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# Tissue Inhibitor of Metalloproteinases-2 Is Expressed in the Interstitial Matrix in Adult Mouse Organs and during Embryonic Development

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Tissue inhibitor of metalloproteinases-2 (TIMP-2) is a member of a family of inhibitors of matrix-degrading metalloproteinases. A better insight into the role of this inhibitor during development and in organ function was obtained by examining the temporospatial expression of TIMP-2 in mice. Northern blot analysis indicated high levels of TIMP-2 mRNA in the lung, skin, reproductive organs, and brain. Lower levels of expression were found in all other organs with the exception of the liver and gastrointestinal tissue, which were negative. In situ hybridization revealed a selective expression in the stromal component of these tissues with complete absence of TIMP-2 mRNA in the epithelium. In the testis, TIMP-2 was present in the Leydig cells, and in the brain, it was expressed in pia matter and in neuronal tissues. TIMP-2 expression in the placenta increased during late gestation and was particularly abundant in spongiotrophoblasts. In mouse embryo (day 10.5–18.5), TIMP-2 mRNA was abundant in mesenchymal tissues that surrounded developing epithelia and maturing skeleton. The pattern of expression significantly differs from that observed with TIMP-1 and TIMP-3, therefore, suggesting specific roles for each inhibitor during tissue remodeling and development.

## INTRODUCTION

Matrix metalloproteinases (MMPs)[1] consist of a family of structurally similar $Zn^{2+}$-binding $Ca^{2+}$-dependent endopeptidases that include collagenases, gelatinases, and stromelysins (Woessner, 1994) and are involved in the degradation of native macromolecules of the extracellular matrix (ECM) such as collagens, proteoglycans, and glycoproteins (Alexander and Werb, 1992). In addition to their structural function, components of the ECM play an important regulatory role in cell growth, differentiation, and apoptosis (Boudreau *et al.*, 1995) and their turnover during tissue remodeling is under the control of a complex combination of factors that include MMPs and tissue inhibitors of MMPs (TIMPs; Birkedal-Hansen *et al.*, 1993). Subtle changes in the balance between MMPs and TIMPs occur in many physiological processes associated with increased remodeling of the ECM such as trophoblastic implantation (Alexander *et al.*, 1996; Lala and Hamilton, 1996), embryonic development (Canete Soler *et al.*, 1995; Harvey *et al.*, 1995), angiogenesis (Liotta *et al.*, 1991), endometrial proliferation (Rodgers *et al.*, 1993), and wound healing (Agren, 1994). In addition to inhibiting MMP activity, TIMPs have been shown to bind to the surface of some cells and to modulate cell growth by acting as growth stimulators (Hayakawa *et al.*, 1992, 1994) or growth inhibitors (Murphy *et al.*, 1993). The nature of this dual function of TIMPs is presently unclear.

Four members of the TIMP family designated TIMP-1 (Docherty *et al.*, 1985), TIMP-2 (Stetler-Stevenson *et al.*, 1989; Boone *et al.*, 1990), TIMP-3 (Apte *et al.*, 1994; Leco *et al.*, 1994), and TIMP-4 (Greene *et al.*, 1996; Leco *et al.*, 1997) have so far been cloned, purified, and character-

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[1] Abbreviations used: ECM, extracellular matrix; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; p.c., post coitus; TIMP, tissue inhibitor of metalloproteinase.

ized in a variety of species. TIMPs share a similar structural feature that includes the presence of 12 cysteine residues in preserved position and a similar function directed toward inhibition of all members of the MMP family. However, these inhibitors differ in many aspects. TIMP-1 and TIMP-2 form preferential complexes with progelatinase B and progelatinase A, respectively (Goldberg *et al.*, 1989, 1992), and although TIMP-1 and TIMP-2 are present in a soluble form, TIMP-3 is associated with the ECM (Leco *et al.*, 1994). Significant differences in the mechanisms that transcriptionally regulate the expression of TIMPs in cells have been reported (Leco *et al.*, 1992). For example, the expression of TIMP-1 is transcriptionally regulated by a large variety of cytokines, hormones, and growth factors such as transforming growth factor-$\beta$, interleukin-1, interleukin-6, retinoic acid, tumor necrosis factor-$\alpha$, proto-oncogenes, and glucocorticoids (Chua and Chua, 1990; Lotz and Guerne, 1991). Similarly, TIMP-3 expression is up-regulated by 12-*O*-tetradecanoylphorbol-13-acetate and transforming growth factor-$\beta$ (Leco *et al.*, 1994), whereas the expression of TIMP-2 is predominantly constitutive. Accordingly, analysis of the human TIMP-2 promoter has indicated the presence of many features typically seen in housekeeping genes (DeClerck *et al.*, 1994; Hammani *et al.*, 1996).

Analysis of the expression of TIMPs in mouse tissues and during murine development has provided additional information on the specific function of TIMPs in tissue remodeling and embryonic development. In particular, these studies have pointed to a specific role for TIMP-1 in bone morphogenesis (Nomura *et al.*, 1989; Flenniken and Williams, 1990) and TIMP-3 in musculoskeletal and cardiac development, in the morphogenesis of certain epithelial structures, and in placental implantation (Apte *et al.*, 1994). However, less is known of the role of TIMP-2 because information on the temporospatial expression of TIMP-2 in mouse tissues and during development has been limited to a few reports that have shown its expression in cells of osteogenic tissues and in cartilage primordia (Mattot *et al.*, 1995; Kinoh *et al.*, 1996).

To further understand the role of TIMP-2 in adult mouse tissues and during murine development, we have examined its expression in a variety of adult organs and in embryos during the second half of gestation. The data reveal a specific pattern of expression that differs in many aspects from TIMP-1 and TIMP-3 and suggest different roles for the three TIMPs.

## MATERIALS AND METHODS

### *Mice*

Female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were used to generate a collection of embryos. Noon on the day of the vaginal plug was designated day 0.5 post coitum (p.c.). Animals were killed on each day of pregnancy, ranging between day 10.5 p.c. and day 19 p.c. (birth). Embryos and placenta were collected separately and fixed for in situ hybridization or snap frozen in liquid nitrogen for RNA extraction. Adult organs and tissues derived from male and female C57BL/6 or B6/CBA mice of 2–4 mo of age were similarly collected and preserved. Additional sections of embryos used for in situ hybridization were purchased from Novagen (Madison, WI).

### *Northern Blot Analysis*

Total RNA was isolated by homogenization in guanidium isothiocyanate with a Polytron, followed by phenol:chloroform extraction and LiCl precipitation (Chomczynski and Sacchi, 1987). Twenty micrograms of total RNA were electrophoretically separated in 1% (wt/vol) agarose gels containing formaldehyde. After transfer to nylon membranes (ICN Biomedicals, Aurora, OH), the blots were sequentially hybridized at 42°C with denatured $^{32}$P-labeled random-primed cDNA probes. The mouse TIMP-1 cDNA was a gift from Dr. D. Denhardt (Rutgers University, Piscataway, NJ). The mouse TIMP-2 and TIMP-3 cDNAs were generously provided by Dr. R. Khokha (Ontario Cancer Institute, Toronto, Ontario, Canada) and Dr. D. Edwards (University of Calgary, Alberta, Canada). To account for differences in loading and transfer of the RNA, a 28S RNA probe (Ambion, Austin, TX) or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (American Type Culture Collection, Rockville, MD) was used. Scanning densitometry analysis of the RNA signals was done on the autoradiography films with a Deskscan II, ScanJet IIcX/T scanner (Hewlett–Packard, Palo Alto, CA) and the Sigmagel/PC software (Sigma, St. Louis, MO). Densitometry values were then used to derive the ratios of TIMP signal over GAPDH signal for each sample.

### *Preparation of Riboprobe*

Plasmid pGEM2 (Promega, Madison, WI) containing a 362-bp *Pst*I–*Kpn*I (positions +303 to +665) fragment of mouse TIMP-2 was generously provided by Dr. R. Khokha. The plasmid was linearized with *Hin*dIII or *Eco*RI restriction enzymes and RNA templates for transcription were generated by using SP6 (antisense) or T7 (sense) RNA polymerase (SP6/T7 transcription kit, Boehringer-Mannheim, Indianapolis, IN). In vitro transcription was performed in the presence of [$\alpha$-$^{33}$P]UTP (DuPont NEN, Boston, MA).

