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In-situ hybridization localized MUC7 mucin gene expression to the mucous acinar cells of human and MUC7-transgenic mouse salivary glands

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Abbreviations: Mucin glycoprotein 2 (MG2); *in-situ* hybridization (ISH); tyramide signal amplification (TSA); immunohistochemistry (IHC); human mucin genes 1–8 (MUC1–8); variable number of tandem repeats (VNTR); polymerase chain reaction (PCR); diamino benzidine (DAB); hematoxylin-eosin (H&E); streptavidin-horse redish peroxidase (SA-HRP); monoclonal antibody (MAb).

MUC7 gene that encodes the low molecular weight human salivary mucin MG2, was previously shown to be expressed in tissue-specific manner in normal human salivary glands and in salivary glands of transgenic mice carrying the MUC7 transgene. The purpose of this study was to examine the cell specificity of MUC7 expression in human and transgenic mice salivary glands. To localize the MUC7 transcripts, we used *in-situ* hybridization in combination with Tyramide Signal Amplification procedure. The results clearly showed that in both the human and transgenic mice salivary gland tissue sections, MUC7 transcripts were localized only in mucous acinar cells; no signals were found in serous acinar cells or any other cell types present in these salivary glands.

Introduction

Mucin glycoprotein 2 (MG2) is a low molecular mass (120–150 kDa) human salivary mucin. Biochemical studies have shown that MG2 is composed of about 30% protein and 70% carbohydrate and its peptide moiety is a single polypeptide chain [1,2]. MG2 has been shown to bind to a variety of oral [3–6] and respiratory [7–9] microorganisms. To date, at least nine mucin genes designated MUC1–4, MUC5AC, 5B and MUC 6–8 have been identified in various human tissues. Interesting feature of most of these mucins is the common central region which consists of repetitive arrays of amino acid sequences, called tandem repeats, flanked on either side by non-repetitive sequences. MG2 contains 6 tandem repeats of 23 amino acid residues. These tandem repeats which are rich in serine and threonine residues are potential sites for O-glycosylation [10].

MG2 protein core is encoded by MUC7 gene. As shown by Northern blot analyses, MUC7 mRNA is about 2.5 kb long and its expression appears to be tissue specific and confined to salivary glands [11,12] and trachea [13]. The MUC7 gene spans 10 kbp of the human genomic DNA and is comprised of three exons and two introns [14]. Its chromosomal localization has been assigned to chromosome 4q13–q21. The entire region encoding the secreted peptide of 357 amino acids residues with a molecular mass of 37 kDa is located on exon 3 [14]. Limited variable number of tandem repeats (VNTR) polymorphism determined by polymerase chain reaction (PCR) and Southern blot analysis have also been reported [13]. In addition to MUC7, MUC1 and MUC4 have been shown to be expressed in human submandibular gland by RT-PCR [13]; and MUC5B by *in-situ* hybridization (ISH) and immunohistochemistry (IHC) [15,16]. Tissue-specific expression of MUC7 gene was also seen in transgenic mice with a 16 kbp MUC7 transgene containing all MUC7 exon and intron sequences (10 kbp) and 3 kbp of the upstream and 3 kbp of the downstream sequences. By northern blot analyses MUC7 transcripts were shown in the sublingual glands of both female and male transgenic mice and in the submandibular glands of female mice [17].

One of the most interesting features of the expression of mucin genes is their degree of tissue and cell specificity

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[18]. While MUC1 and MUC4 appear to be expressed by several tissues, MUC2, MUC3 and MUC7 display a more confined pattern of tissue-specific expression, although the cell specificity of MUC7 expression was controversial. Originally, IHC studies using monoclonal antibody 1/F-9 localized MG2 to mucous acinar cells of normal human salivary glands [19,20]. Recently, using another monoclonal antibody, PANH3, MG2 has been reported to be localized only in a sub-population of serous acinar cells [21]. Serous cells and demilunes but not mucous cells also hybridized with MUC7 RNA probe in ISH assays using salivary tissue sections, indicating that MG2 was synthesized in serous cells [15]. However, these findings do not explain, why MUC7 was not expressed in parotid gland which mainly consists of serous cells. In the present study we are reporting the cell localization of MUC7 transcripts in normal human salivary glands and salivary glands of transgenic mice carrying the MUC7 transgene. The localization was performed by ISH in combination with chromogenic Tyramide Signal Amplification (TSA) procedure which significantly enhances chromogenic detection of the biotin labeled riboprobes. TSA procedure uses conjugates of streptavidin-horseradish peroxidase (SA-HRP) in order to bind to biotin labeled riboprobes, which are amplified by the deposition of numerous biotin labeled tyramides onto tissue sections. Amplified signals are then detected by the addition of SA-HRP followed by horseradish peroxidase catalyzed chromogen, diaminobenzidine (DAB).

