Cell-specific metallothionein gene expression in mouse decidua and placentae

SWAPAN K. DE\textsuperscript{1}, MICHAEL T. McMASTER\textsuperscript{1}, SUDHANSU K. DEY\textsuperscript{2} and GLEN K. ANDREWS\textsuperscript{1}

\textsuperscript{1}Department of Biochemistry and Molecular Biology, and \textsuperscript{2}Departments of Obstetrics - Gynecology and Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, KS 66103, USA

Summary

Oligodeoxyribonucleotide excess solution hybridization, Northern blot and \textit{in situ} hybridization were used to analyze metallothionein gene expression in mouse decidua and placentae during gestation. Metallothionein (MT) -I and -II mRNA levels were constitutively elevated, 11- and 13-fold, respectively, relative to the adult liver, in the deciduum (D8), and decreased coordinately about 6-fold during the period of development when the deciduum is replaced by the developing placenta (D10-16). Coincident with this decline, levels of MT mRNA increased dramatically in the visceral yolk sac endoderm.

\textit{In situ} hybridization established that MT-I mRNA was present at low levels in the uterine luminal epithelium (D4), but was elevated at the site of embryo implantation exclusively in the primary decidual zone by D5, and then in the secondary decidual zone (D6—8). Although low levels of MT mRNA were detected in total placenta RNA, \textit{in situ} hybridization revealed constitutively high levels in the outer placental spongiotrophoblasts. Analysis of pulse-labeled proteins from decidua and placentae established that these tissues are active in the synthesis of MT. The constitutively high levels of MT mRNA in decidua were only slightly elevated following injection of cadmium (Cd) and/or zinc (Zn), whereas in placentae they increased several-fold. MT mRNA levels were equally high in decidua and experimentally induced deciduomata (D8) which establishes that decidual MT gene expression is not dependent on the presence of the embryo or some embryo-derived factor.

Although the functional role of MT during development is speculative, these results establish the concept that, from the time of implantation to late in gestation, the mouse embryo is surrounded by cells, interposed between the maternal and embryonic environments, which actively express the MT genes. This suggests that MT plays an important role in the establishment and maintenance of normal pregnancy.

Key words: metallothionein mRNA, developmental expression, \textit{in situ} hybridization, decidua, placental spongiotrophoblasts, cadmium.

Introduction

Metallothioneins (MTs) are cysteine-rich, heavy-metal-binding proteins present in eukaryotes. Two isoforms of MT (MT-I and MT-II) exist which, in the mouse, differ slightly in amino acid sequence and net charge (Searle \textit{et al.} 1984). Expression of mammalian MT genes can be regulated by a variety of factors, such as essential and toxic metal ions, glucocorticoids, and cytokines (for reviews see: Karin, 1985; Hamer, 1986; Palmiter, 1987; Kagi and Schaffer, 1988). Although little is known about expression of MT genes during early mammalian development, substantial variations in expression of these genes in the fetal and neonatal period have been noted (reviewed by Webb, 1987). Specifically, MT-I and -II are known to be expressed at high levels in the visceral yolk sac endoderm, and later in fetal and neonatal liver (Andrews \textit{et al.} 1984; see Webb, 1987).

The precise functions of MT are unknown (see Karin, 1985). However, the ability to bind to, and be induced by, metals suggests that MT plays a role in homeostasis of essential metals (Zn, Cu) and may provide protection from toxic metals such as Cd. This later hypothesis is supported by the observation that cells that overexpress MT, due to preexposure to metals or to amplification of MT genes, are more resistant to Cd toxicity (Crawford \textit{et al.} 1985). Alternatively, cells that express the MT genes poorly are more sensitive to Cd toxicity (Compere and Palmiter, 1981). In the adult, the liver, kidney and pancreas are the major sites of synthesis of MT following Cd injection, and these are the organs that preferentially accumulate and retain this metal (see Hamer, 1986; Kagi and Schaffer, 1988). Chronic retention of Cd by the kidney results in nephropathy. Thus, protection of the whole animal from Cd toxicity occurs at the expense of renal function.