### *In Situ Hybridization*

Embryos, placenta, and adult mouse tissues were immersed into fresh phosphate-buffered saline (PBS: 137 mM NaCl, 2.6 mM $Na_2HPO_4$, and 1.7 mM $KH_2PO_4$, pH 7.4) containing 4% (wt/vol) paraformaldehyde and fixed overnight at 4°C. For a better fixation of critical organs such as heart, liver, and blood vessels, an anesthetized animal was perfused with fixative by intracardiac puncture to allow the solution to distribute more uniformly in each organ. After fixation, the samples were washed in PBS, dehydrated through a graded series of ethanol washes, cleared in xylene, and embedded in paraffin. Serial sections (6 $\mu$m thick) were collected onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and dried overnight at 42°C. Sections were dewaxed in two changes of xylene (10 min each), then rehydrated through an ethanol series of decreased concentrations for 2 min each, and rinsed in two changes of PBS. Sections were incubated with 5 $\mu$g/ml proteinase K in 100 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0, and 50 mM EDTA for 30 min at 37°C, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and acetylated by a 10-min incubation in a freshly prepared solution of 0.1 M triethanolamine hydrochloride, pH 8.0, containing 0.25% (vol/vol) acetic anhydride. The sections were then washed in PBS, dehydrated in ethanol series, and air dried before being incubated overnight at 50°C in the hybridization buffer [50% (vol/vol) formamide, 0.3 M

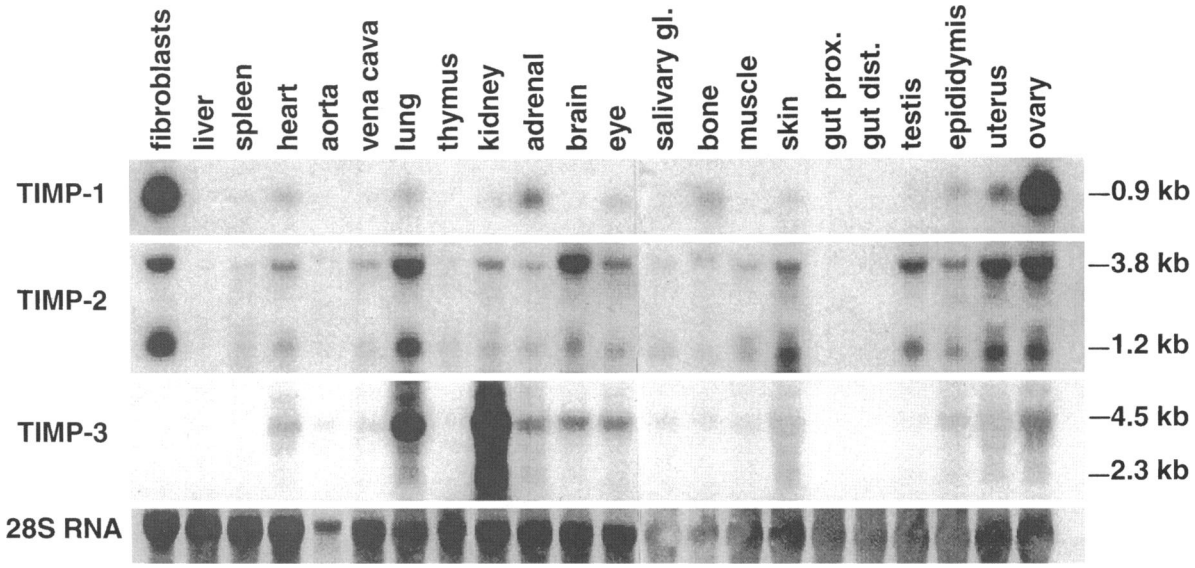


**Figure 1.** Expression of TIMPs in adult mouse tissues. Northern blot analysis of total RNA (20 μg/lane) extracted from a series of adult mouse organs and tissues as indicated at the top. The blots were hybridized sequentially with mouse cDNA probes for TIMP-1, TIMP-2, and TIMP-3. The size of the TIMP transcripts is indicated on the right. A 28S RNA probe was used as control to assess the amount of RNA loaded and transferred. RNA extracted from mouse fibroblasts was used as control for TIMP-1 and TIMP-2.

NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1× Denhardt's solution, 250 μg/ml tRNA, 10% (wt/vol) dextran sulfate], containing the $^{33}$P-labeled riboprobe. For each tissue examined, six adjacent sections were used, three being incubated with an antisense probe and three others with a sense probe. After hybridization, the sections were washed by successive incubations first in 4× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate) at 50°C; secondly at 63°C in 50% (vol/vol) formamide, 0.3 M NaCl, and 20 mM Tris-HCl, pH 8.0; and third at 37°C in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA, pH 8.0, containing RNase A (20 μg/ml). After final washes in 2× SSC and 0.1× SSC at 50°C, the sections were dehydrated in an ethanol series of increased concentrations containing 0.3 M ammonium acetate. The dried slides were dipped in photographic emulsion (Hypercoat LM-1, Amersham Life Science, Arlington Heights, IL) and exposed for 1 to 2 wk at 4°C. After exposure, the slides were developed and counterstained with eosin and Mayer's hematoxylin or with Mayer's hematoxylin alone.

## RESULTS

### *TIMPs Expression in Adult Organs*

The expression of TIMP-1, TIMP-2, and TIMP-3 genes in adult mouse organs was examined by Northern blot analysis (Figure 1). Two TIMP-2 mRNAs of 3.8 and 1.2 kb were detected in a large variety of organs. The highest levels of expression were observed in lung, skin, testis, epididymis, uterus, ovary, and brain. Low levels of TIMP-2 mRNA were present in the heart, vena cava, kidney, adrenal gland, eye, bone, and muscle, and a very weak level of expression was detected in the spleen, aorta, thymus, and salivary gland. An absence of expression was noted in the liver and gastrointestinal tissue. In contrast, the expression of TIMP-1 and TIMP-3 was more restricted. TIMP-1 was identified as a single 0.9-kb mRNA strongly present in the ovary, uterus, and adrenal gland and weakly detected in the heart, lung, eye, bone, skin, and epididymis. TIMP-3 was detected predominantly as a 4.5-kb mRNA expressed at high level in the lung and kidney and present at a lower level in the heart, adrenal gland, brain, eye, skin, and ovary. Thus although the three TIMPs were coexpressed in several organs, in particular in the heart, lung, and female reproductive organs, some major differences in expression were seen in other organs. In particular, in the vena cava, brain, muscle, skin, testis, and epididymis, TIMP-2 was the predominant or the only TIMP expressed, whereas as previously reported by others, TIMP-1 and TIMP-3 were found more specifically expressed in the bone (Nomura *et al.*, 1989; Flenniken and Williams, 1990) and kidney (Apte *et al.*, 1994; Leco *et al.*, 1994), respectively. The absence of any TIMP expression in the liver and gastrointestinal tissue was noteworthy.

### *Localization of TIMP-2 mRNA within Adult Mouse Tissues*

We performed in situ hybridization by using an antisense RNA probe on tissue sections to further define the expression of TIMP-2 in positive organs. Organs of the female (ovary and uterus) and male (testis and epididymis) reproductive systems were initially examined (Figure 2). In the ovary, TIMP-2 mRNA was diffusely detected in the stroma surrounding the follicles and was absent in the granulosa cells (Figure 2,