Materials and methods

The following materials were obtained from the indicated sources: Wizard plus minipreps DNA purification kit (Promega, Madison, WI); RNA biotin labeling kit, and Renaissance TSA-indirect chromogenic kit (NEN Life Sciences, Boston, MA); ProbeQuant G-50 micro columns (Pharmacia Biotech, Piscataway, NJ); Diaminobenzidine (DAB) peroxidase substrate system (Amresco, Solon, OH); Hematoxylin (Polyscientific, Bay Shore, NY); Immunon permanent mounting medium (Shandon/Lipshaw, Pittsburgh, PA); Staining slides, dishes, and other reagents (Sigma, St. Louis, MO or Fisher Scientific, Pittsburgh, PA).

Tissue fixation, embedding and sectioning

Human salivary gland specimens (submandibular, minor and parotid glands) as well as other tissue samples (such as adjacent lymphoid tissue and liver to serve as negative tissue controls) were received through the tissue procurement facility of the Department of Pathology at Roswell Park Cancer Institute (RPCI), Buffalo, NY. Written consent of the donors and pathologist was obtained according to the IRB. The tissues were immediately fixed in 10% neutral buffered formalin for 24 hours, and embedded in paraffin for conventional processing. Five micron sections were cut and mounted on aminoalkylsilane precoated slides. Mice salivary glands (submandibular/sublingual complex) were obtained from the female and male MUC7-positive and negative mice, and processed as described above in order to mount the tissue sections on slides. Normal histological structures of the salivary glands were confirmed by light microscopic examination of the hematoxylin-eosin (H&E) stained slides.

Preparation of riboprobe

PCRII plasmid vector with T7 and SP6 RNA polymerase promoter sites containing 433 bp of the 5′ end portion of the MUC7 cDNA (designated MG2-5-5), of which first 96 nucleotides are untranslated [12], was used as a template for preparation of riboprobes. Plasmid DNA, purified using Promega's Wizard Plus miniprep kit, was first linearized to produce run-off transcripts derived from the entire insert sequences; for generation of the antisense probe, with *Sac* I, and the sense probe, with *Xba* I. Digestion was confirmed by agarose gel electrophoresis and RNA transcripts were then synthesized using biotin labeled nucleotides and T7 or SP6 RNA polymerase according to NEN's protocol. Biotin labeled transcripts were further purified by ProbeQuant G-50 spin column. Size (~430 bases) and purity of RNA transcripts were confirmed by 1% agarose gel electrophoresis.

Hybridization of RNA probe on human and transgenic mice salivary gland tissues

Prior to hybridization, tissue sections were deparafinized and rehydrated in xylene and ethanol of decreasing concentration according to established procedures. In order to make cellular RNA more accessible to antisense RNA probe, tissue sections were treated with 0.2 N HCl for 5 minutes at room temperature (RT); washed with RNase free (RF) water for 5 minutes; digested with pronase or proteinase K (100 µg/ml) for 10 minutes at RT; and washed in RF water for 10 minutes. In order to reduce the background level, positively charged free amino groups that can bind to probe non specifically, were neutralized by immersing the tissue sections in 0.1 M triethanol-amine-HCl (pH 8.0) and by adding acetic anhydride while stirring to a concentration of 0.25% (V/V). After washing with RF water, sections were dehydrated in ethanol of increasing concentrations for hybridization with the biotin labeled RNA probe.

Hybridization was performed by incubating the sections with 100 µl of hybridization mixture from a 10 ml stock solution which contained: 50% deionized formamide/ 1X Denhardt's solution/ 1X SSC/ 1 mM EDTA/ 10 mM phosphate buffer/ 1 mg salmon sperm DNA/ 1 mg yeast tRNA/ 1 g dextran sulfate. Biotin labeled antisense (for target mRNA) and sense (control) probes (1 µg/ml) were added just prior to hybridization. For other controls, only the hy-

bridization mixture without any probe was used. Sections were incubated at 45 °C in a humidified flat container for overnight. After hybridization, the sections were rinsed and washed with 2X SSC for 20 minutes at RT. Post hybridization wash was done at 50% formamide/ 2XSSC at 50 °C for 10 minutes. Non-specific binding of RNA probe was also removed by incubating the sections with the above solution containing 10 mg/ml RNase at 37 °C for 10 minutes. Sections were washed 3 times in RF water, and further processed for TSA procedure.