One of the profound effects of Cd is on the process of embryonic development. Cd has well-documented teratogenic and embryotoxic effects in a large variety of species including man (reviewed by Barlow and Sulli-
van, 1982; Vallee and Ulmer, 1972). Cd also affects the placenta which rapidly degenerates following Cd exposure (Barlow and Sullivan, 1982). Placental transfer of Cd has been studied in the rat, mouse, and hamster (reviewed by Dencker et al. 1983). Apparently, there is limited transfer of Cd across the placenta (0.02% of the total maternal dose). Therefore, the placenta is thought to protect the developing embryo from this toxic metal (Barlow and Sullivan, 1982; Dencker et al. 1983). The mechanism by which the placenta restricts the entry of Cd into the embryonic environment, and the effects of heavy metals on placental MT gene expression are unknown.

Normal development of the embryo is also influenced by the availability of essential metals, such as Zn and copper (Cu), in the maternal diet. A deficiency of Zn is teratogenic and leads to abnormal growth and morphogenesis (see Webb, 1987; Rogers and Hurley, 1987). Zn has a wide array of physiological functions, many of which can be attributed to changes in the activity of Zn metalloenzymes (Cousins, 1986). Zn and Cd interactions have also been described. Zn deficiency leads to heightened Cd toxicity, and Cd reduces the placental transport of Zn (Webb, 1987; Barlow and Sullivan, 1982). It has also been documented that the presence of high levels of Zn can completely prevent the teratogenic and embryotoxic effects of Cd (Warner et al. 1984). The mechanisms by which metal homeostasis is achieved in the early embryo are unknown as are the mechanisms by which Zn protects from Cd effects on the embryo.

Given the likely role for MT in essential metal homeostasis and protection from metal toxicity, cell-specific expression of the MT genes during the early post implantation period was studied. Results are presented which establish, for the first time, that from the time of implantation to late in gestation the embryo is surrounded by cells in the deciduum, placenta and visceral yolk sac, which actively express the MT genes. These cells are positioned between the maternal and embryonic environments and are responsive to exogenous metals. MT in the extraembryonic tissues may, therefore, provide a mechanism for homeostasis of essential metals, and may explain the mechanism by which the deciduum and placenta restrict Cd from access to the embryo. These studies provide a foundation for analysis of regulation of expression of the mouse MT genes during pregnancy.

Materials and methods

Animals

Female CD-1 mice (48 days old; Charles River Laboratories) were mated, and the gestational age of the embryo calculated from the day on which a vaginal plug was detected (designated D1 of gestation). Mice were injected subcutaneously with Zn and/or Cd chloride salts or with vehicle alone (normal saline), and tissue samples recovered 4h after injection. Samples for RNA extraction were obtained as follows: decidua (D8) and decidua/placentae (D10) were dissected free from the uterus, and the embryo and extraembryonic membranes were removed; the visceral yolk sac (D9 to D14) was recovered free of amnion and parietal yolk sac; placenta (D12 to D16) were dissected free from the uterus and extraembryonic membranes. For each experiment, tissues from at least three animals were pooled. Pseudopregnancy was induced by mating with vasectomized males, and the decidual reaction (deciduomata) was induced on D4 by injection of 100 μl of sesame oil into the uterus--tubular junction. Deciduomata tissue was harvested on D7. Samples were quick frozen and stored at −80°C. Samples for in situ hybridization were processed as described below.

Hybridization probes

A mouse MT-I cDNA clone was provided by Dr R. D. Palmiter (University of Washington, Seattle, WA) and a rat placental lactogen-II cDNA clone was provided by M.L. Duckworth (University of Manitoba, Winnipeg, Manitoba). These cDNA clones were inserted into the SP6 vectors (Promega Biotech, Madison, WI) and used as templates for the synthesis of 32P or 35S-labeled RNA probes as described by Melton et al. (1984). A MT sense strand probe was also synthesized. Probes had specific activities of about 2×106 disintegrations min⁻¹ μg⁻¹. Oligonucleotide monomers (27 nucleotides) complementary to 3' untranslated regions of mouse MT-I and MT-II were synthesized with the following sequences: MT-I, 5'-GGGTGAACTGTATAGGAAGACGCTGG-3'; MT-II, 5'-GGTCTATTTCACAGATGTGGGGACCC-3' (Searle et al. 1984). These probes were 32P 5' end-labeled using T4 polynucleotide kinase (Bethesda Research laboratories, Gaithersburg, MD) according to the manufacturers' suggestions. These probes had a specific activity of about 2×106 disintegrations min⁻¹ μg⁻¹.