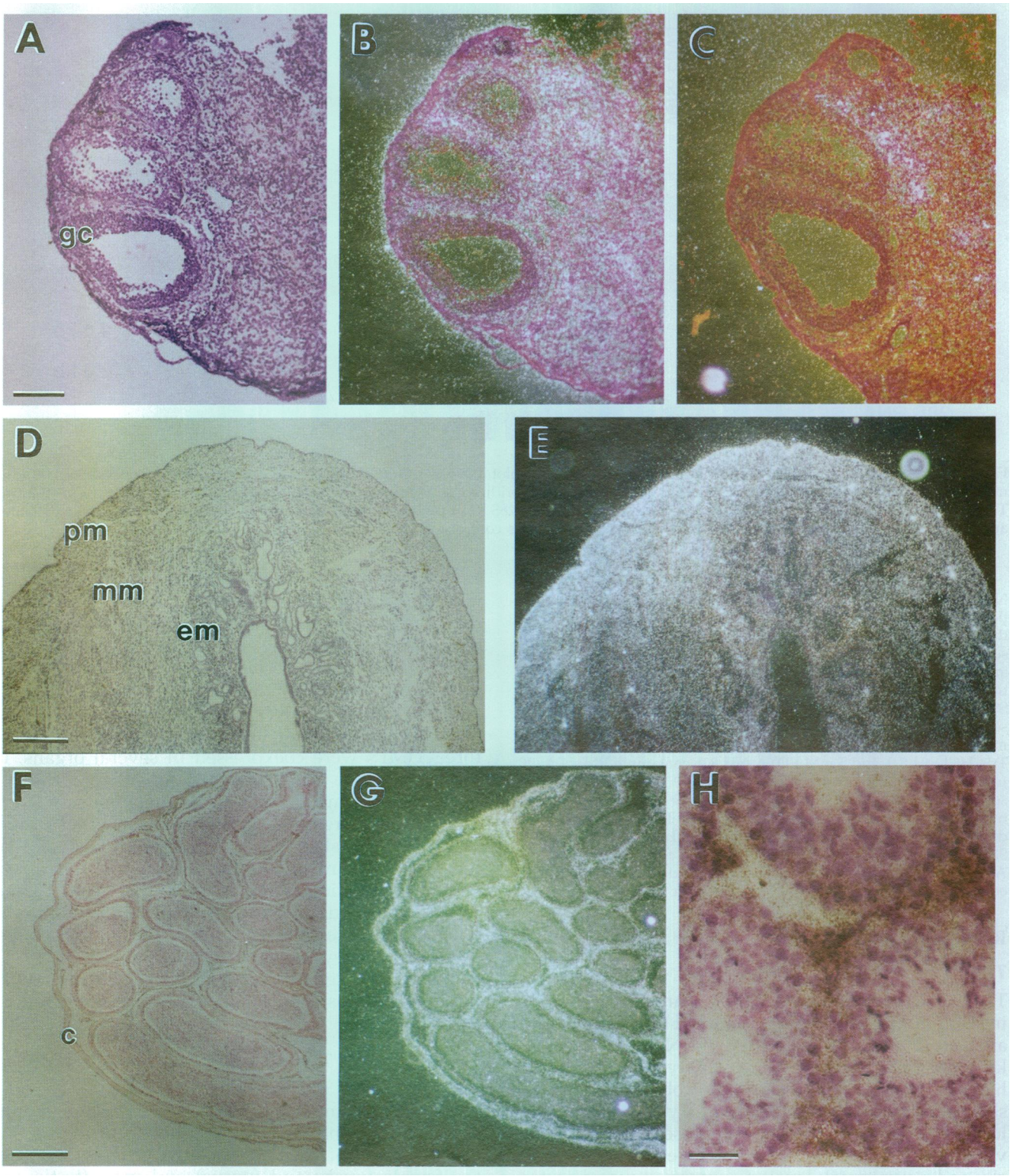


**Figure 2.** Localization of TIMP-2 expression in adult reproductive organs. Analysis of TIMP-2 gene expression by in situ hybridization in the ovary (A–C), uterus (D and E), epididymis (F and G), and testis (H). Corresponding bright-field and dark-field images of a same section are presented in A and B, D and E, and F and G, respectively. (A and B) Sagittal section of an ovary hybridized with the TIMP-2 antisense probe. Note that the signal is present in the ovarian stroma but absent in the granulosa cells (gc) of the follicles. (C) Section adjacent to B hybridized with a TIMP-2 sense probe. (D and E) Transverse section of the uterus showing the hybridization signal in the parametrium (pm)

A and B). Although the stage of the estrus cycle was not determined at the time the ovary was obtained, a similar pattern of expression was observed in three separate specimens. No signal above background was obtained with a sense probe (Figure 2C). In the uterus, TIMP-2 mRNA was predominantly present in the parametrium and the myometrium but was absent in the luminal and the glandular epithelia of the endometrium (Figure 2, D and E). In the epididymis, a strong expression of TIMP-2 mRNA was detected in the capsule and in the smooth muscle cells adjacent to the ductus epididymis (Figure 2, F and G). In contrast, the tubular epithelium and the sperm cells were entirely negative. A similar pattern of expression was observed in the testis, where interstitial Leydig cells present in between the seminiferous tubules expressed a strong signal for TIMP-2 mRNA whereas the germinal epithelium and the Sertoli cells were negative (Figure 2H). In all the reproductive organs, the expression of TIMP-2 was restricted to the interstitial tissues surrounding epithelial structures, which were systematically negative.

In situ hybridization was also performed on sections of vessels, heart, and lung to examine TIMP-2 expression in the cardiovascular and respiratory systems (Figure 3). Sections through the femoral artery and femoral vein revealed a predominant expression of TIMP-2 mRNA in the adventitial and medial layers of the artery and little expression in the vein (Figure 3, A and B). Striated muscle cells around these vessels were negative. In the heart, the expression of TIMP-2 was restricted to the pericardium, in particular around the auricle and was undetected in the myocardium and endocardium (Figure 3, C and D). In the lung, TIMP-2 mRNA was detected in particular in the pleura (mesothelium) and the interalveolar septa (Figure 3, E and F) but was absent in the epithelium forming the respiratory bronchioles (Figure 3G). The predominant expression of TIMP-2 in interstitial tissues rich in elastin such as the arterial vessel wall and the lung was noteworthy.

Of particular interest was the analysis of TIMP-2 expression in the brain. On coronal sections (Figure 4A), a well-defined TIMP-2 signal was observed not only in the meninges and the choroid plexus but also within the neural tissue at the level of the neocortex, the septum, and the piriform olfactory cortex (Figure 4B). In the lateral ventricles, the TIMP-2 mRNA signal was particularly intense in the invaginated folds of pia matter forming the choroid plexus (Figure 4, C and D). In the neocortex, the TIMP-2 mRNA signal was specifically expressed in the second (external granular) and the fourth (internal granular) layers that contain neurons and glial cells (Figure 4, E and F).

In the skin, the TIMP-2 mRNA signal was present in connective tissue cells of the dermis and in particular around the hair follicles (Figure 5, A and B). No TIMP-2 mRNA was detected in the epidermis and the subcutaneous tissue. In the gut and the liver, which were negative for TIMP-2 expression by Northern blot analysis, no TIMP-2 mRNA was detected by in situ hybridization. Sections through the small intestine did not reveal a TIMP-2 signal above background in the epithelium of the villi or the crypt and in the muscularis mucosae (Figure 5, C and D). Similarly, in the liver no TIMP-2 mRNA signal was detected in the parenchyma, the central vein (Figure 5, E and F), or the portal space.

Thus, with the exception of the brain, in all organs examined by in situ hybridization, the selective and abundant expression of TIMP-2 in the stroma has been a consistent observation, suggesting a predominant role for this inhibitor in controlling the homeostasis of the interstitial matrix. To further explore whether TIMP-2 participates in the remodeling of the ECM associated with organ maturation, we examined its expression in murine placenta and whole embryos obtained at various stages of the second half of gestation.

### *TIMPs Expression in Placenta during Gestation*

Northern blot analysis performed on RNA extracted from whole murine placenta obtained from day 10.5 p.c. to day 18.5 p.c. indicated significant differences in TIMP expression during placental maturation (Figure 6A). The amount of TIMP-2 mRNA in the placenta was relatively low from day 10.5 p.c. to day 14.5 p.c., rapidly increased by 4.5-fold on day 16.5 p.c., and remained at this higher level until term (Figure 6B). In contrast, the expression of TIMP-3 mRNA more gradually increased from day 10.5 p.c. to day 17.5 p.c. On day 18.5 p.c., a sudden decrease in expression to levels in the range of those observed on day 16.5 p.c. was seen (Figure 6C). No expression of TIMP-1 was detected in placenta obtained from midgestation to term.

### *Localization of TIMP-2 mRNA within Placenta*

In a day 11.5 p.c. placenta, TIMP-2 mRNA was detected in the decidua and to a lesser degree in the junctional zone (Figure 7A). As TIMP-2 expression increased, a relative decrease in expression in the decidua was seen on day 17.5 p.c., whereas the expres-

**Figure 2 (cont).** and in the myometrium (mm), and its absence in the glandular and luminal epithelia of the endometrium (em). (F and G) Sagittal section of the epididymis (portion of the cauda), showing the hybridization signal in the capsule (c) and in the stroma surrounding the ductus epididymis and its absence in the tubular epithelium and the sperm cells. (H) High magnification (200×) of a sagittal section of the testis, showing the hybridization signal restricted to Leydig cells. Bars: A–C, 158 μm; D–G, 400 μm; H, 50 μm.

