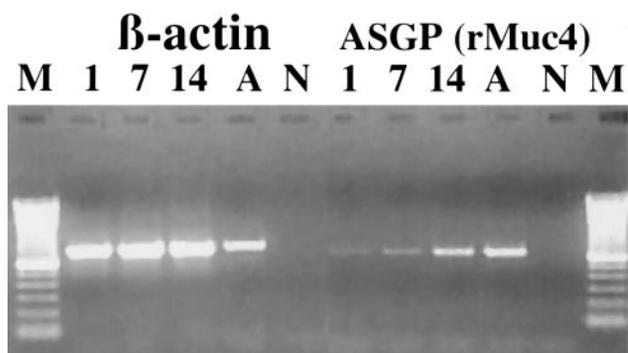


FIGURE 1. Representative example of RT-PCR analysis of ASGP (rMuc4) mRNA in developing rats. ASGP and  $\beta$ -actin, which was used as the housekeeping gene control, were amplified separately by PCR using aliquots of the same RT products. ASGP was detected by RT-PCR by 1 day after birth. The ratio of ASGP to  $\beta$ -actin increased during development with a major increase by day 14. M, 100-bp DNA marker. 1, 7, and 14 indicate days after birth. A, adult rat; N, negative control without cDNA.

rMuc5AC tandem repeat region has been described.<sup>24</sup> Hybridization was performed overnight at 52°C using  $1 \times 10^6$  cpm/ml of <sup>35</sup>S-uridine triphosphate-labeled sense or antisense riboprobe transcribed from plasmid Bluescript SK(-) (Stratagene, La Jolla, CA) using T3 or T7 RNA polymerase (Boehringer Mannheim, Indianapolis, IN) for rMuc5AC and from pGEM-T vector using T7 or SP6 RNA polymerase for ASGP. The sections were counterstained with hematoxylin and eosin.

## RESULTS

Two litters consisting of 9 and 13 rat pups were used for mRNA and in situ hybridization analyses at days 1, 7, and 14 of postnatal development. Eyelids were open at day 14. For in situ hybridization studies of ASGP localization just before eyelid opening, additional pups from a third litter were used. These pups developed more slowly, and in some, eyelids were not yet open at day 15.

At 1 day after birth, the conjunctival epithelium was very thin with only one cell layer. Goblet cells were not obvious in histologic sections of conjunctiva from rats taken 1 day after birth. By 7 days of postnatal development, the conjunctival epithelium was still thin but was composed of two cell layers. The presence of clusters of mature goblet cells was still not obvious; only an occasional cell in the fornix stained with PAS. At 14 days goblet cells were observed, and the conjunctival epithelium was thicker with three to four cell layers. Stratification of the conjunctival epithelium appeared earliest and was most pronounced at the lid margin. In the adult, the number of epithelial cell layers was five to six, and clusters of goblet cells were clearly observed.

### ASGP RT-PCR

Low levels of mRNA for the membrane-spanning mucin ASGP were detected by RT-PCR at 1 and 7 days after birth (Fig. 1). Comparison of the amount of ASGP mRNA with that of  $\beta$ -actin at 1, 7, and 14 days of development showed a major increase in the membrane-spanning mucin gene transcripts by day 14 (Fig. 1). By day 14 of development, the mRNA had not reached adult levels.

### ASGP In Situ Hybridization

In situ hybridization using <sup>35</sup>S-labeled riboprobes that recognize a region in the 3' end of ASGP designated as ASGP-2 resulted in no detectable specific expression of ASGP in the ocular surface at 1 (Figs. 2A, 2B) and 7 (Figs. 2C, 2D) days after birth. The expression of ASGP was clearly first detected at the lid margin where the most stratification of epithelium was seen and along the adjacent palpebral conjunctiva at 14 days after birth. The corneal epithelium did not show label (Fig. 2F). This pattern was seen in rats in which the eyelids were not yet open but appeared to be about to open (Figs. 2E, 2F). In rats in which eyelids were already opened at 14 days after birth, ASGP mRNA was diffusely spread in the apical cell layer of both conjunctival (Figs. 2G, 2H) and corneal epithelia (Figs. 2I, 2J). Although in the 14-day-old rat with open eyelids ASGP mRNA was detectable over the entire ocular surface, mRNA signal was not as obvious as that in adult rat conjunctiva and cornea (Figs. 2K through 2N). The sense sequence of the same region of ASGP, used as a negative control probe, did not show binding

to sections from developing or adult rat ocular surface (Figs. 2O, 2P).