Amplification of MUC7 transcripts

TSA procedure was followed according to NEN's protocol provided along with TSA-Indirect kit. Briefly, sections were washed 3 times for 5 minutes in TNT wash buffer (0.1 M Tris-HCl, pH 7.5/0.15 M NaCl/0.05% Tween 20). Sections were first incubated with TNB blocking buffer (0.1 M Tris-HCl, pH 7.5/0.15 M NaCl/0.5% Blocking reagent, supplied in the kit) for 30 minutes at 37 °C, and then with streptavidin-horse redish peroxidase (SA-HRP) for another 30 minutes at RT. Sections were washed 3 times in TNT buffer for 5 minutes at RT, and incubated with biotinyl tyramide for 7 minutes at RT. After washing with the TNT buffer 3 times for 5 minutes at RT, sections were again incubated with SA-HRP for 30 minutes at 37 °C. Sections were washed in TNT buffer 3 times for 5 minutes at RT, and then in water. For TSA control, tissue sections after hybridization were treated only with SA-HRP and no biotinyl tyramide.

Chromogenic detection and counter staining for visualization

For the chromogenic detection, sections were incubated with 0.5% DAB solution (prepared according to Amresco's instruction) for 10 minutes at RT in dark. After the substrate deposition, sections were washed with water, and counter stained by hematoxylin. Sections were mounted with imunon permanent mounting medium.

Cell localization of MUC7 transcripts was accomplished by comparing the *in-situ* hybridized slides with their parallel H&E stained slides examined under a light microscope. Photographs were taken with a video camera interfaced to it.

Results

Human samples

Tissue sections from 3 individuals in 3 different experiments were examined and gave the identical results. The results of the representative samples are shown in Fig. 1 and Fig. 2. Human liver sections, treated with the same probe simultaneously and under identical conditions, were used as negative control. As revealed in Fig. 1B, no MUC7 transcripts were found in any cell type of liver sections. Fig. 1A shows an H&E stained section of submandibular gland and points out the mucous acinar cells (red arrow), serous acinar cells (black arrow) and serous demilunes (green arrow). As shown in Fig. 1C, only mucous acinar cells of submandibular gland were positive for the MUC7 transcripts, while no such transcripts were observed in the serous demilunes and serous acini. The submandibular gland contains approximately 10–20% mucous acinar cells and, as shown in figure 1D, most of them, if not all, show the MUC7 transcripts. Fig. 2A shows the minor salivary gland with the adjacent lymphoid tissue (which actually served as an internal control). As revealed in this figure, MUC7 signals are present in mucous cells of the minor salivary gland, where as the lymphoid tissue is completely negative. Fig. 2B shows, at higher magnification, MUC7 signals in the cytoplasm of mucous acini of minor salivary gland. Parotid gland mainly consists of serous cells, however, in rare situations, very few mucous secreting cells and serous demilunes can be found. Fig. 2C shows the scarcity of these cells in one of the parotid gland sections. Interestingly, all these mucous cells showed positive signals with the MUC7 probe, while the majority cells, which were identified at higher magnification as serous in nature, were all negative. Fig. 2D shows, at higher magnification, those rare mucous acinar cells of parotid gland with MUC7 transcripts. This observation is in agreement with our previous finding, where expression of MUC7 was detected by RT-PCR in parotid gland (unpublished results). Parallel controls as described in material and methods showed no transcripts (not shown).

Collectively, our results show that in the salivary glands examined, the MUC7 transcripts were found only in mucous acinar cells. Further, the quantity of MUC7 transcripts can be correlated with the proportion of the mucous and serous acinar cell content in these salivary glands. Submandibular gland, which contains significant number of mucous cells, showed moderate number of the signals; minor salivary gland, containing purely mucous cells, showed highest number of signals; and parotid gland, with rare mucous cells, showed the least number of signals.