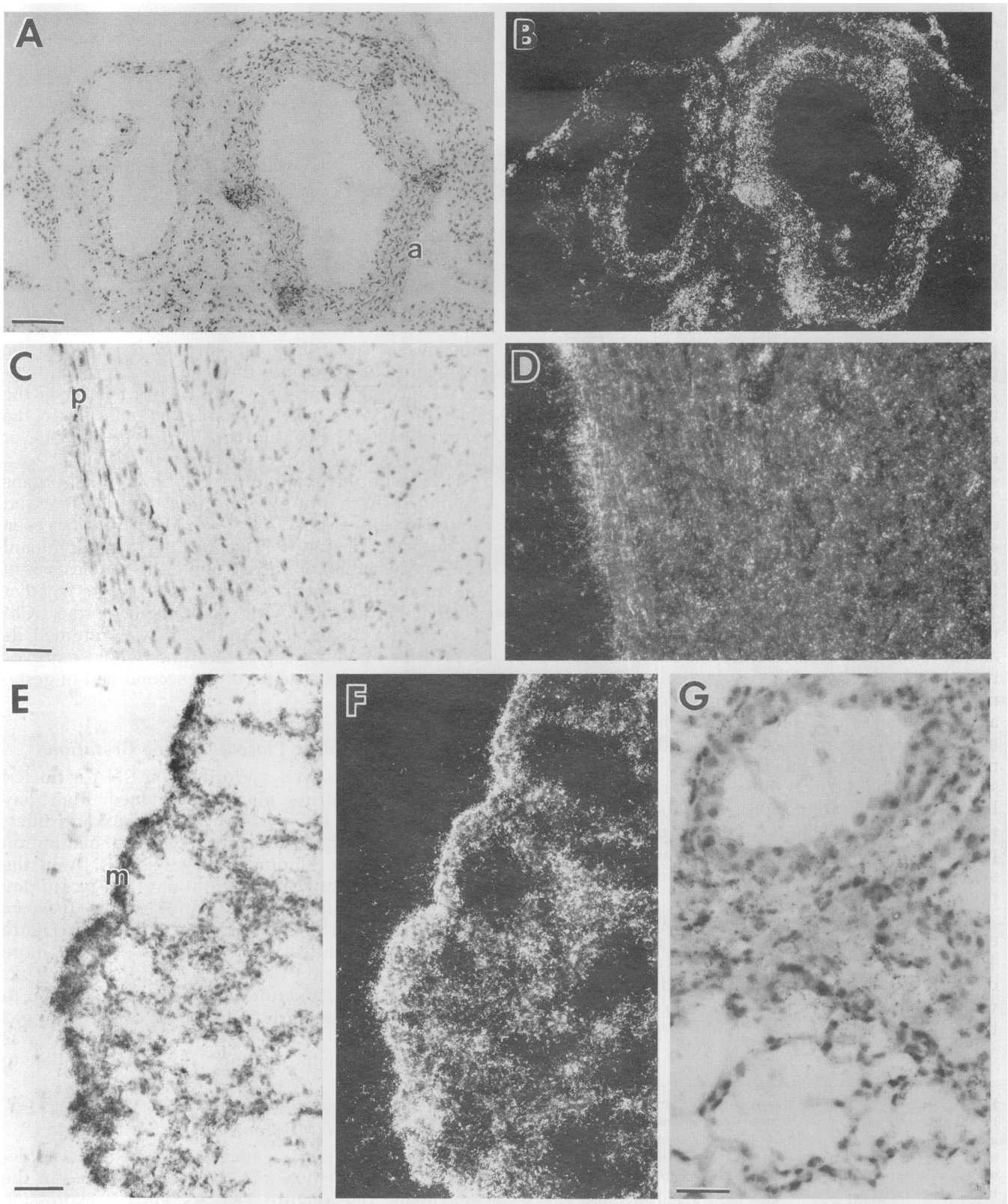


**Figure 3.** Localization of TIMP-2 expression in adult cardiovascular and respiratory systems. Analysis of TIMP-2 gene expression by in situ hybridization in the vessels (A and B), heart (C and D), and lung (E–G). Photographs of sections hybridized with an antisense TIMP-2 probe

sion in the junctional zone dramatically increased (Figure 7B). No signal was detected in this zone with a sense riboprobe (Figure 7C). In the decidua, the expression of TIMP-2 was confined to cells surrounding the central artery and maternal blood vessels (Figure 7, D and E). In the junctional zone (Muntener and Hsu, 1977) that separates the labyrinth, rich in fetal blood vessels and maternal blood sinuses, from the zone of giant cells, TIMP-2 was intensively expressed in the spongiotrophoblasts (Figure 7, F–I).

### *Localization of TIMP-2 mRNA during Embryonic Development*

To examine the spatial expression of TIMP-2 during mouse development, in situ hybridization was performed on midsagittal sections of whole embryos obtained during the second half of gestation. The data indicated a similar pattern of TIMP-2 mRNA expression at all stages of development from day 10.5 p.c. to day 18.5 p.c. This pattern of expression is shown for day 12.5 p.c. and day 15.5 p.c. on Figure 8, A–C, and revealed an intense TIMP-2 mRNA signal in the snout, oropharynx, nasopharynx, tongue, diaphragm, bladder, spine, and brain ventricles. In the snout, TIMP-2 expression was found abundant in the connective tissues surrounding the nasal and oral epithelia (Figure 8, B and C). An intense signal was observed in the forming palate, muscles of the tongue, and connective tissues around the forming nasal, oropharyngeal, laryngeal, and esophageal epithelia, which were negative (Figure 8D). An intense signal was also detected in the perichondral tissues surrounding the developing vertebral bodies and cartilage primordium of the basioccipital bone. The thymus and hypophyseal gland shown on this section were negative as were other glandular organs such as the thyroid gland and salivary glands. In the chest and upper abdomen (Figure 8E), TIMP-2 mRNA was expressed in the diaphragm, pericardium, auricle, and lung pleura, whereas the liver was negative. In the lower abdomen (Figure 8F), the kidney parenchyma and bladder wall showed a positive TIMP-2 signal, whereas the glandular epithelium of the pancreas, gut epithelium, and adrenal gland did not express TIMP-2 mRNA. In the kidney, liver, and adrenal gland, the capsule of these organs showed a strong TIMP-2 signal. In the head, TIMP-2 mRNA was expressed in the developing skull as well as in the ventricles and the choroid plexus (Figure 8G). No mRNA was detected in the forming cortex, striatum, medulla oblongata, and cerebellum. An intense TIMP-2 mRNA signal was also observed in the perichondrial tissue surrounding the developing primitive bone of the vertebrae (Figure 8H) and the foot (Figure 8, I and J).

**Figure 3 (cont).** were taken with either bright-field (A, C, E, and G) or dark-field (B, D, and F) illumination. (A and B) Transverse section of the femoral artery and femoral vein, with a strong expression of TIMP-2 mRNA in the adventitia (a). (C and D) Sagittal section of the heart showing the hybridization signal limited to the pericardium (p). (E and F) Transverse section of the lung showing high expression in the mesothelium (m). (G) Detail of the lung parenchyma showing the presence of the TIMP-2 signal in the connective tissue surrounding the airways and its absence in the cuboidal epithelium of the bronchus. Bars: A and B, 350 μm; C–E and F, 70 μm; G, 35 μm.