### rMuc5AC Quantitative RT-PCR

To quantitate the amount of rMuc5AC gene expression in developing ocular surface epithelium, competitive RT-PCR was performed with rMuc5AC-CRS. A weak level of expression of rMuc5AC was detected by RT-PCR in ocular surface epithelium in rat pups 1 day after birth (Fig. 3). Quantitative RT-PCR showed a low level of rMuc5AC RNA expression in conjunctiva of 1-, 7-, and 14-day-old rats followed by a large increase in expression between 14 days and adulthood (Fig. 3). At 1 day, the amount of rMuc5AC present was  $5.16 \times 10^{-2}$  femtomoles/ $1 \mu\text{g}$  total RNA. At 7 days, the amount was  $3.62 \times 10^{-1}$ ; at 14 days it was  $6.16 \times 10^{-1}$ ; and the adult's was 13.6 femtomoles/ $\mu\text{g}$  total RNA.

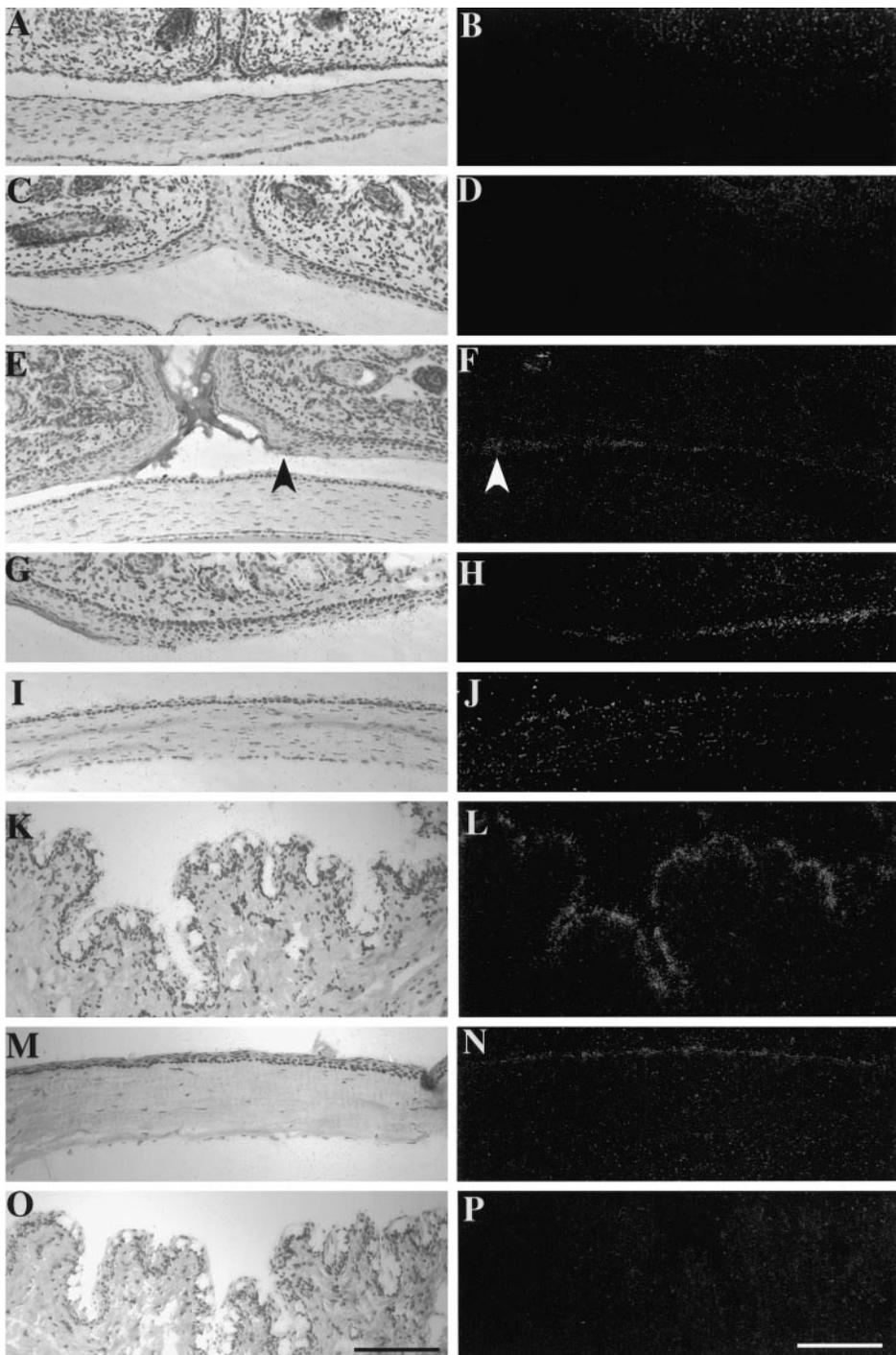
### rMuc5AC In Situ Hybridization and PAS Staining of Goblet Cells