Transgenic mice samples

Tissue sections from 2 female and 2 male MUC7-positive and negative mice were analyzed. The results of the representative samples are shown in Fig. 3. Fig. 3A and 3C, respectively, show at low magnification, the *in-situ* hybridization results with the submandibular-sublingual gland complex of female and male transgenic mice. As revealed in these figures, significant number of MUC7 transcripts were localized only in the sublingual portion of the gland complex. Fig. 3 B and 3D show the sublingual portions of the same gland complexes with intercalated ducts at higher magnification. MUC7 signals were found only in the mu-

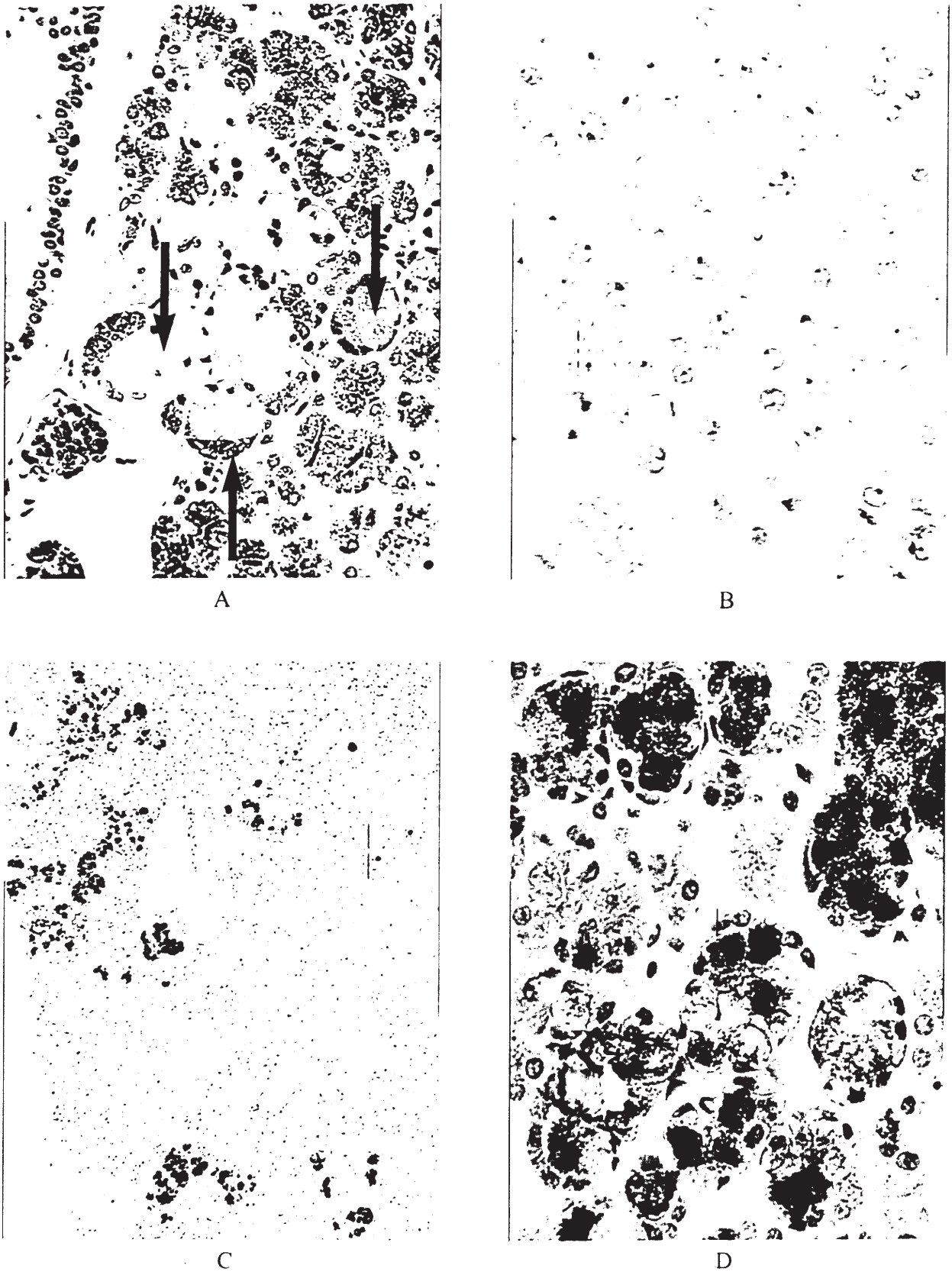


Figure 1. (A) Hematoxylin and eosin stain, and (B–D)*In-situ* **hybridization of human tissue sections counterstained with hematoxylin. A:** *Submandibular gland (400X);* mucous acinar cells (red arrow), serous acinar cells (black arrow) and serous demilunes (green arrow). **B:** *Liver (400X);* negative control tissue showing cords of hepatocytes and hepatic sinusoids with no MUC7 transcripts. **C:** *Submandibular gland (65X);* showing approximately 20% of the mucous acinar cells compared to 80% of the serous cells. Most of the 20% of these mucous cells show MUC7 transcripts. No MUC7 transcripts are found in serous cells. **D:** *Submandibular gland (400X);* showing an intense MUC7 signal in the mucous acinar cells of the secretory end pieces. The serous demilunes and the serous acini failed to show MUC7 transcripts.

