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Gene Expression of Cytokeratin *endo A* and *endo B* during Embryogenesis and in Adult Tissues of Mouse

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We have examined the pattern of gene expression of mouse cytokeratin *endo A* and *endo B* during postimplantational development and in adult organs by Northern blot and *in situ* hybridization analyses. Both mRNAs localized in the ectoplacental cone, trophoblastic giant cells surrounding the parietal yolk sac, trophoblast cells in placenta, visceral yolk sac, and simple epithelium of the embryo during postimplantational development and in simple or transitional epithelial tissues in adult organs. These results indicate that *endo A* and *endo B* are coexpressed and may play some roles in these tissues. © 1991 Academic Press, Inc.

INTRODUCTION

Cytokeratins are epithelium-specific intermediate filaments comprising more than 19 different proteins [1-5]. According to their immunological cross-reactivities, peptide maps, and amino acid sequence relationships, cytokeratins are grouped into two subfamilies; type I keratins are generally smaller and more acidic than type II keratins on the average. At least one from each subfamily is necessary to form the fundamental heterotypic tetramers representing the cytokeratin subunit complex and resulting intermediate filaments [5-8]. Southern hybridization studies using type I and type II epidermal keratin cDNAs with genomic DNA have suggested that the different keratins can be grouped into two distinct types composed of approximately 10 genes each [9-11]. These data show that cytokeratins represent two multigene families.

Mouse Endo A and Endo B are type II and type I keratin intermediate filament proteins and these correspond to No. 8 and No. 18 cytokeratins in human, respectively [2, 12, 13]. During embryogenesis, the initial expression of both types at the 4- to 8-cell stage and enrichment in the trophectoderm of blastocyst stage embryos have been detected by immunological [14-17] and RNA [18, 19] analyses. From these observations, *endo A* and *endo B* are considered to be among the early

gene products expressed temporally and tissue-specifically during mouse development. The expressions of *endo A* and *endo B* are induced at the mRNA level during the differentiation processes of the F9 embryonal carcinoma cell line by retinoic acid treatment [18, 20-22]. Both Endo A and Endo B seem to be essential for intermediate filament formation in the parietal endoderm such as in embryonal carcinoma cell line PFHR9 [23]. *In vitro* assembly studies imply that roughly equivalent levels of type I and type II keratins are involved in filament formation [8, 24, 25]. These results suggest that the expressions of *endo A* and *endo B* genes are regulated coordinately. However, there have been few observations concerning the pattern of *endo A* and *endo B* mRNA distribution *in vivo* after implantation.

In this study, we have examined the tissue distribution of *endo A* and *endo B* mRNA in postimplantation embryos and in adult organs by Northern blot and *in situ* hybridization analyses to gain some insight into the coordinate expressions of *endo A* and *endo B* genes. We found that both *endo A* and *endo B* were strongly expressed in trophoblast cells, extraembryonic endoderm cells during postimplantational development, and in simple or transitional epithelial cells in embryos and in some adult tissues. Moreover, the tissues expressing *endo A* also expressed *endo B* in most cases, but the ratio of *endo A* to *endo B* mRNA varied in each organ.

MATERIALS AND METHODS

Embryos and organs. Mouse embryos were removed from 129/Sv-ter mice. The day on which the vaginal plug was observed was designated Day 1 of gestation. Organs for Northern blot analysis were collected from 25- to 26-week-old 129/Sv-ter mice, except for skin which was removed from a 19-day-old fetus.

Isolation of total RNA and Northern blot analyses. For Northern blot analysis, a 3' *Bam*HI/*Eco*RI fragment of *endo A* cDNA [13] and a 5' *Eco*RI/*Eco*RI fragment of *endo B* cDNA [26] were used as probes. Tissues were homogenized in 3 ml of 4 M guanidine thiocyanate, 250 mM sodium citrate, and 0.1 M β -mercaptoethanol, using a tissue homogenizer. The RNA was then purified by ultracentrifugation (SW4.1 Ti rotor, 35,000 rpm, 12 h) through a 1.2-ml cushion comprising 5.7 M cesium chloride in 0.1 M EDTA [27]. Samples containing 10 μ g of total RNA were separated on a 1% agarose gel containing 17% formaldehyde in a 1 $\times$ Mops (3-N-morpholinopropanesulfonic acid) buffer (40 mM Mops, 10 mM sodium acetate, 1 mM EDTA (pH 8.0)) after denaturation with formaldehyde and deionized formamide. The

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RNA was transferred to Nitroplus 2000 (Micron Separations Inc.) and the filters were baked for 2 h at 80°C. The membranes were prehybridized in 50% formamide, 4× SSC, 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 0.2% SDS, 120 µg/ml salmon sperm DNA for 1 h at 42°C. The heat-denatured, random prime labeled cDNA probes were added to the prehybridization mixture and hybridization was performed at 42°C overnight. Specific activities of the *endo A* and *endo B* probes are almost the same, as described in the legends to the figures. The membranes were washed three times in 0.1× SSC, 1% SDS at 55°C for 15 min and then exposed to Kodak X-OMAT AR5 X-ray film for varying lengths of time at -70°C using intensifier screens [28].