rMuc5AC expression was not detected by in situ hybridization using <sup>35</sup>S-labeled riboprobes for the tandem repeat region in the ocular surface at 1 day after birth (Figs. 4A, 4B). First detectable expression was found in a few cells at 7 days after birth (Figs. 4C, 4D). One or two labeled cells were present in the fornical area. In some eyes, these early goblet cells were on the palpebral side of the fornix (Figs. 4C, 4D), and in others, they were present on the bulbar side (data not shown). They were not clustered in the angle of the fornix. This localization correlated with first appearance of PAS staining at 7 days. Only one or two cells in the fornical region stained with PAS (data not shown). By 14 days many goblet cells showed rMuc5AC expression not only in the fornix, but in palpebral (data not shown) and bulbar conjunctiva as well (Figs. 4E, 4F). This labeling pattern correlated with PAS staining pattern (data not shown). In the adult, binding of the rMuc5AC was present along all parts of conjunctiva, and the signal was very intense (Figs. 4G, 4H). The sense sequence of the same region of rMuc5AC, used as a negative control probe, did not show any binding to sections from adult or developing tissues (Figs. 4I, 4J).

## DISCUSSION

A major conclusion of this study is that the developmental expression of the membrane-spanning mucin ASGP (rMuc4) and the gel-forming mucin rMuc5AC were regionally and temporally separated. Expression of membrane-spanning mucin began in the stratified epithelium at the eyelid margin just before the eyelids opened at days 14 to 15, and expression of the gel-forming mucin began at the fornix at 7 days after birth. Although expression of both mucins could be detected by RT-PCR at 1 day after birth, they were insufficiently expressed to be detectable by in situ hybridization. These data suggest that the membrane-spanning mucin is primarily a differentiation product of the stratified epithelia, whereas the gel-forming mucin is a differentiation product of the goblet cell, and that goblet cell differentiation appears before differentiation of the stratified epithelia.

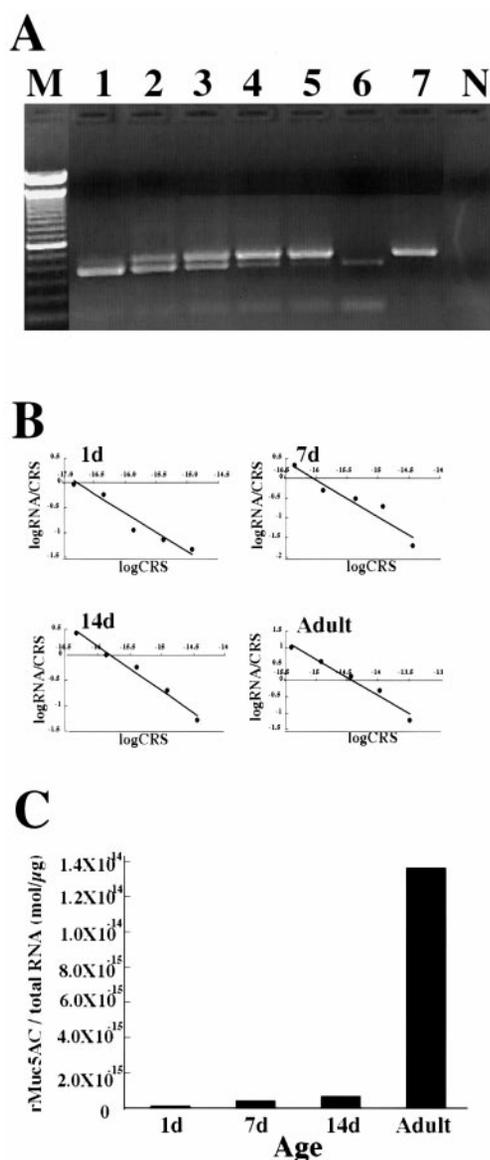
A second major finding of this study is that, unlike humans, rodents expressed ASGP (rMuc4) in the corneal