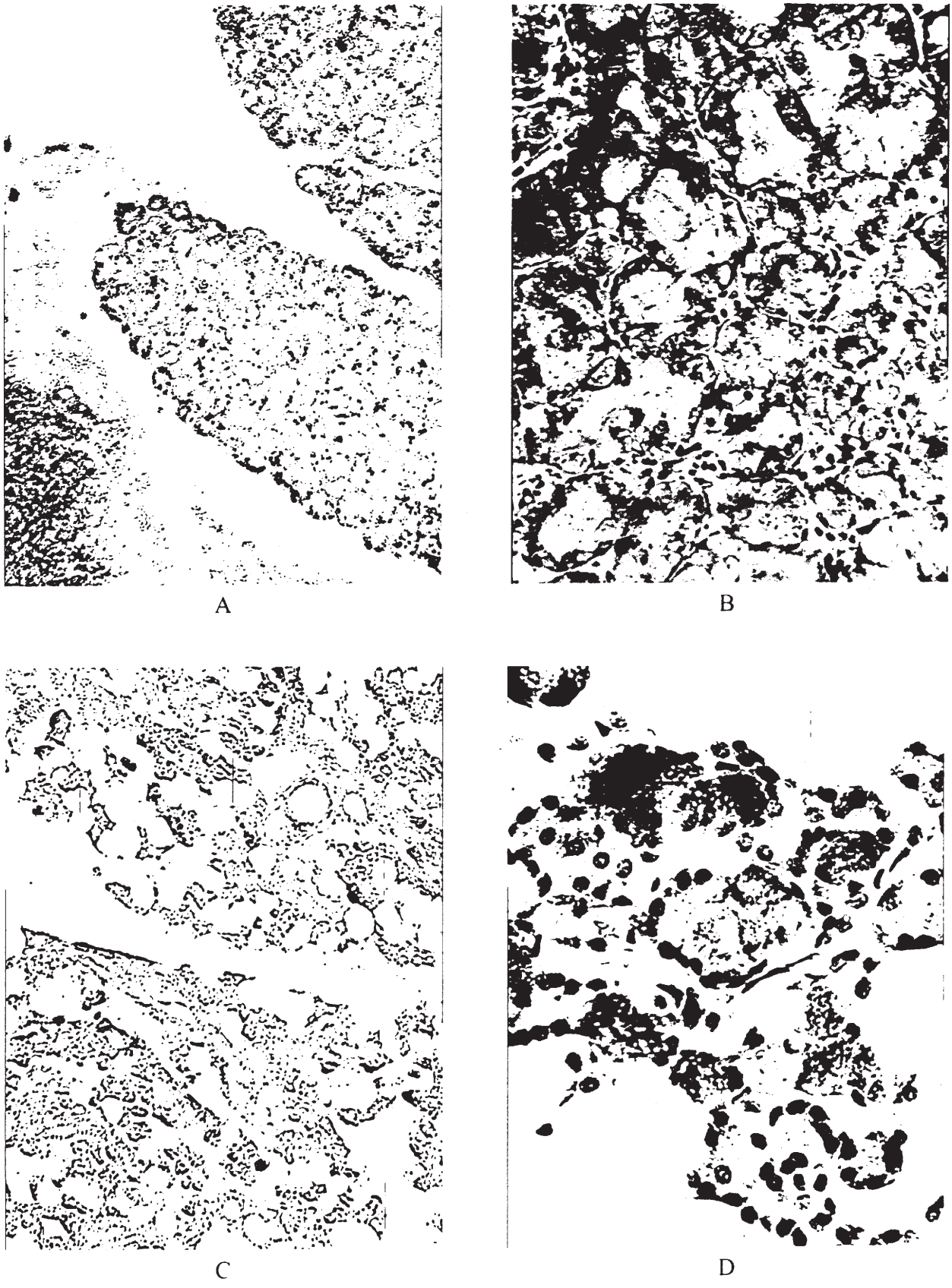
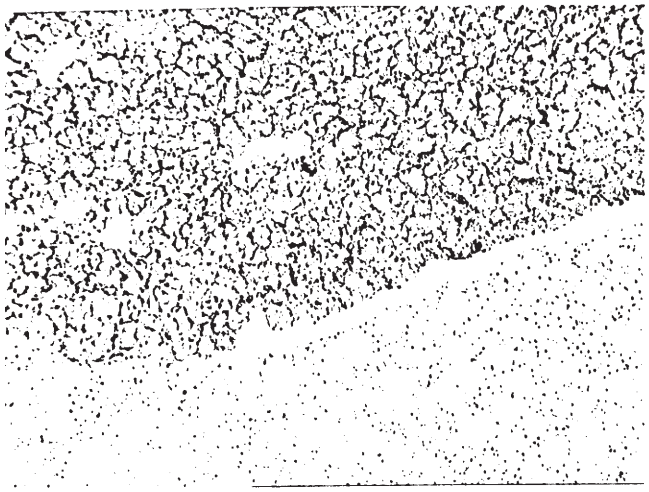