## DISCUSSION

Our analysis of the expression of TIMP-1, TIMP-2, and TIMP-3 in adult mouse organs indicated that TIMPs are coexpressed in several organs, in particular the skin, lung, and female reproductive organs. As previously reported in human cells (Stetler-Stevenson *et al.*, 1990), two TIMP-2 mRNAs of 1.2 kb and 3.8 kb were identified by Northern blot analysis in all tissues examined (Figure 1), including the placenta (Figure 6). In general the 3.8-kb mRNA was more abundant than the 1.2-kb mRNA. We have recently demonstrated that these two mRNAs differ by the selection of their polyadenylation signal sites but have a very similar half life (Hammani *et al.*, 1996). The expression of TIMPs in organs rich in connective tissues such as the skin and lung or in organs in which significant tissue remodeling occurs such as the uterus and ovary is not unexpected. In fact many studies have shown that MMPs and TIMPs play an active role during ovulation, endometrial proliferation, menstruation, and gestation (Marbaix *et al.*, 1992; Rodgers *et al.*, 1993; Polette *et al.*, 1994; Inderdeo *et al.*, 1996). The expression of TIMP-2 in addition to TIMP-1 and TIMP-3 in these organs suggests an active participation of these three inhibitors in the intense tissue remodeling associated with these processes. However, in situ hybridization indicated a significant difference in cell-specific expression in the ovary and the uterus. TIMP-1 and TIMP-3 expression in the ovary is confined to the oocytes, granulosa cells, and corpus luteum (Nomura *et al.*, 1989; Inderdeo *et al.*, 1996). In contrast, our observations on TIMP-2 indicate an absence of expression in the ovarian follicles and corpus luteum and a selective expression in the ovarian stroma surrounding the follicles. These observations suggest that TIMPs might have different physiological roles in the ovarian cycle. TIMP-1 and TIMP-3 may regulate the maturation and regression of the corpus luteum (Inderdeo *et al.*, 1996), whereas TIMP-2 may control the turnover of the stromal tissue during the normal ovulatory cycle. In our study we did not examine whether the levels of TIMP-2 expression varied during the ovarian cycle; however, similar levels of expression were detected by Northern blot analysis and in situ hybridization in three independent specimens. Stable levels of TIMP-2 expression during the ovarian cycle were also re-

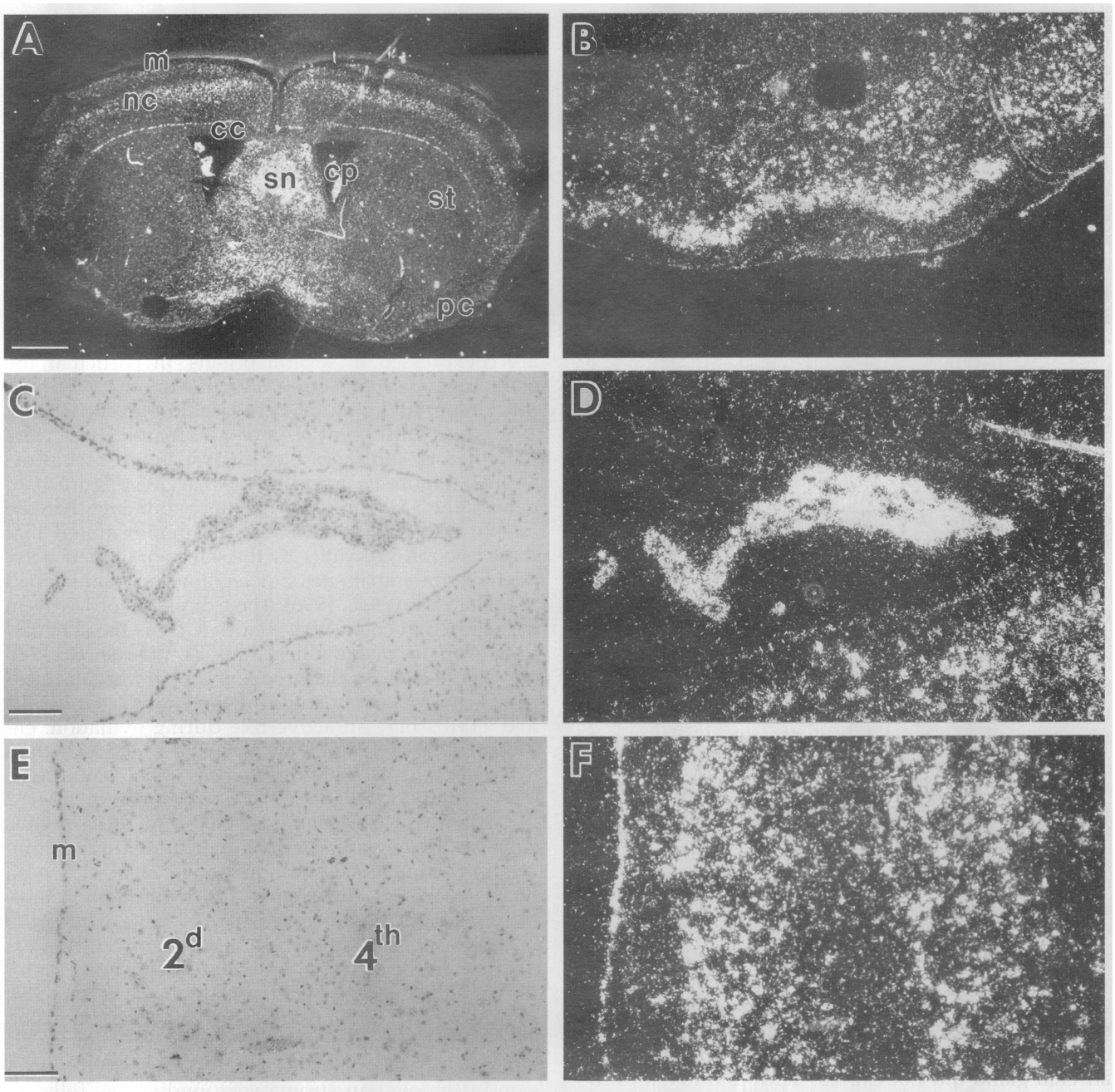


**Figure 4.** Localization of TIMP-2 expression in adult central nervous system. Analysis of TIMP-2 gene expression by in situ hybridization on coronal sections of the brain. Photographs were taken with a bright-field (C and E) or a dark-field (A, B, D, and F) illumination. (A) Entire view of the brain. TIMP-2 is present in the meninges (m), neocortex (nc), choroid plexus (cp), piriform cortex (pc), and septal nucleus (sn) but is absent in the striatum (st) and corpus callosum (cc). (B) Detail of the TIMP-2 hybridization signal in the piriform cortex. (C and D) Detail of the lateral ventricle with the strongly positive signal in the choroid plexus. (E and F) Detail of the neocortex. TIMP-2 mRNA is expressed in the meninges (m) and in the second (2d) and fourth (4th) cortical layer. Bars: A, 700 $\mu$m; B, 350 $\mu$m; C–F, 140 $\mu$m.

ported (Inderdeo *et al.*, 1996). The expression of TIMP-2 in the uterus was restricted to nonepithelial tissues. High levels of MMP-7 expression in epithelial cells lining the lumen and in some glandular structures in the endometrium have been reported (Wilson *et al.*, 1995). Our data suggest no involvement of TIMP-2 in the remodeling of the epithelial tissues and a preponderant role in the turnover of the interstitial matrix. The high level of expression of TIMP-2 in the testis and epididymis was unexpected. Expression of