In situ hybridization. *In situ* hybridization was performed according to the method of Wilkinson *et al.* [29] and Cox *et al.* [30] with the following modifications. Briefly, embryos were fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline) at 4°C overnight and then washed, dehydrated, and embedded in paraffin wax. Sections 6 µm thick were cut, transferred to egg-albumin-coated and glutaraldehyde-fixed slides, and allowed to dry at 50°C overnight. Wax was removed and sections were rehydrated, fixed with 4% paraformaldehyde in PBS for 20 min at 4°C, and washed twice with PBS for 5 min each. Sections were treated with 20 µg/ml Proteinase K in PBS for 10 min at 37°C, rinsed with PBS, and further fixed in 4% paraformaldehyde in PBS for 5 min at room temperature. Following this, the slides were washed with PBS twice for 5 min each, treated with acetic anhydride, washed with PBS twice for 5 min, and dehydrated as described. For *in situ* hybridization, a 5' 1.4-kb *EcoRI/BamHI* fragment of *endo A* cDNA and a 0.7-kb *EcoRI/HindIII* fragment of *endo B* cDNA were subcloned into pGEM-3Z and pGEM-4Z (Promega). [α -³⁵S]UTP-labeled single-strand sense and antisense RNA probes were prepared by standard procedures [31] using T7 polymerase to transcribe the linearized template DNA. Following the removal of unincorporated nucleotides on a Sephadex G-50 column, the probes were degraded to an average length of 100 nucleotides and ethanol precipitated. The probes were resolved at a final concentration of 5×10^4 – 10^5 cpm/µl of hybridization solution (50% formamide, 0.3 M sodium chloride, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's, 0.5 mg/ml *Escherichia coli* tRNA, 20 mM DTT (dithiothreitol)). Aliquots (10 µl) of this mixture were used to hybridize each section, which were then covered with siliconized coverslips. Hybridization was performed at 50°C overnight, and the slides were then washed and dehydrated. Autoradiography using Kodak NTB-2 nuclear track emulsion was performed essentially as described by Angerer and Angerer [32]. Finally, sections were stained with Mayer's hematoxylin and mounted. Photographs were taken under bright-field and dark-field illumination.

RESULTS

Expression of *endo A* and *endo B* during Postimplantational Development

Cytokeratin Endo A and Endo B were identified as trophectoderm-specific markers. To observe the expression of *endo A* and *endo B* genes during postimplantational development, we determined the transcript level by Northern blot analysis and localization by an *in situ* hybridization experiment.

Northern blot analysis was performed using total RNA prepared from the entire conceptus at 7, 9, and 10 days of gestation and from the embryonic proper at 12, 14, and 16 days of gestation. As shown in Fig. 1, about 1.8 kb of *endo A* and 1.5 kb of *endo B* mRNA were first detected at 7 days of gestation, although *endo A* mRNA

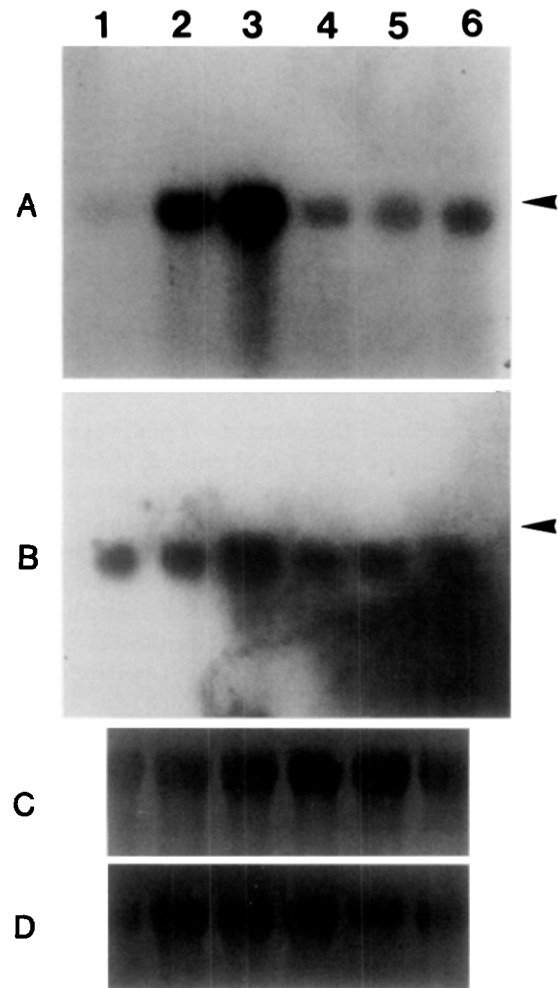


FIG. 1. Northern blot analyses of postimplantational stage. Total RNA (10 µg) from 7 (lane 1), 9 (lane 2), 10 (lane 3), 12 (lane 4), 14 (lane 5), and 16 (lane 6) days of gestation was loaded onto each lane. Seven- to ten-day-old embryos containing the entire conceptus and 12- to 16-day-old embryos containing only the embryonic proper were examined. Specific activities of the probes used were 3.6×10^8 cpm/µg of *endo A* cDNA and 4.0×10^8 cpm/µg of *endo B* cDNA. (A and B) The blotting with *endo A* and *endo B* probes, respectively. (C and D) Methylene blue staining of 28 S rRNA on the blotting filters to control for levels of RNA in A and B, respectively. Arrowhead in each panel indicates the size of 18 S rRNA. Exposure was for 5 days.

was faint. Their sizes were the same as those of PYS-2 cells from which these cDNAs were cloned. The amounts of both mRNAs significantly increased at 10 days of gestation (Fig. 1, lane 3). After 12 days of gestation, the signal intensities of both mRNAs of the embryonic proper slightly increased (Fig. 1, lanes 4, 5, and 6). These observations indicate that both *endo A* and *endo B* mRNAs accumulated at each stage of development, although their quantities were different.