Figure 2. *In-situ* **hybridization of human salivary gland sections counterstained with hematoxylin. A:** *Mucous minor salivary gland (65X)* with adjacent lymphoid tissue. Intense MUC7 signals can be seen in all the mucous cells of salivary gland, whereas cells in the lymphoid tissue show no signals. **B:** *Mucous minor salivary gland (400X)* showing intense MUC7 signals in the cytoplasm of mucous acini. **C:** *Parotid gland (65X)* showing rare presence of serous demilunes and mucous secreting cells. This figure shows an intense signal only in mucous cells. **D:** *Parotid gland (400X)* showing mucous cells with intense MUC7 signals.



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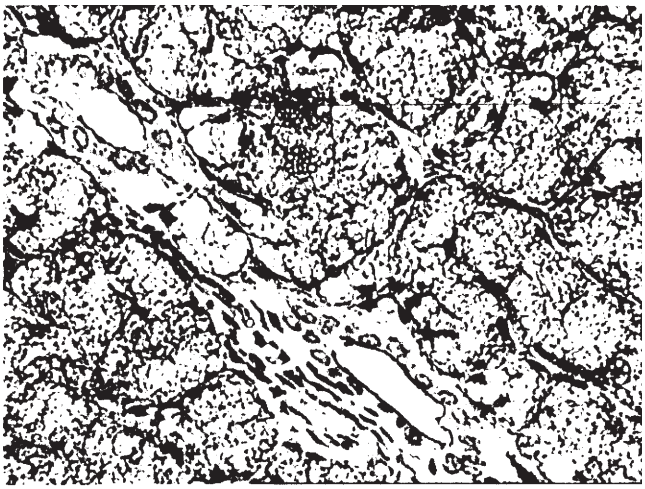

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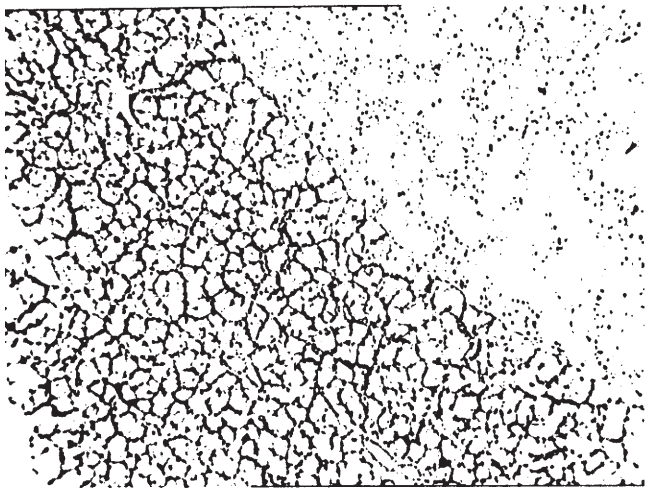

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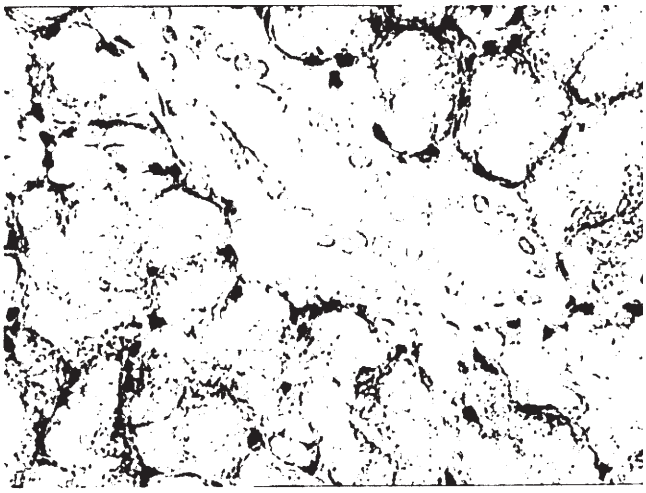