**FIGURE 2.** Localization of ASGP (rMuc4) mRNA in developing and adult ocular surface tissues by in situ hybridization using  $^{35}\text{S}$ -labeled riboprobes for the 3' end region of ASGP. Sections of rat ocular surface tissue hybridized with antisense probe (A through N) and sense probe as control (O, P). (A) and (B) show 1-day-old rat tissue by bright and dark field microscopy, respectively. No ASGP expression was detected in either conjunctival or corneal epithelium. (C) and (D) show 7-day-old tissue by bright and dark field, respectively. No ASGP expression was detected. (E) and (F) show 14-day-old rat eye taken just before eyelid opening by bright and dark field, respectively. ASGP expression was detected in the conjunctival epithelium at the lid margin where the most stratification of the epithelium was seen and along the palpebral conjunctival epithelium. *Arrows* in micrographs (E) and (F) show same position along lid. (G, H) Sections of 14-day-old ocular surface lid margin and palpebral conjunctiva after eyelid opening by bright and dark field, respectively. Expression of ASGP was present along the entire conjunctival epithelium. (I) and (J) are from the same 14-day-old eye shown in (G) and (H). These micrographs show spotty signal all along the apical corneal epithelium of the newly opened eye. (K, L) Sections of adult conjunctiva by bright and dark field, respectively. The signal was very strong in the apical layer of ocular surface. (M, N) ASGP mRNA localization in adult cornea by bright and dark field, respectively. ASGP mRNA was detected in the apical layers of the corneal epithelium. (O, P) Labeling with sense probe, which was used as control on sections of adult conjunctiva by bright and dark field, respectively. Binding of the sense probe was not detected. All micrographs are of the same magnification; bar, 50  $\mu\text{m}$ .

epithelium. Recently, the 3' end of human MUC4 was partially sequenced, and a comparison of the 3' end of MUC4 to the 3' end of the rat ASGP shows high homology (80%).<sup>11</sup> We previously reported that the human conjunctival epithelium shows positive binding of the  $^{35}\text{S}$ -labeled and DIG-labeled oligoprobes to the tandem repeats of MUC4.<sup>5</sup> The expression of MUC4 was detected in all layers of the conjunctival epithelium but was not detected in human limbal or corneal epithelium.<sup>5</sup> In rats, however, the expression of ASGP was detected in the apical layers of both conjunctival epithelium and corneal epithelium. Price-Schiavi et al.<sup>17</sup> have recently reported that ASGP-2 protein was present in

abundance in rat corneal tissue and in conjunctiva. Antibodies to ASGP showed binding in corneal samples and more faintly in conjunctival samples by immunoblot analysis using a monoclonal antibody that recognizes the transmembrane subunit ASGP-2. (This is the same region from which our cDNA probe was made.) They reported that ASGP (sialyl mucin complex) was localized in the apical epithelial layers in cornea and conjunctiva. Our in situ hybridization pattern of rat ASGP mRNA correlates to this antibody-binding pattern. Although, MUC4 and ASGP have a high homology at their 3' domains, their function in the ocular surface may differ between human and rat. It is tempting to correlate the



**FIGURE 3.** Quantification of rMuc5AC mRNA in developing and adult ocular surface tissue by competitive RT-PCR. (A) A representative example of rMuc5AC competitive RT-PCR using adult rat conjunctiva. Three-fold serial dilutions of CRS, starting with 33 femtomoles at *lane 1*, were titrated (*lane 2* through *lane 5*) against a constant amount of total RNA (0.25 μg) and amplified as described in the Methods section. Starting amounts varied with stage of development. The upper bands are the wild-type PCR products, and the lower bands are CRS PCR products. Wild-type rMuc5AC and CRS PCR products are 470 bp and 368 bp, respectively. M, 100 bp DNA marker, *lane 6*; PCR product of 10 femtomoles CRS without mRNA, *lane 7*; PCR product of rat stomach mRNA without CRS. N, negative control. (B) Plots of quantitative data obtained after the PCR. The gel was photographed and subjected to scanning densitometry. The values of 1-, 7-, and 14-day-old rats and adults were plotted as the logarithm of the wild-type band densities to the corrected CRS RNA band densities (see the Methods section for details of analysis). (C) shows values for rMuc5AC obtained in (B) for 1-, 7-, and 14-day-old and adult tissues. A large increase in rMuc5AC expression occurred between tissue obtained 14 days after birth and adult tissues.