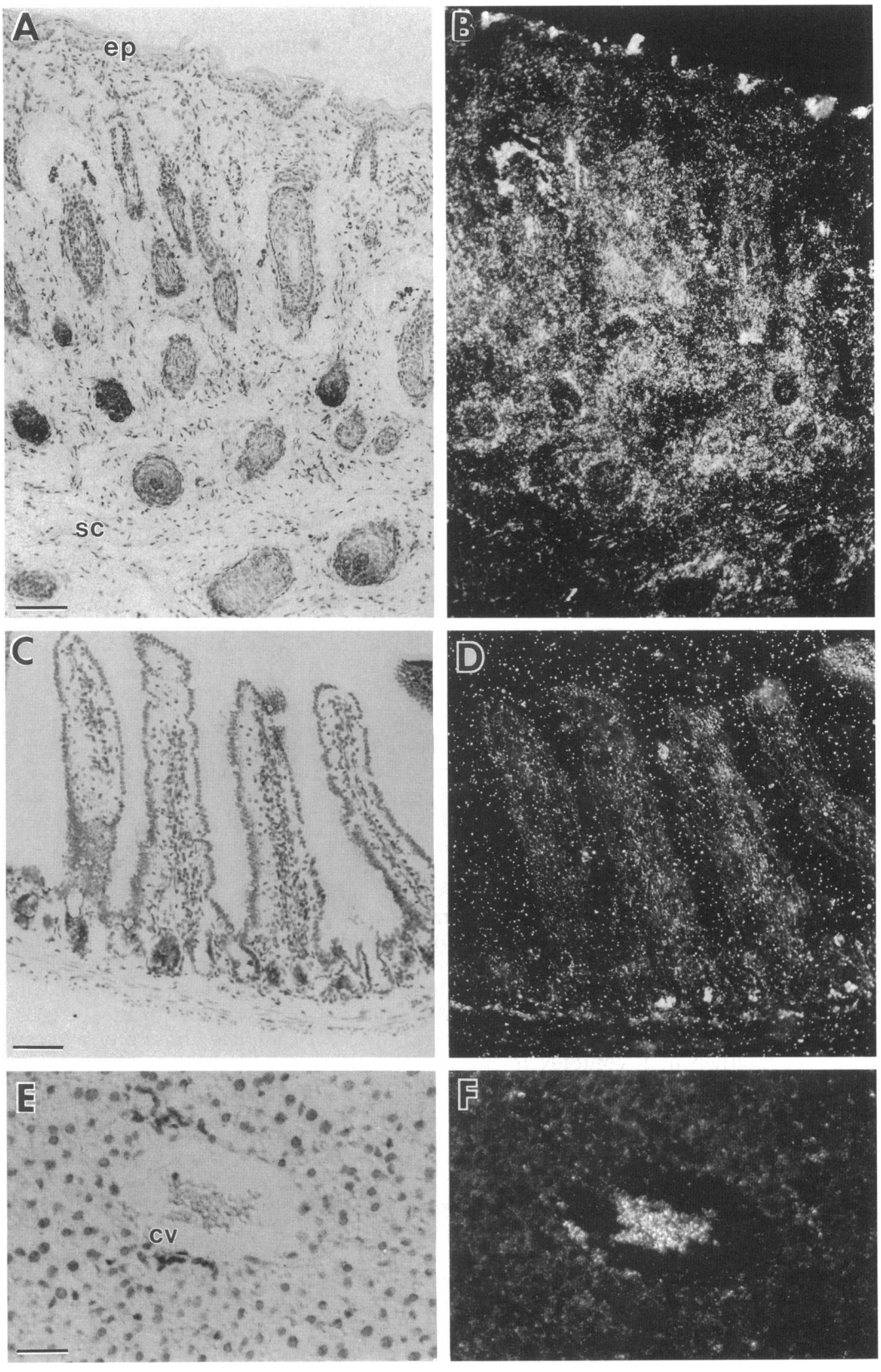


**Figure 5.** Localization of TIMP-2 expression in adult skin, liver, and gastrointestinal tissue. Analysis of TIMP-2 gene expression by in situ hybridization on transverse section of the skin (A and B), small intestine (C and D), and liver (E and F). Photographs of sections hybridized with an antisense TIMP-2 probe were taken with either bright-field (A, C, and E) or dark-field (B, D, and F) illumination. (A and B) In the skin TIMP-2 mRNA is present in the dermis around the hair follicles and absent in the epidermis (ep) and subcutaneous tissue (sc). (C and D) No signal was detected in the gastrointestinal tissue. (E and F) In the liver, only red blood cells present in the lumen of the central vein (cv) gave an artifactual signal. Bars: A and B, 140 $\mu$m; C and D, 100 $\mu$m; E and F, 50 $\mu$m.

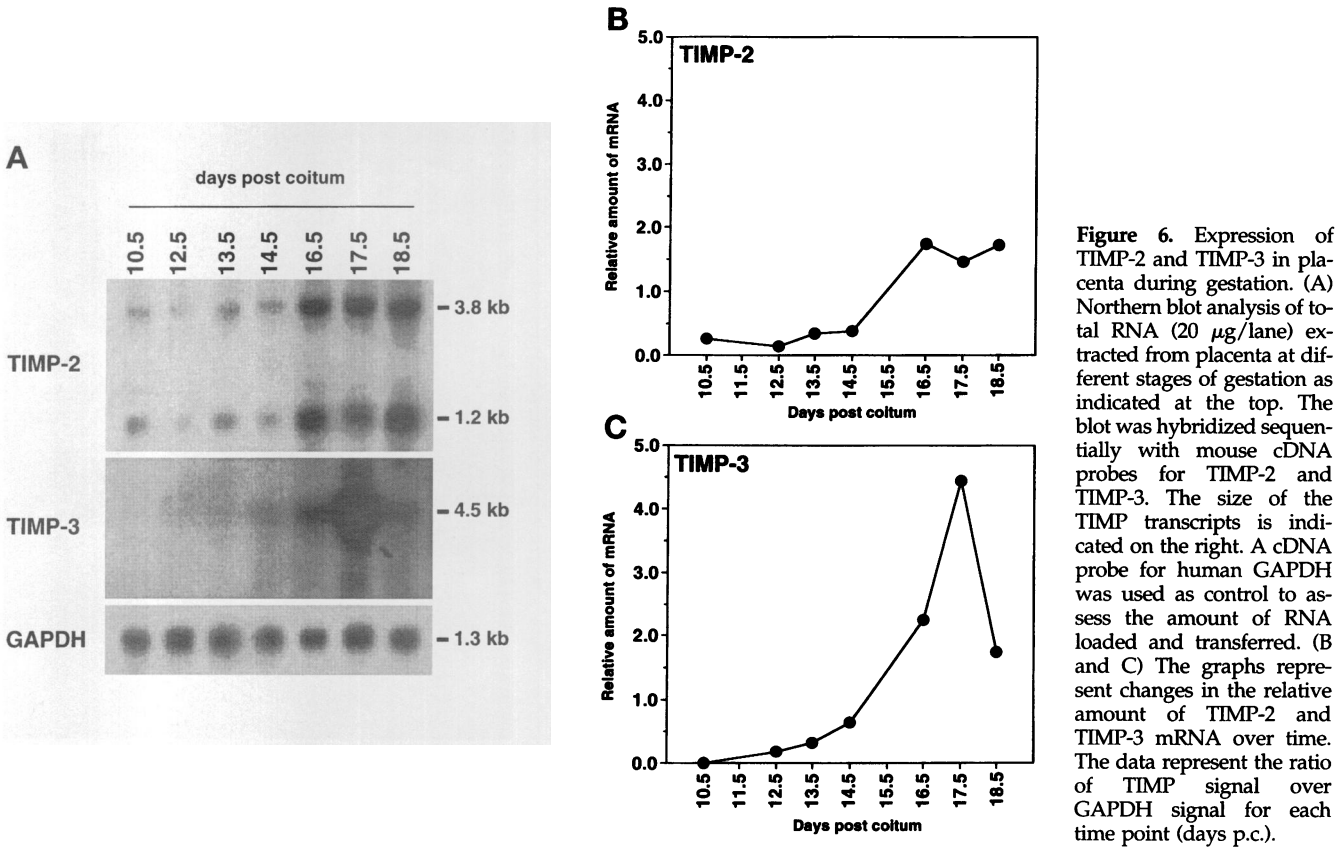


**Figure 6.** Expression of TIMP-2 and TIMP-3 in placenta during gestation. (A) Northern blot analysis of total RNA (20 μg/lane) extracted from placenta at different stages of gestation as indicated at the top. The blot was hybridized sequentially with mouse cDNA probes for TIMP-2 and TIMP-3. The size of the TIMP transcripts is indicated on the right. A cDNA probe for human GAPDH was used as control to assess the amount of RNA loaded and transferred. (B and C) The graphs represent changes in the relative amount of TIMP-2 and TIMP-3 mRNA over time. The data represent the ratio of TIMP signal over GAPDH signal for each time point (days p.c.).

MMP-7 in the epithelial cells lining the efferent ducts, which transport sperm from the testis to the epididymis, as well as in epithelial cells of the ductus epididymis has been documented, suggesting a potential role for this protease in sperm maturation (Wilson *et al.*, 1995). The expression of TIMP-2 in the testis and epididymis was confined to the interstitium between epithelial structures, suggesting a function not related to spermatogenesis. Increased expression of TIMP-1 and TIMP-2 in prepubertal rat Sertoli cells in primary culture has been reported (Ulisse *et al.*, 1994), and a steroidogenesis-stimulating protein identified as a TIMP-1–procathepsin L complex in human and rat Sertoli cells stimulated by follicle-stimulating hormones has been described (Boujrad *et al.*, 1995). We found that TIMP-2 expression in the mature testicle was restricted to the interstitial Leydig cells and found no expression in Sertoli cells.

The presence of TIMP-2 in the lungs and in large arterial blood vessels rich in elastin is interesting. In the lung, TIMP-2 expression was particularly intense in the viscera pleura, which contains several layers of elastic fibers, and less intense in the septa of the alveoli. It was absent in the bronchial epithelium. In large vessels, the TIMP-2 signal was also predominant in the elastin-rich adventitia and tunica media that contain smooth muscle cells. Since elastin can be degraded by MMPs (Senior *et al.*, 1991; Shapiro *et al.*, 1992), the data suggest that TIMP-2 participates in preserving the integrity of elastic fibers in these tissues.