Isolation of total RNA

RNA was extracted using the sodium dodecylsulfate (SDS)–phenol–chloroform procedure described in detail by Andrews et al. (1987a). Frozen samples were homogenized in 0.5% SDS, 25 mm-EDT A, 75 mm-NaCl, pH8.0 (SDS-buffer), extracted with phenol, and then with phenol/chloroform:isoamyl alcohol (24:1 v/v). RNA was precipitated from the aqueous phase with 3 m-ammonium acetate, and reprecipitated twice more to remove DNA. The RNA pellet was dissolved in water and precipitated with ethanol.

Northern blot hybridization

RNA (20 μg) was denatured for 5 min at 60°C, in a solution of 1× 3-(4-morpholino) propane sulfonic acid (MOPS) buffer (20 mM-MOPS, 5 mM-sodium acetate, 1 mM-EDTA, pH 7.0) containing 50% formamide and 2.2 M-formaldehyde. Denatured RNA (6 μg in 15 μl) was separated by electrophoresis in a 1.5% agarose gel (5×7.5 cm) containing 1× MOPS buffer and 2.2 M-formaldehyde (Lerch et al. 1977). During electrophoresis, gels were submerged in running buffer (2.2 M-formaldehyde and 1× MOPS). Electrophoresis was carried out by applying a constant voltage (45 V) across the gels. Following electrophoresis, gels were soaked for 40 min in 10 mM-sodium phosphate (pH 7.0), and transferred to nitrocellulose in the presence of 20×SSC (3 M-NaCl and 0.3 M-sodium citrate, pH 7.4) as described by Thomas (1980). Following transfer, the filters were baked in a vacuum oven at 75°C, for 5 h. Northern blots were prehybridized, hybridized, and washed as described in detail by Andrews et al. (1987a). In all experiments, duplicate gels were stained with acridine orange to ensure integrity of the RNA sample and to confirm that equal amounts of RNA had been loaded onto each lane.
Oligodeoxyribonucleotide excess hybridization

Solution hybridization was used to quantify MT-I and MT-II mRNA levels according to the methods of Durnam and Palmiter (1983) as modified by Omiecinski et al. (1985). Total RNA (0 to 40 μg) was dissolved in 30 μl of buffer (0.75 M-NaCl, 150 mM-Tris, pH 8.0, 10 mM-EDTA) containing 2 ng ml⁻¹ of ³²P-labeled oligodeoxyribonucleotide (20 000 cts min⁻¹). This mixture was incubated under a drop of silicone oil at 60°C for 18 h. S1 nuclease resistant hybrids were assayed by dilution of the reaction mixture with 1 ml of buffer (0.75 M-NaCl, 70 mM-NaAcO, pH 4.5, 3 mM-ZnSO₄, 100 μg ml⁻¹ sonicated calf thymus DNA) containing 16 units ml⁻¹ of S1 nuclease (Bethesda Research laboratories, Gaithersburg, MD). This mixture was incubated at 37°C for 1 h, and the amount of S1 resistant radioactivity determined as described previously (Durnam and Palmiter, 1983). In the absence of RNA about 1% of the probe was S1 resistant, and maximal hybridization was 75 to 97% of the input radioactivity. RNA from control liver and Zn-treated liver served as comparative standards for basal and high levels of MT mRNA, respectively.

In situ hybridization

The methods for in situ hybridization have been adapted from procedures published by Angerer and colleagues (Angerer and Angerer, 1981; Deleon et al. 1983; Cox et al. 1984; Angerer et al. 1984). Pregnant mice, on the indicated days of gestation, were anesthetized with Avertin, and uteri fixed by perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS). Implantation sites or placentae were further fixed at 4°C for 2 h, washed twice in PBS, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Paraffin blocks were serially sectioned at 7 μm, and sections were mounted on poly-L-lysine-coated slides. Within a given experiment, sections from all experimental samples were mounted onto the same microscope slide, and several slides were prepared. Sections were deparaffinized, rehydrated, and then acetylated (Hayashi et al. 1978). Slides were incubated at room temperature for 10 min each in: PBS containing 5 mM-MgCl₂; and 0.25 mM-Tris, 0.1 mM-glycine, pH 7. After incubation at 37°C in 50% formamide, 2×SET (1×SET=150 mM-NaCl, 5 mM-EDTA, 10 mM-Tris-HCl, pH 8.0), slides were hybridized for 5 h at 42°C in a humidified environment with 100 μl of buffer containing; 2×SET, 10×Denharts' solution (0.2% each of bovine serum albumin, ficoll and polyvinylpyrrolidone) (Denhart, 1966), 250 μg ml⁻¹ yeast tRNA, 50% formamide, 100 mM-DTT, 10% dextran sulfate, and about 0.05 μg ml⁻¹ of ³⁵S-labeled cRNA probe. After hybridization, the siliconized coverslip was removed by washing in 4×SSC. Slides were incubated at 37°C for 30 min with RNase A (20 μg ml⁻¹ RNase A, 3×SET, 100 μg ml⁻¹ BSA), washed in a large volume of 1×SSC, and then subjected to a final 30 min wash at 50°C in 0.2×SSC, 0.1% mercaptoethanol. Autoradiography was with Kodak NTB-2 liquid emulsion for 4 days and slides were poststained lightly in hematoxylin.