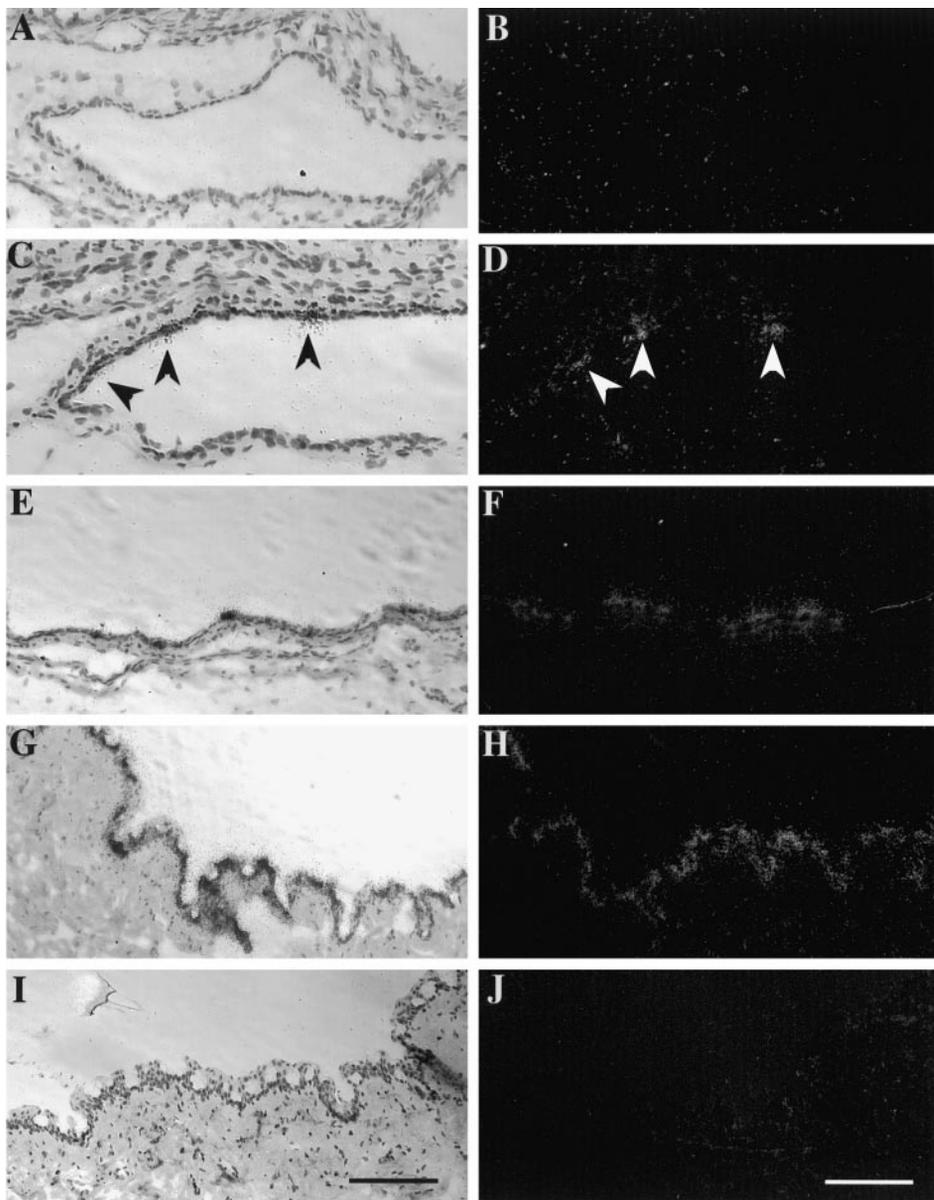
wider ocular surface distribution of expression of rMuc4 in rodents to the increased stability of the tear film in rodents and to their concomitant low blink rate compared with that of humans.<sup>28</sup>