In the extraembryonic proper including the placenta, maternal decidual tissue, part of the parietal yolk sac, visceral yolk sac, and amnion, both mRNAs were detectable after 9 days of gestation, increased drastically af-

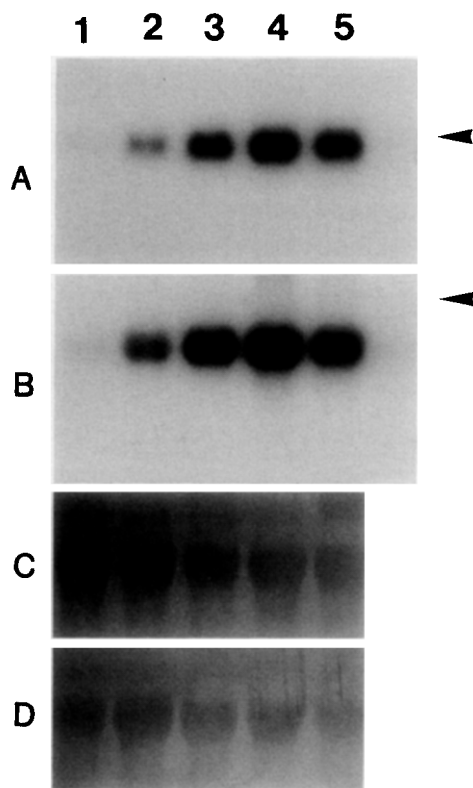


FIG. 2. Northern blot analyses of extraembryonic proper of postimplantational development. Total RNA (10 μ g) from extraembryonic proper from 9-day (lane 1), 10-day (lane 2), 12-day (lane 3), 14-day (lane 4), and 18-day (lane 5) embryos was loaded onto each lane. Specific activities of the probes used were about 6.2×10^8 cpm/ μ g (*endo A*) and 6.8×10^8 cpm/ μ g (*endo B*). (A and B) The hybridization with *endo A* and *endo B* probes, respectively. (C and D) 28 S rRNA staining of the blotting filters as in Fig. 1. Arrowhead indicates 18 S rRNA. Exposure was for 24 h.

terward, and were most intense at 14 days of gestation (Fig. 2). These transcripts were more abundant in extraembryonic tissues than in the embryonic proper (Fig. 1, lanes 4, 5, and 6 vs Fig. 2, lanes 3, 4, and 5).

To define the cell types expressing *endo A* and *endo B* mRNA more precisely, we examined sections by *in situ* hybridization. [α - 35 S]UTP-labeled sense and antisense RNA probes were hybridized to tissue sections from various embryonic stages. No hybridization was observed with the sense probe at any stages of development (data not shown).

The *endo A* transcripts were detected only in a subset of trophoblast cells of the ectoplacental cone region at 7 days of gestation. Parietal endoderm cells, visceral endoderm cells, trophoblastic giant cells, embryonic proper, and maternal decidual tissue showed no hybridization signals at this stage (Figs. 3A and 3B). Hybridization signals of *endo A* in sections at 7 days of gestation were detected in trophoblast cells in the ectoplacental cone, trophoblastic giant cells, and some parietal endoderm cells. No signals were detected in maternal deci-

dual tissue or embryonic proper (data not shown) at this stage (Figs. 3C and 3D). *endo B* transcripts were also detected in the same cell types in which *endo A* signals were detected at 7 and 9 days of gestation.

Abundant *endo B* transcripts were localized in trophoblastic giant cells, trophoblast cells, and visceral yolk sac at 10 days of gestation. Amnion showed weak hybridization signals, and no significant signals were observed in maternal decidual tissue. In 10-day-old embryos, *endo B* transcripts were detected in the epithelial cell layer surrounding the body (Figs. 4A and 4B). At 12 days of gestation, transcripts of *endo B* were detected in trophoblast cells in the placenta, trophoblastic giant cells in the parietal yolk sac (data not shown), and visceral yolk sac. In 12-day-old embryos, *endo B* mRNA in liver and in the inner layer of the gut was weakly detected (Figs. 4C and 4D). At 14 days of gestation, *endo B* transcripts were detected prominently in trophoblast cells (including spongio- and labyrinthine-trophoblasts) in placenta (Fig. 5E), trophoblastic giant cells in the parietal yolk sac, and visceral yolk sac endoderm cells. At this stage, *endo B* transcripts were also detectable in some epithelial cells of the nasal cavity, hypophysis, trachea (Figs. 5A and 5B), umbilical cord, duodenum, gut, urogenital tract, lung, liver, and kidney of embryonic proper (Figs. 5C and 5D). Hybridization with *endo A* showed the same localization as *endo B* at each stage of development (data not shown). These observations indicated that *endo A* and *endo B* genes are expressed mainly in cells of trophoblastic lineage during development and weakly in epithelial layers of some organs after 12 days of gestation.

Expression Pattern of *endo A* and *endo B* in Adult Organs

In postimplantational development, *endo A* and *endo B* were found to be expressed predominantly in trophoblast cells and trophoblastic giant cells and weakly in epithelial tissues of the embryo proper in later stages. However, little is known about their gene expression in adult organs. To determine which tissues express *endo A* and *endo B* in adults, we performed Northern blot analysis and *in situ* hybridization.

As shown in Fig. 6, *endo A* and *endo B* transcripts were detected in the small intestine, stomach, urinary bladder, ovary, uterus, lung, kidney, and liver (for longer exposure; data not shown), and their sizes were the same as those of the embryos. Intense expression of *endo A* mRNA was detected in the small intestine, stomach, and urinary bladder (Fig. 6A). Abundant *endo B* transcripts, as many as *endo A*, were also detected in the urinary bladder, but the amount was less than that of *endo A* transcripts in the small intestine and stomach (Fig. 6B).

We performed *in situ* hybridization to identify the cell types that express *endo A* and *endo B* in adult organs.