Determination of relative rates of MT synthesis

Decidua and placentae were dissected as described above and tissues were teased apart in phosphate-buffered saline (PBS). Samples (7 to 14 decidua or placenta) were transferred into cysteine-free Minimal Essential Medium (3 ml) containing 150 μCi ml⁻¹ ³⁵S-cysteine (specific activity of 1000 Ci mm⁻¹; New England Nuclear, Boston, MA) and incubated at 37°C for 3 h. After labeling, the samples were washed twice in PBS and homogenized in 700 μl of buffer (Hamer and Walling, 1982) containing 1% Nonidet P40, 50 mM-Tris (pH 7.4), 100 mM-

![Fig. 1. MT-I mRNA levels in mouse decidua, placenta and visceral yolk sacs. RNA was extracted from decidua or placenta (A), and visceral yolk sacs (B) on the indicated days of gestation (D1=vaginal plug). Total RNA (6 μg) was fractionated by formaldehyde–1.5% agarose gel electrophoresis, and analyzed by Northern blot hybridization using a mouse MT-I cRNA probe. In A samples were as follows: lane 1, D8 deciduum; lane 2, D10 deciduum/placenta; lane 3, D12 placenta; lane 4, D14 placenta; lane 5, D16 placenta. For comparative purposes, normal adult liver (lane 6, AL) total RNA, which contains basal levels of MT mRNA, was also analyzed by Northern blotting. In all experiments, duplicate gels were stained with ethidium bromide to ensure integrity of the RNA sample and to confirm that equal amounts of RNA had been loaded onto each lane.](image-url)
NaCl, 5 mM dithiothreitol (DTT). The soluble proteins were recovered by centrifugation at 15,000 g for 15 min at 4°C, and the proteins in the supernatant were carboxymethylated by incubation with 0.5 volumes of 0.3 M-iodoacetic acid in 1 M-Tris (pH 8.4) for 45 min in the dark at room temperature. The samples were then incubated at 80°C for 8 min and the heat-stable proteins recovered in the supernatant following centrifugation at 15,000 g for 20 min at 4°C. An equal volume of 2x sample buffer (125 mM-Tris (pH 6.8), 2.5% β-mercaptoethanol, 25% glycerol, 0.5% bromophenol blue) was added and equal amounts (80,000 cts min⁻¹) of trichloroacetic acid-precipitable radioactivity were separated by 20% polyacrylamide gel electrophoresis. Gels were fixed and stained with Coomassie blue, soaked for 1 h in Enlightening (New England Nuclear), dried and exposed to Kodak XAR-5 film at -70°C. Purified carboxymethylated MT served as a reference standard.

Table 1. Metallothionein-I and -II mRNA levels in decidua and placentae during gestation

<table>
<thead>
<tr>
<th>Age*</th>
<th>MT Isoform†</th>
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<tr>
<td>8</td>
<td>11.0 13.4</td>
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<tr>
<td>10</td>
<td>10.0 8.9</td>
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<td>12</td>
<td>4.6 4.9</td>
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<tr>
<td>14</td>
<td>3.0 2.2</td>
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<tr>
<td>16</td>
<td>N.D.</td>
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* Age refers to day of gestation. † Data represent fold-increase in MT-I and MT-II mRNA levels relative to those in control adult liver, as determined by oligodeoxyribonucleotide excess solution hybridization. Liver RNA, obtained 5 h following injection of 100 μmol kg⁻¹ of ZnCl₂ contained 43.0- and 23.0-fold more MT-I and MT-II mRNA than control liver, respectively. Deciduum (D8), deciduum/placenta (D10) and placenta (D12 to 16) were obtained from the conceptuses from 3 to 6 pregnant females, and the results represent the average of three determinations. N.D. means not determined.