In the skin, TIMP-2 expression was detected in the dermis and more specifically in stromal cells surrounding the hair follicles. This is in contrast to what has been reported for TIMP-1, which is specifically expressed in the Henle's layer of the inner root shaft during the growing phase of the hair cycle and expressed at a low level in few connective tissue cells (Kawabe *et al.*, 1991). The complete absence of expression of all three TIMPs in organs rich in epithelial tissues such as the liver and the gastrointestinal tract was also remarkable. A similar ob-

**Figure 7 (facing page).** Localization of TIMP-2 expression in placenta. Analysis of TIMP-2 gene expression by in situ hybridization on midsagittal sections of the placenta. Photographs were taken with either bright-field (D, F, and H) or dark-field (A–C, E, G, and I) illumination. (A) The hybridization TIMP-2 signal at 11.5 d p.c. is mainly present in the decidua (d). (B) The hybridization signal at 17.5 d p.c. is detected in the decidua and in the junctional zone (jz).(C) Adjacent section of B hybridized with the TIMP-2 sense probe. (D and E) Detail of the decidua of day 17.5 placenta showing TIMP-2 mRNA present in cells surrounding the central artery(ca) and the maternal blood vessels. (F–I) Detail showing TIMP-2 mRNA

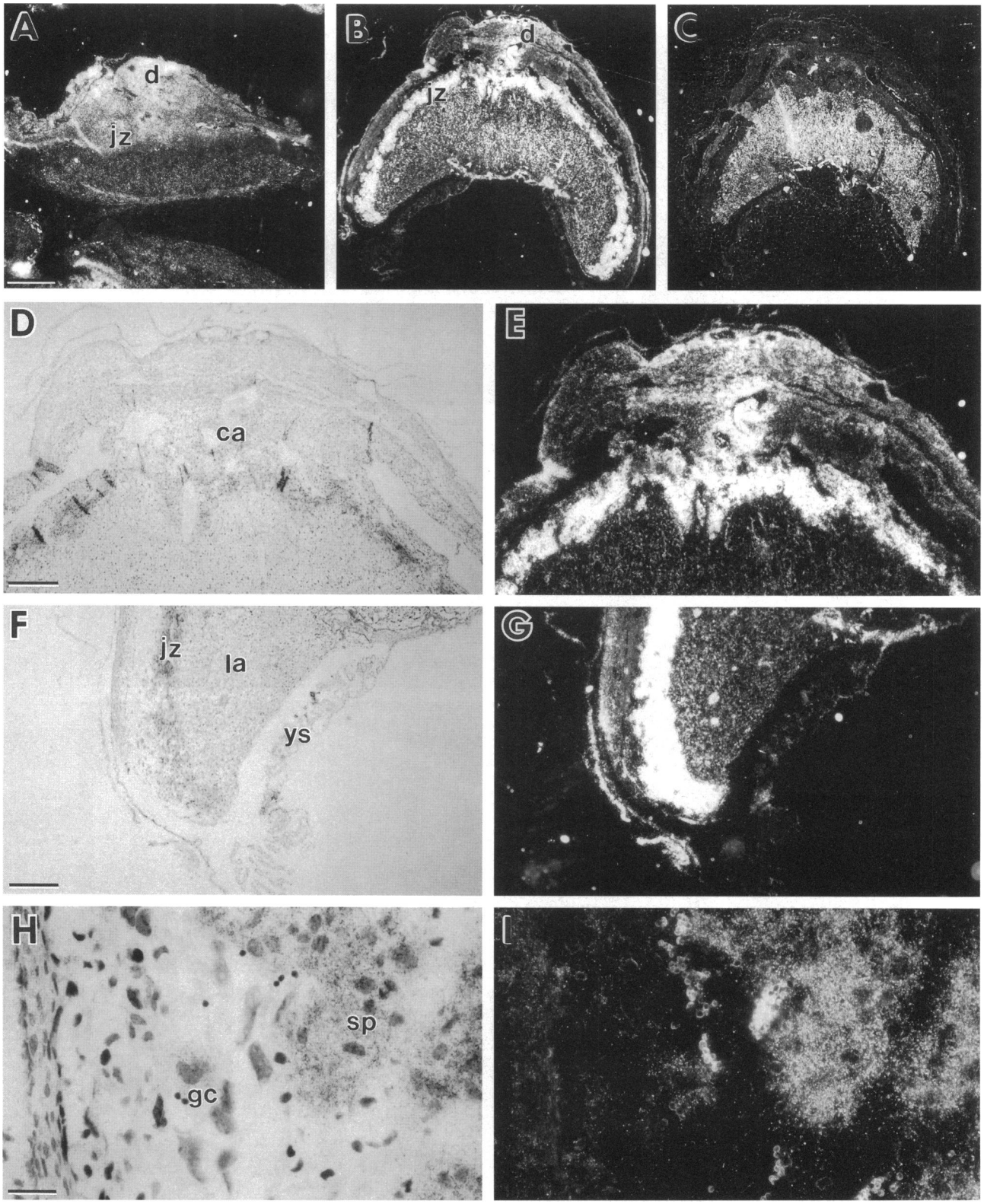


**Figure 7 (cont).** intensively expressed in the spongiotrophoblasts (sp) of the junctional zone which separates the labyrinth (la) from the zone of giant cells (gc). The yolk sac is negative (ys). Bars: A–C, 470 $\mu$m; D–G, 350 $\mu$m; H and I, 35 $\mu$m.