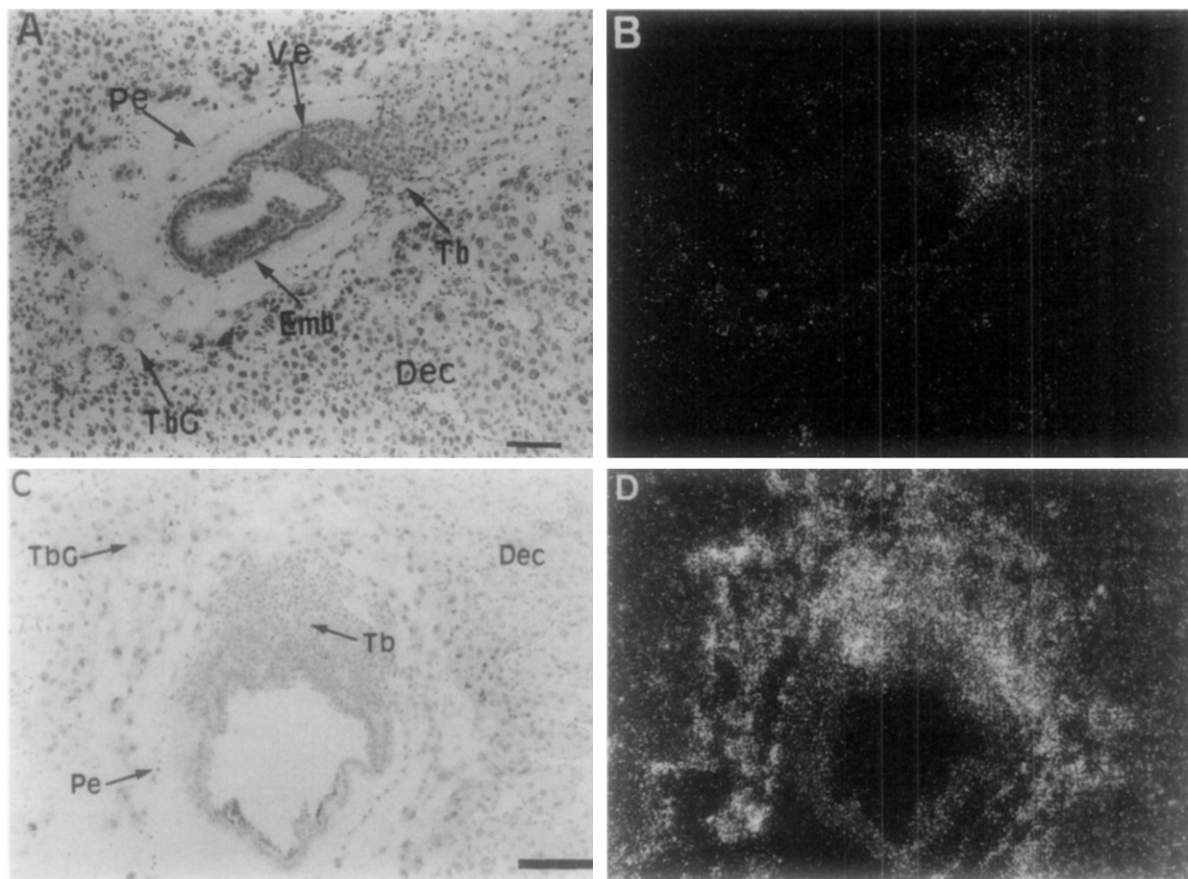


FIG. 3. Localization of *endo A* mRNA in 7-day egg cylinder (A, B) and 9-day ectoplacental cone region in maternal decidual tissue (C, D). Nine-day exposure of sagittal sections of 7-day-old and 9-day-old embryos photographed under bright-field (A, C) and dark-field (B, D) illumination. Tb, trophoblast cell; TbG, trophoblastic giant cell; Dec, maternal decidual tissue; Emb, embryonic proper; Pe, parietal endoderm; Ve, visceral endoderm. Grid bar represents 0.1 mm (A) and 1 mm (C).

We detected these transcripts uniformly in the simple epithelium of the small intestine (*endo A*; data not shown) and of the oviduct (*endo B*; Figs. 7A and 7B), but not in other tissues. In the urinary bladder, we observed *endo B* signals in transitional epithelium but not in smooth muscle cell layers (Figs. 7C and 7D). These results showed that *endo A* and *endo B* genes are expressed in simple and transitional epithelia in adult organs.

DISCUSSION

Mouse cytokeratin *endo A* and *endo B* are first detected at the 4- to 8-cell stage of development, and then the mRNAs are enriched exclusively in the trophectoderm of blastocysts. The purpose of this study was to determine where *endo A* and *endo B* are expressed during postimplantational development. Northern blot analyses have shown that *endo A* and *endo B* are expressed mainly in the extraembryonic tissues. Localization of these mRNAs was determined in cells derived from trophectoderm cell lineage: trophoblast cells in the

ectoplacental cone at 7 days of gestation, trophoblast cells and trophoblastic giant cells at 9 and 10 days of gestation, and trophoblast cells in placenta at 12 and 14 days of gestation by *in situ* hybridization. These results clearly demonstrate that *endo A* and *endo B* are expressed throughout the trophectoderm cell lineage during postimplantational development.