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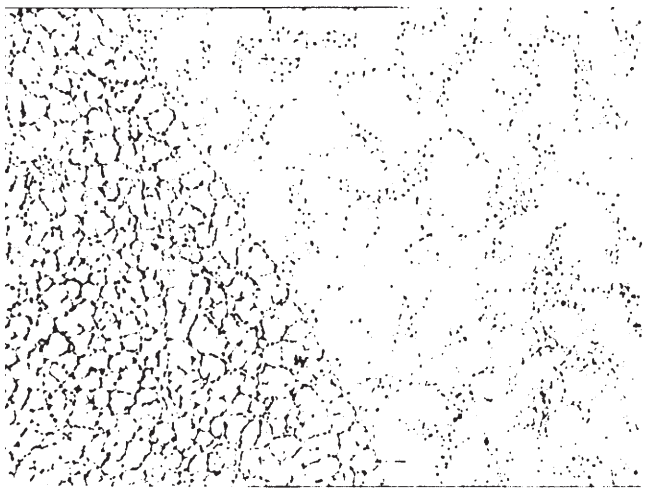

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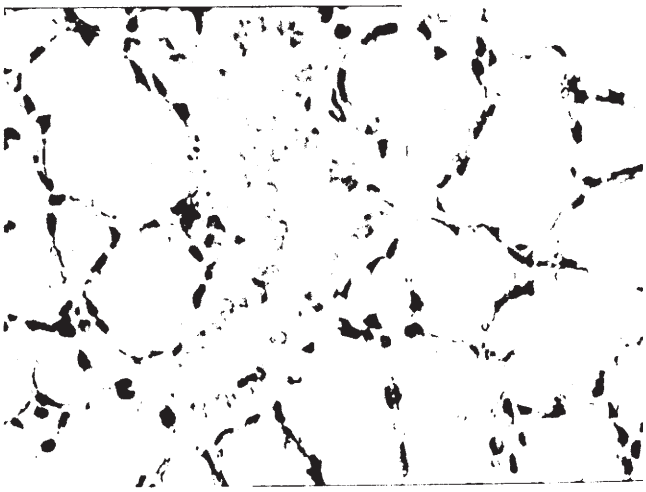

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cous acinar cells; no transcripts were found in the intercalated ducts, signifying the specific localization of MUC7 transcripts. Fig. 3E and 3F show the results with the non-transgenic mice salivary gland tissue sections. No MUC7 transcripts were found either in sublingual or submandibular portions of these glands. Other parallel controls with no probe revealed no transcripts (not shown).

Discussion

Human samples

Present finding are in agreement with one of the previous reports by Cohen et al. [19,20] where MG2 as well as MG1 glycoproteins were shown to be localized in mucous acinar cells of normal human salivary glands by IHC. However, these findings are not in agreement with the results of Nielsen et al. [15,21] who showed the presence of MG2 glycoprotein and of MUC7 transcripts in a sub-population of serous cells. The authors of the latter reports have put much emphasis on the differential expression of human salivary mucins which was based on the cell specificity, however, they could not explain the absence of MG2 glycoprotein and MUC7 transcripts in serous enriched parotid gland. Localization of MG2 glycoprotein and of MUC7 transcripts either in serous or mucous acinar cells could be explained by virtue of the specificity and sensitivity of the probes and antibodies used in those assays. Specifically, in case of MUC7 transcripts localization, the discrepancy may be related to the length of the probe, the method by which the probe was labeled, and to what section of the gene the probe represents. As described in Materials and Methods, we have prepared run-off transcripts of ~430 bases in length, derived from the 5′ portion of the non-repetitive region of the MUC7 cDNA. On the other hand, Nielsen et al. [15,21] used 30-mer antisense alkaline phosphatase-conjugated DNA oligonucleotide probe complementary to the tandem repeats of MUC7 transcripts. Smaller probes require different hybridization conditions, and moreover, raise a possibility of non-specificity and lower sensitivity [22]. In case of MG2 glycoprotein localization, monoclonal antibody (MAb) 1/F-9 used for MG2 localization by Cohen et al. [19] was shown to bind to MG2 of ~120 kDa in Western blot analysis of human saliva, while Mab PANH3 used for MG2 localization by Nielsen et al. [21] that was raised against a synthetic peptide derived from the MG2 (MUC7) sequence, was shown to bind in Western blot analysis of concentrated saliva to native molecule of ca. 200–210 kDa.