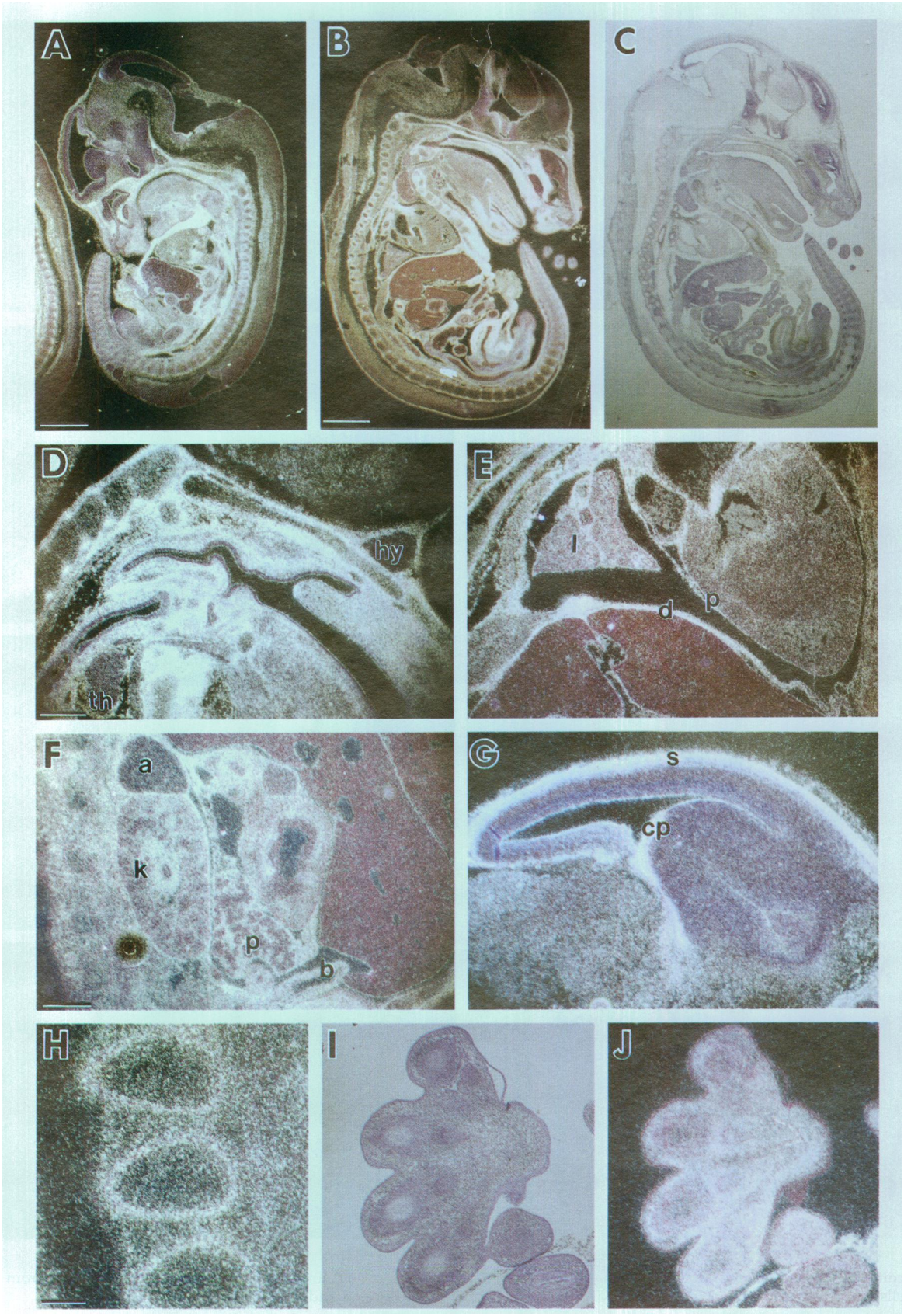


**Figure 8.**

servation in the liver has been made by Northern blot analysis (Waterhouse *et al.*, 1993; Leco *et al.*, 1994) but no studies on the digestive system have been previously reported. Although we did not observe significant levels of expression of all three TIMPs in the spleen and the thymus, low levels of TIMP-1 expression in the spleen (Leco *et al.*, 1994) and of TIMP-1 and TIMP-2 in the thymus (Waterhouse *et al.*, 1993) have been previously reported.

Another intriguing and unexpected observation has been the expression of TIMP-2 in the brain. The pattern of expression was highly specific and the mRNA was restricted to the highly vascularized structures of the arachnoid space in the pia matter and the choroid plexus, in neural tissues, and in particular in the second and fourth layers of the neocortex and in the piriform cortex. Although very low levels of MMP-2 expression in murine brain (Reponen *et al.*, 1992) and expression of MMP-9 in neurons of the human hippocampus (Backstrom *et al.*, 1996) have been described, studies on TIMP expression in the adult brain have not been reported. Our observations of the expression of TIMP-2 in the arachnoid, the pia matter and the choroid plexus suggest that TIMP-2 may play a role in maintaining the integrity of the ECM in these highly vascularized structures. However, the expression of this inhibitor in neural tissue of the neocortex and the piriform cortex remains presently unexplained. In particular, it is unknown whether TIMP-2 is expressed by neurons or by glial cells present in neuronal tissues. Further studies are underway in our laboratory to address this specific point.

Our data on the expression of TIMPs during placental maturation brings some interesting insight into the role of these inhibitors during a physiologically invasive process. Consistent with the work of others (Waterhouse *et al.*, 1993), we did not detect TIMP-1 expression during the second half of gestation. The murine hemochorial type placenta is much more invasive and actively penetrates maternal tissues up to the midsecond week of gestation (Lala and Graham, 1990). MMPs (gelatinases A and B) and TIMPs have been shown to actively participate in the invasive process taking place during the first half of gestation (Reponen *et al.*, 1995; Alexander *et al.*, 1996). Interestingly, we observed a steady increase in TIMP-2 and TIMP-3 expression after the midsecond week as the placenta becomes noninvasive. The data indicate that TIMP-2 may play an active role in preventing further invasion into the maternal tissue. This possibility was further supported by our in situ analysis that revealed a high level of TIMP-2 mRNA expression in the junctional zone of the spongiotrophoblasts between maternal and embryonic tissues at day 17.5 p.c. Interestingly, spongiotrophoblast cells, which are of embryonic origin, belong to the invasive organ. Overexpression of TIMP-2 in these cells during late gestation suggests, therefore, the existence of a self-limiting invasive process in the placenta. Although TIMP-3 expression was not examined by in situ in our studies, a similar pattern of expression for this inhibitor has been reported (Apte *et al.*, 1994). The drop of TIMP-3 expression on day 18.5 (close to term) is currently unexplained, although a shift of the MMPs–TIMPs balance in favor of the MMPs at that stage may be important to allow the placenta to be separated from maternal tissues during delivery.

In situ hybridization analysis of TIMP-2 expression in whole embryo sections revealed an abundant expression in connective tissues surrounding the respiratory and upper digestive epithelium. This is in significant contrast to TIMP-3, which was found to be expressed in oral and nasal epithelium and in some tubular and secretory epithelia of the developing bronchial tree, esophagus, colon, urogenital sinus, and bile duct (Apte *et al.*, 1994). Thus, an important observation made from our studies is the significant difference between TIMP-2 and TIMP-3 expression in embryonic tissue, with predominant expression of TIMP-2 in the mesenchyme and TIMP-3 in the epithelia. Interestingly, a very similar pattern of mesenchymal expression of MMP-2 in 10- to 15-d mouse embryos has been reported (Reponen *et al.*, 1992). As we observed with TIMP-2, these investigators found that MMP-2 was intensively expressed in mesenchymal cells and absent in epithelial tissues of developing organs. They also found no temporal and spatial changes during the advancement of epithelia in branching morphogenesis. These observations further support the concept of a functional interaction between TIMP-2 and MMP-2.

The presence of high levels of TIMP-2 in perichondrial tissues surrounding the cartilage primordia of the spine, foot, and craniofacial bones is noteworthy. Very small levels of TIMP-2 expression around the dorsal ribs at day 13 of mouse development was reported (Mattot *et al.*, 1995); however, our studies revealed much higher levels of expression in the peri-

**Figure 8 (facing page).** Localization of TIMP-2 expression during embryonic development. Analysis of TIMP-2 gene expression by in situ hybridization in embryos. (A–C) Midsagittal sections of embryos at 12.5 (A) and 15.5 (B and C) d p.c. (D) Detail of the oral cavity of a 14.5 d p.c. embryo showing the strong signal surrounding the nasopharyngeal epithelia, and forming bones. Note absence of expression in the thymus (th) and hypophyseal gland (hy). (E) Detail of the section of the chest and upper abdomen of a 15.5 d p.c. embryo. TIMP-2 mRNA is strongly expressed in the diaphragm (d), pericardium (p), and lung (l). (F) Detail of the section of the lower abdomen of a 14.5 d p.c. embryo, showing TIMP-2 expression in the kidney (k), bladder (b), the capsule of the kidney, liver, adrenal (a), and around the glandular epithelium of the pancreas (p). (G) Section of the head of a 14.5 d p.c. embryo showing the presence of TIMP-2 mRNA in the choroid plexus (cp) of the lateral ventricle and in the developing skull (s). (H–J) Sections of the developing spine (H) and developing foot (I and J), showing the presence of TIMP-2 mRNA in the perichondrial tissue. Bars: A, 560 μm; B and C, 800 μm; D–J, 350 μm.

chondrial tissues. This difference may be due to the fact that a human riboprobe was used in the studies of Mattot *et al.* (1995), and a murine TIMP-2 probe was used in our studies. TIMP-2 expression around the cartilage primordia of the mandible, maxilla, basioccipital bone, and ribs of embryos from day 13 to day 16 has been recently reported (Kinoh *et al.*, 1996). TIMP-1 is expressed in areas of new bone formation and in the perichondrium and periosteum (Nomura *et al.*, 1989; Flenniken and Williams, 1990), and TIMP-3 is expressed in hypertrophic chondrocytes of the vertebral cartilage (Apte *et al.*, 1994). In contrast, TIMP-2 expression was absent in hypertrophic chondrocytes of cartilage primordia but present in perichondrial tissue. Because bone development involves the production of MMPs by osteoblasts (Chambers *et al.*, 1985), hypertrophic chondrocytes, and osteoclasts (Blavier and Delaisse, 1995), it is not surprising that TIMPs play an important role in the control of the delicate balance between bone formation and bone resorption. Interestingly, our data suggest a complementary role of the three TIMPs during the bone formation. TIMP-1 seems to play a predominant role in restricting bone resorption during the formation of the bone marrow cavity (Nomura *et al.*, 1989; Flenniken and Williams, 1990), TIMP-3 appears important during endochondral ossification, and TIMP-1 and TIMP-2 may play a particular role in limiting the activity of MMPs present around the forming bone.

Thus, our studies on the temporospatial expression of TIMP-2 in the mouse indicate a specific pattern of expression that significantly differs from that of TIMP-1 and TIMP-3. The data suggest that each TIMP has a specific role during development and in maintaining the integrity of the various ECM. They point to a predominant role of TIMP-2 in the control of the homeostasis of the interstitial matrix. Studies on transgenic mice in which TIMP-2 has been knocked out should be informative in further defining the function of this inhibitor in development.

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