The functions of trophoblast cells are thought to include nutrition, invasion, and hormone production [33, 34], which involve not only the uptake of food materials outside of the embryo but also the secretion of proteolytic enzymes, some peptides, and steroid hormones. It would be interesting to learn how cytokeratin networks composed of Endo A and Endo B are related to these functions of trophoblast cells. Since we found that those cells express *endo A* and *endo B* mRNA continuously, mouse trophoblast cells are thought to synthesize both proteins as shown in rat trophoblastic giant cells by the immunological method described previously [35]. Therefore, it is possible that cytoskeletal filaments containing cytokeratins participate to express these functions. Moreover, prominent expression of *endo A* and *endo B*

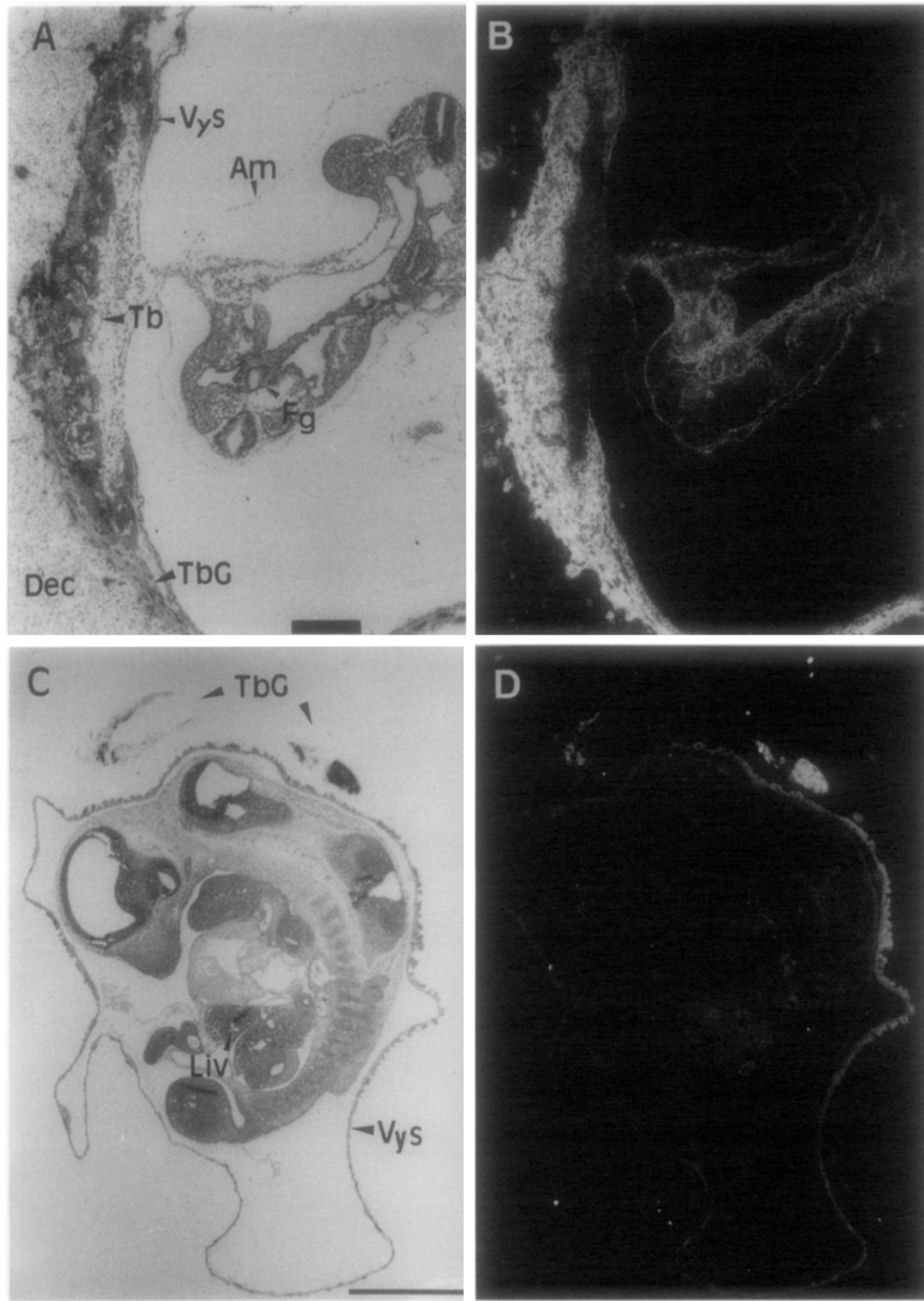
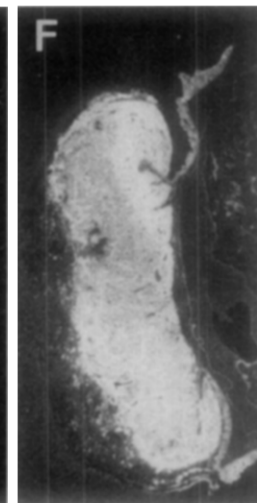
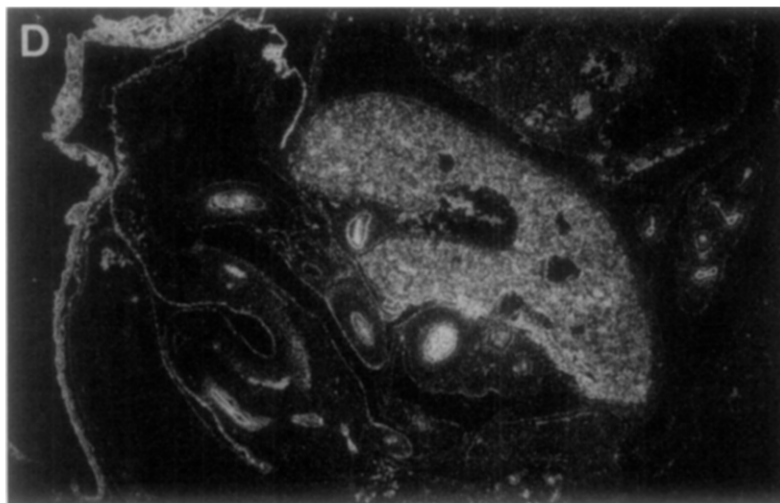
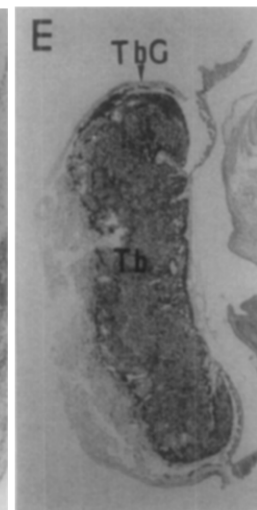
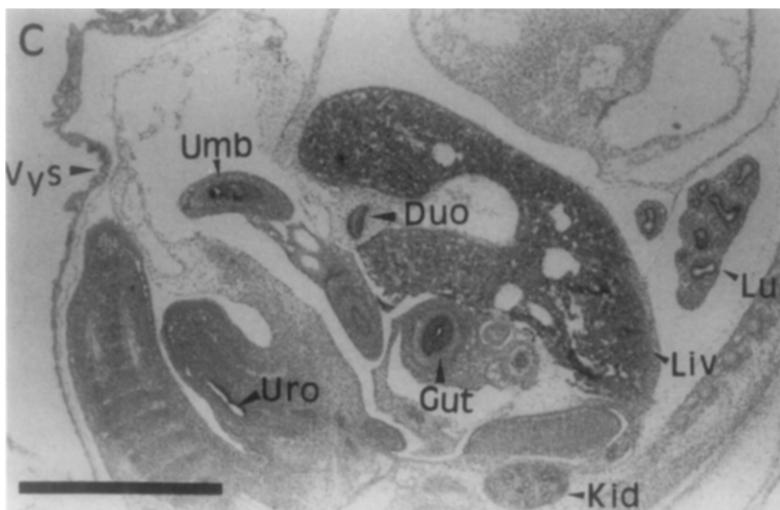
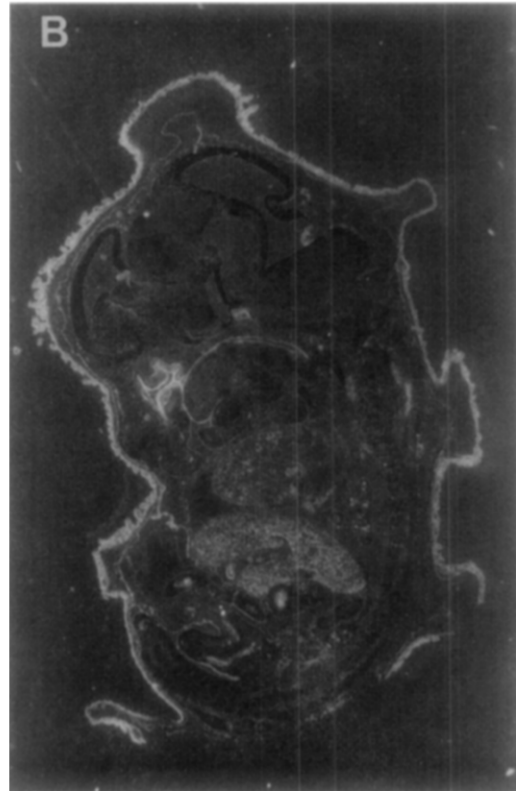
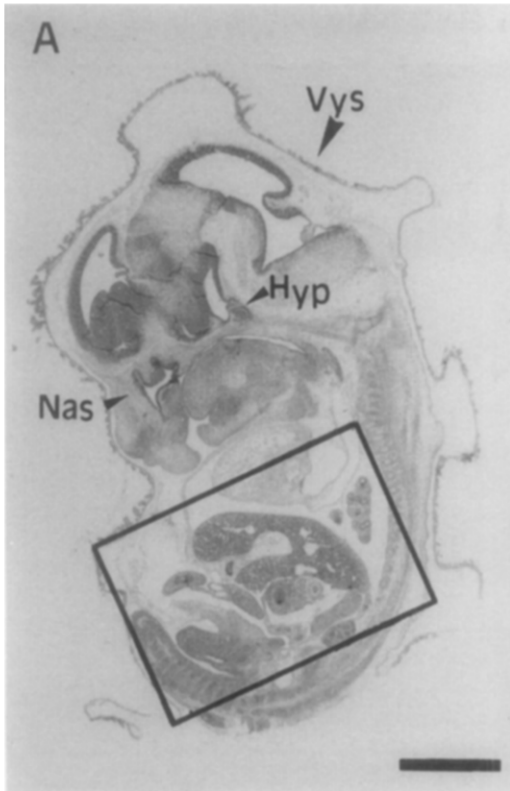