ISH has been used for examining the expression of other mucin genes. For example, one study examined the level of the expression and the cell localization of MUC2–4, 5B & 5AC in human normal respiratory, digestive and reproductive tracts. The results of that study enabled the investigators to define a specific pattern of mucin gene expression for each tissue, thus describing the "normal" status of these mucin gene expression [16]. Another study used ISH to examine the developmental expression of mucin genes MUC3-8 in human fetal gastrointestinal system to establish the localization and age of onset of expression of each mucin gene during human development [23]. An intriguing point is whether the specificity of mucin gene expression is maintained in different pathological conditions, and what prognostic implications it may have to manage certain diseases including cancer. Interestingly, it is suggested that quantitative and qualitative alterations of mucins (both at the level and pattern of mRNA and protein expression) are associated with malignant transformation, tumor progression and metastasis [10 and references within]. Whether these changes are due to cellular differentiation in normal development and/or in tumors is still unclear, however, examining a cell specific expression of mucin genes and defining their status in normal tissues may possibly lead to the understanding of neoplastic transformation of a normal tissue. To study the possible cell specificity and to define the status of MUC7 expression in normal human salivary glands, in the present report we used ISH/TSA procedure. Our results clearly localized MUC7 transcripts only in mucous acinar cells of human normal salivary glands. Our future studies will be directed toward determining any quantitative or qualitative alterations of MUC7 as well as of other mucin gene expression in a variety of salivary gland tumors. To date such studies have not been done and may have prognostic significance.

Transgenic mice samples

Present results complement our recent report where expression of MUC7 gene (16 kbp fragment, containing all MUC7 exon and intron sequences (10 kbp) and 3 kbp of the upstream and 3 kbp of the downstream sequences) in transgenic mice was shown to be tissue-specific by northern blot analysis [17]. One of the transgenic lines showed high levels of MUC7 transgene transcripts in the sublingual

Figure 3. *In-situ* **hybridization of transgenic and non-transgenic mice salivary gland tissue sections counterstained with hematoxylin. A and C:** *Female and male transgenic mice (carrying MUC7 gene) sublingual/ submandibular gland complexes (65X).* MUC7 RNA transcripts are shown as specifically localized in the sublingual gland. Adjacent submandibular glands show no such transcripts. **B and D:** *Female and male transgenic mice sublingual glands (400X).* MUC7 RNA transcripts are localized in mucous cells. No transcripts are found in intralobular ducts of sublingual glands. **E:** *Male control mouse (carrying no MUC7 gene) sublingual/submandibular gland complex (65X).* No MUC7 RNA transcripts can be seen in either sublingual or submandibular gland of the MUC7 negative mouse at low magnification. **F:** *Male control mouse (carrying no MUC7 gene) sublingual gland (400x).* Mucous cells of control mouse at high magnification show no MUC7 RNA transcripts.

glands of both males and females and indicated low levels of MUC7 transcripts in the submandibular glands of females. At that time, we could only suggest the possible reasons for the observed differences in the expression of MUC7 in male and female mice. One of these pointed out to the possibility of the imperfect separation of submandibular glands from the sublingual glands in female mice, thus contaminating the submandibular gland RNA with the sublingual gland RNA. The present study, using ISH to salivary gland tissue sections, and the probe from the same region of MUC7 cDNA as in ref. 17 resolved this discrepancy conclusively. It showed the presence of MUC7 transcripts in the mucous acinar cells of the sublingual glands of both male and female mice, and the absence of MUC7 transcripts in the submandibular glands of both male and female mice. These results also indicate that the regulatory sequences required for both the tissue- and cell-specific expression of MUC7 gene are within the MUC7 transgene, preferably in the 5′-flanking region, and are faithfully conserved in transgenic mice. In the future, we would like to use the MUC7 promoter and a strategy of targeted oncogenesis in transgenic mice for isolation of novel mouse cell lines capable of MUC7 expression. Such cell lines, currently not available, would be quite useful for studying regulation of MUC7 gene expression.

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