A third finding of this study was the correlation of initial expression of ASGP detectable by in situ hybridization, with eyelid opening. We previously developed a monoclonal antibody, rat ocular surface glycocalyx antibody (R339), that binds to the apical squamous cells of the ocular surface epithelium of the rat.<sup>29,30</sup> This antibody recognizes a sugar epitope on a high-molecular-weight, highly glycosylated glycoprotein found in the ocular surface glycocalyx. We have reported that binding of the R339 antibody was prominent at the tip of micro-plicae where the mucus layer is prominent. Interestingly, just as with ASGP, the developmental expression of the R339 antigen coincides with the eyelid opening, with initial binding occurring in the stratified epithelium at the eyelid margin. This study shows that the R339 binding pattern was localized only in the palpebral conjunctiva near the lid margin at days 5 to 7 after birth and rapidly extended to the cornea after eyelid opening.<sup>31</sup> Artificial opening of the lids induces R339 expression. The similarity of developmental expression between ASGP and R339 suggests that the epitope recognized by the R339 is perhaps a sugar epitope on ASGP. We previously hypothesized that R339 antigen is required for mucus spread. In this study, we show that ASGP may be correlated to R339 expression, and because these differentiated products appear after rMuc5AC and at eyelid opening, they may play a key role in facilitating the spread of gel-forming mucin after eyelid opening.

Our in situ hybridization data showing that the goblet cell-specific mucin is first detectable in the fornical region of the developing rat supports the hypothesis of Wei et al.<sup>26</sup> that stem cells that give rise to goblet cells reside in the fornix. Cell kinetic investigations using incorporation of tritiated thymidine into the conjunctival epithelium have shown that the fornical zone is a site enriched by slow-cycling, label-retaining cells in neonatal and adult SENCAR mice,<sup>25</sup> leading to the hypothesis of Wei et al. that the conjunctival epithelial stem cells are located in the fornical region. In addition to the fact that our rMuc5AC in situ hybridization data support their hypothesis, the data show that differentiation of goblet cells occurred before differentiation of the apical cells of the stratified epithelium. If ASGP is the gene product of differentiated stratified cells, the appearance of this differentiation product later than rMuc5AC and its presence initially at the lid margin may suggest a different inducer of differentiation of the stratified epithelial cells than that of goblet cells.

The sensitivity of quantitative RT-PCR allowed the detection of transcripts for the goblet cell marker rMuc5AC at 1 day after birth, but these cells were not detected by in situ hybridization. Quantitative RT-PCR using an RNA CRS is a very sensitive technique that allows precise quantitation of gene expression in very few cells (100–1000).<sup>27</sup> Quantitative RT-PCR has the advantage of the ability to quantitate RNA more accurately than quantitative PCR,<sup>27</sup> because it includes an internal standard at the beginning of the RT reaction and thus can avoid a difference of RT reaction efficiency. Quantitative RT-PCR could be used to quantitate even low levels of rMuc5AC expression. Because this is a more highly sensitive method than in situ hybridization, very low transcript levels can be detected that initiate before overt differentiation of cells. Use of these methods may facilitate studies of effectors of mucin gene expression.

In summary, we examined the expression of mucin genes ASGP (rMuc4) and rMuc5AC in developing rat ocular surface



**FIGURE 4.** Localization of rMuc5AC in developing and adult conjunctiva by in situ hybridization using  $^{35}\text{S}$ -labeled riboprobes for tandem repeat region of rMuc5AC. Rat conjunctival epithelium hybridized with antisense probe (A through H) and sense probe as control (I, J). (A, B) show sections from 1-day-old eyes by bright and dark field microscopy, respectively. (C, D) show sections from 7-day-old eyes by bright and dark field, respectively. A couple of cells showing rMuc5AC expression were detected in the fornix area (*arrowheads*). (E, F) Fourteen-day-old conjunctiva by bright and dark field, respectively. All goblet cells showed rMuc5AC expression, not only in the fornix, but in palpebral and bulbar conjunctiva as well. (G, H) Adult conjunctiva by bright field, respectively. All goblet cells showed rMuc5AC expression, not only in the fornix, but in palpebral and bulbar conjunctiva as well. (G, H) Adult conjunctiva by bright field, respectively. Many goblet cells expressing rMuc5AC were present, along with whole parts of conjunctiva, and the binding of probe was very strong. (I) and (J) show labeling with sense probe used as control in adult conjunctiva by bright and dark field, respectively. No binding was detected for all in situ runs when sense control was used. All micrographs are of the same magnification; bar, 50  $\mu\text{m}$ .

epithelia. By in situ hybridization, expression of the membrane-spanning mucin ASGP (rMuc4) was determined to begin at an eyelid margin just before eyelid opening, and expression of the gel-forming mucin rMuc5AC began earlier at the fornix at 7 days of postnatal development. These data indicate that the membrane-spanning and gel-forming mucins have independent, temporal, and regional patterns of development. The correlation of expression of the membrane-spanning mucin rMuc4 with eyelid opening suggests that it has a role in spread of the gel-forming mucin at the time of initial need of a stable tear film.

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