FIG. 4. Localization of *endo B* mRNA in 10-day embryo in maternal decidual tissue (A, B) and in sagittal section of 12-day embryo in visceral yolk sac (C, D). (A, C) Bright-field illumination. (B, D) Dark-field illumination. Tb, trophoblast cells; TbG, trophoblastic giant cells; Dec, maternal decidual tissue; Vys, visceral yolk sac; Am, amnion; Liv, liver. Grid bar represents 0.3 mm (A) and 1 mm (C). Exposure was for 14 days.

mRNAs in simple epithelium of the small intestine and stomach, which show vigorous digestion and secretion in adults, supports this possibility. On the other hand, Northern blot analysis has shown that the embryo

proper expressed low levels of *endo A* and *endo B* in comparison with extraembryonic tissues. In 14-day-old embryos, *endo B* mRNAs were detected weakly in epithelial layers of the lung, gut, liver, kidney, and urogenital



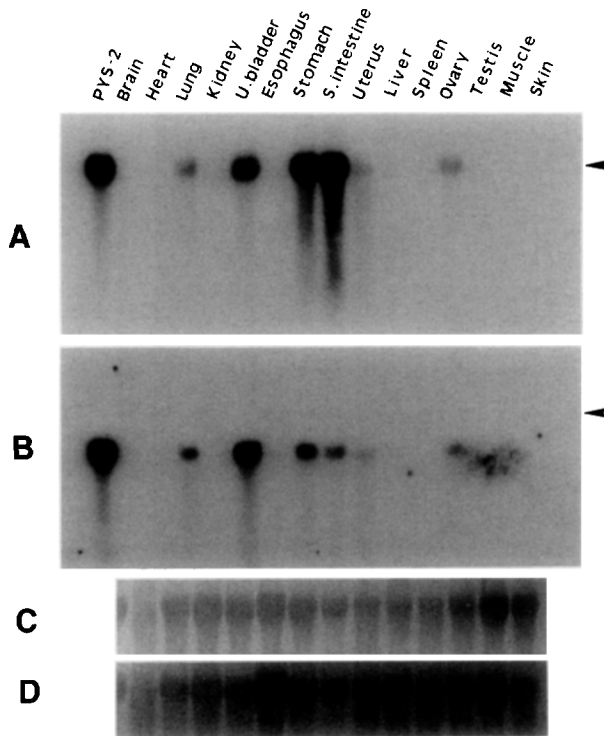


FIG. 6. Northern blot analyses of adult mouse organs. Total RNA (20 μ g) from each organ was loaded onto each lane. (A and B) Hybridization with *endo A* and *endo B* probes, respectively. (C and D) 28 S rRNA of the filters as in Fig. 1. Arrowheads represent the 18 S rRNA. The specific activities of the probes were the same as those in Fig. 1. Exposure was for 7 days. U.bladder, urinary bladder; S.intestine, small intestine.

tract by *in situ* hybridization, but these epithelial cells seem unlikely to play functions as in the adult. These results suggest that the increase in transcriptional activities of *endo A* and *endo B* genes correlates with functional differentiation of the epithelium. To test these possibilities, further studies will be necessary.

Cytokeratin filaments have been observed to associate with desmosomes inside the epithelial cell membrane [36]. It is thought that cyto keratin filament assembly correlates to desmosome formation [37, 38]. Desmosomes are observed to localize between syncytio- and cytotrophoblast and between cells of the cytotrophoblast [39]. In our results, *endo A* and *endo B* mRNA were localized prominently in the trophoblast cells. Thus, it is suggested that there is some relationship between desmosomes and cyto keratin in trophoblast cells. In this context, cyto keratin filament formation is closely associated in time and topography with the ap-

pearance of desmosomal structures in trophoctoderm cells as well as in trophoblast cells [40–42]. Moreover, a reduction in Endo A and Endo B protein synthetic rates, cyto keratin filament network formation, and desmosome formation has been correlated with failure of blastocyst formation in mouse embryos homozygous for the t^{12} mutation [16, 42]. On the other hand, disruption of the cyto keratin network by microinjection of anti-Endo A antibody, TROMA-1, into a preimplantation embryo had no effects on the morphological development of the embryo into blastocysts, although the effects on desmosome formation have not been examined [43]. Therefore, it seems unlikely that the formation of cyto keratin filaments is necessary to form blastocysts, whereas cyto keratin networks may play some role in the maintenance of desmosomes in differentiated trophoblast cells.

Xenopus keratin *endo B* (XK *endo B*) gene has been identified as a notochord-specific sequence and its expression is concentrated in the notochord [44]. Mouse notochord also expresses Endo A, as determined by immunohistochemistry using TROMA-1 [45]. However, we could detect neither *endo A* nor *endo B* in the notochord by *in situ* hybridization. It is possible that undetectable levels of these mRNAs are present, while the protein can be detected in the notochord.

As shown in this study, the level of expression between *endo A* and *endo B* was not necessarily equivalent. Especially in the small intestine and stomach, the amounts of *endo A* mRNA were significantly greater than those of *endo B*. In human small intestine, three types of cyto keratins, No. 8 (Endo A), No. 18 (Endo B), and No. 19, which has been found to be identical to Endo C [46], have been identified by two-dimensional gel electrophoresis [2]. We have also found prominent expression of No. 19 (*endo C*) mRNA in the small intestine and stomach of mice [47]. By immunohistochemical analysis, Endo A distribution was detected uniformly in the epithelium, while Endo B expression was found in goblet cells in the adult duodenum [48]. These results suggest that Endo A makes a filamentous structure with Endo B in some cell types containing goblet cells and assembles with Endo C in other epithelial cells of the gut and stomach, although the possibility that these three types of cyto keratins assemble in a cell cannot be excluded. In the *in vitro* experiments, indeed, transformed fibroblasts expressing No. 8 alone, No. 8 and No. 18, No. 8 and No. 19, and No. 8, No. 18, and No. 19 keratin proteins were isolated [49].

FIG. 5. Localization of *endo B* mRNA in 14-day embryo (A–D) and placenta (E, F). (C and D) Higher magnification views of the area shown in A. (A, C, E) Bright-field illumination. (B, D, F) Dark-field illumination. Vys, visceral yolk sac; Nas, nasal cavity. Hyp, hypophysis; TbG, trophoblastic giant cell; Umb, umbilical cord. Uro, urogenital tract; Duo, duodenum; Gut, gut; Kid, kidney; Liv, liver; Lu, lung. Grid bars represent 1 mm (A, C). Exposure was for 14 days.

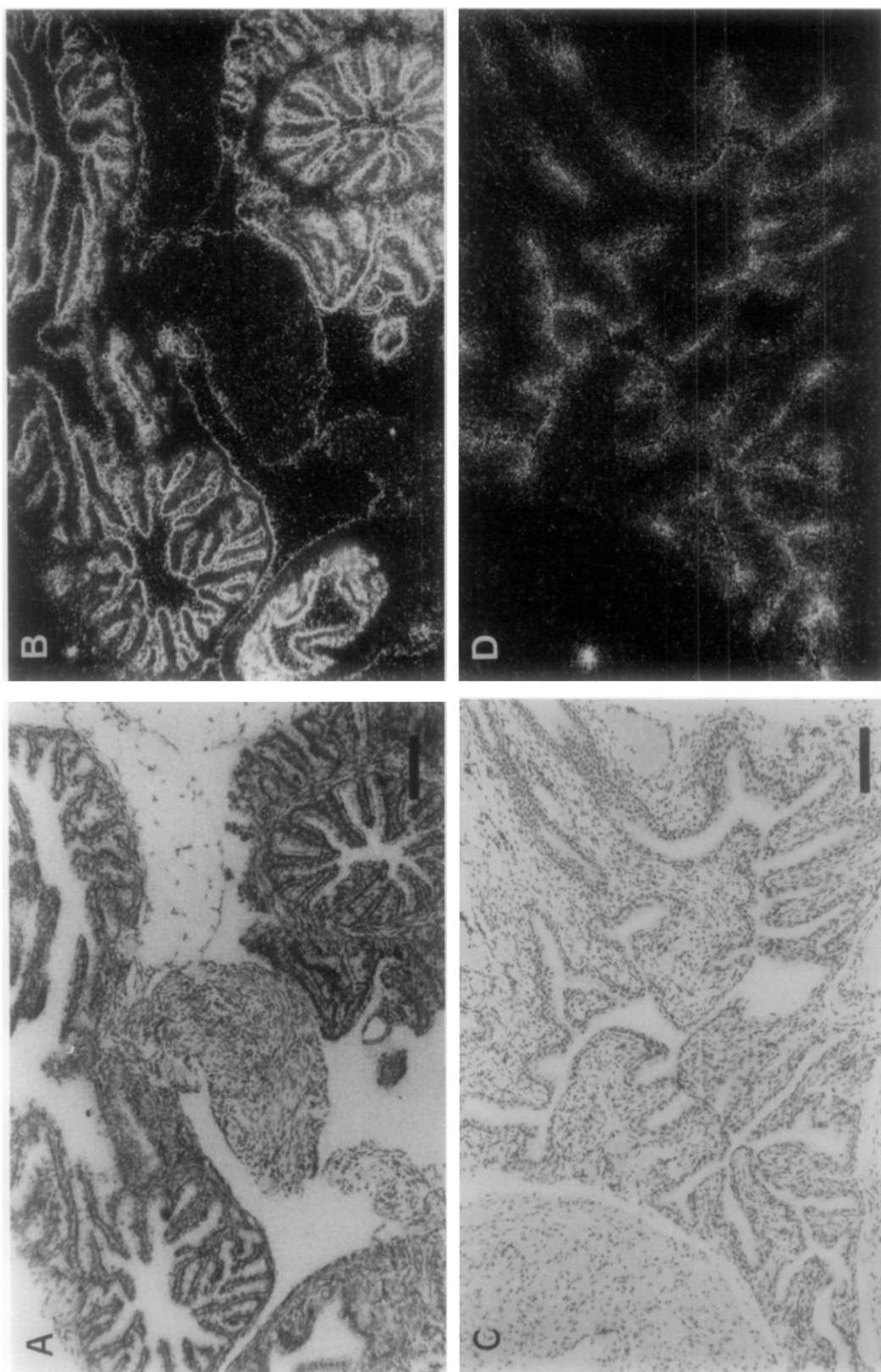


FIG. 7. Localization of *endo B* mRNA in adult oviduct (A and B) and urinary bladder (C and D). (A, C) Bright-field illumination. (B, D) Dark-field illumination. Grid bar represents 0.1 mm (A) and 0.3 mm (C). Exposure was for 14 days.

Many simple and transitional epithelia expressed both *endo A* and *endo B*. From these findings, we expect that homologous upstream sequences of these genes may play some role in the expression of the two genes. Some bovine epidermal cytokeratin genes located in tandem on a chromosome have a common nucleotide sequence upstream of the TATA box and their expressions are coordinately regulated in some tissues [50]. In the case of *endo A* and *endo B* genes, two concentrated homologous sequences around the respective TATA box-like sequences have been found [26]; however, whether these similarities are significant in the regulation of these genes remains to be examined. Alternatively, another possible mechanism for regulating the synthesis of type I and type II cytokeratins was provided by coordinated gene transfection studies, in which the expression of a human type II epidermal keratin gene in mouse fibroblasts gave rise to the appearance of an endogenous type I epidermal keratin [51]. Moreover, antisense *endo B* mRNA not only reduces *endo B* cytokeratin but also inhibits the expression of *endo A* mRNA and protein in teratocarcinoma differentiation systems [52]. In SV40-transformed fibroblasts, the CK 18 gene is constitutively transcribed into translatable mRNA but that protein is rapidly degraded in the absence of its complex partner, CK 8 [53]. From these data, the quantity of one type of cytokeratin protein may regulate the level of mRNA expression of the other.

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