Fig. 2. MT synthesis in mouse decidua and placentae. Explants of decidua and placentae were pulse-labeled with [³⁵S]cysteine. Soluble proteins were carboxymethylated, and the heat-stable proteins (80,000 cts min⁻¹) separated by 20% native polyacrylamide gel electrophoresis and detected by fluorography (Hamer and Walling, 1982). Samples were as follows: lane 1, D8 deciduum; lane 2, D10 deciduum/placenta; lane 3, D12 placenta; lane 4, D14 placenta. The electrophoretic migration of purified carboxymethylated rat MT is indicated. The isoforms of MT migrated very near each other in these gels.

Fig. 3. Effects of heavy metals on MT-I mRNA levels in mouse decidua and placenta. Pregnant females were injected subcutaneously with metals on D8 or D14 of gestation, and 4 h later RNA was extracted from decidua (A) and placenta (B). Total RNA (6 μg) was fractionated by formaldehyde-1.5% agarose gel electrophoresis, and analyzed by Northern blot hybridization using a mouse MT-I cRNA probe. For comparative purposes, normal adult liver (AL) total RNA, which contains basal levels of MT mRNA, was also analyzed. (A) lane 1, (C) control; lane 2, (Cd) 50 μmol cadmium kg⁻¹ body weight (teratogenic dose); lane 3, (Cd/Zn) 50μmol cadmium kg⁻¹ plus 100 μmol zinc kg⁻¹ body weight (all embryos developed normally); lane 4, (Zn) 100 μmol zinc kg⁻¹ body weight. The upper band of hybridization in the autoradiograph (A) may represent MT-I mRNA which was aggregated with 18S ribosomal RNA during electrophoresis or unprocessed MT-I nuclear RNA. (B) Samples of RNA from the D14 placenta were as follows; lane 1, control (0); lane 2 through 5, the indicated dosages of cadmium (μmol kg⁻¹ body weight) were injected. Injection of 75 μmol cadmium kg⁻¹ body weight resulted in rapid embryonic death followed by maternal lethality within 24 h. Lane 6, control adult liver.
Metallothionein mRNA in decidua and placenta

Results

MT gene expression in decidua, placentae and visceral yolk sac

Northern blot hybridization demonstrated that decidua contained high levels of MT-I mRNA on D8 through D10, but as the placenta developed and replaced the deciduum, MT-I mRNA levels decreased several-fold (Fig. 1A). By D16, placental MT-I mRNA levels were similar to those found in normal adult liver (Fig. 1A). Solution hybridization, using oligodeoxyribonucleotides complementary to MT-I or MT-II mRNAs, showed that both messengers were elevated in decidua, and they were coordinately reduced during placental development (Table 1). In comparison with control adult liver RNA, D8 decidual MT-I and MT-II mRNA levels were elevated 11- and 13.4-fold respectively (Table 1). The basal levels of MT-I mRNA in the adult liver reflect about 150 to 350 molecules per cell (Durnam and Palmiter, 1981, 1983). Thus, both MT-I and MT-II mRNAs were in high abundance in decidua. During formation and maturation of the placenta, levels of MT-I and MT-II mRNAs declined about 6-fold (Table 1).

The visceral yolk sac, by D14 of gestation, contained high levels of MT-I mRNA (Fig. 1B). This expression, which has been reported previously, is restricted to the visceral endoderm (Andrews et al. 1984). During the early stages of embryogenesis (D9), MT-I mRNA levels were low in the visceral yolk sac (Fig. 1B) as were MT-II mRNA levels (data not shown). Therefore, incomplete dissection of the visceral yolk sac could not account for the MT-I and -II mRNA levels detected in decidual RNA. Furthermore, RNA samples from the embryo and the uterus on D9 and D11 do not contain significant levels of MT mRNAs (data not shown). These results establish that the mouse MT genes are coordinately expressed in a tissue-specific and temporal manner during gestation: first in the decidua, and later in the visceral endoderm.

To determine whether synthesis of MT occurs in the decidua and placentae, tissue explants were pulse-labeled with [35S]cysteine. Newly synthesized MT was preferentially labeled under these culture conditions due to its high cysteine content (32 mol%) (Hamer, 1986). Labeled cytosolic proteins were carboxymethylated, and the heat-stable proteins were separated on a 20% polyacrylamide gel (Hamer and Walling, 1982) and detected by fluorography (Fig. 2). Under these conditions, MT has a characteristic mobility which is faster than other cysteine-rich proteins in these extracts (Hamer and Walling, 1982; Andersen et al. 1983; Andrews et al. 1987a). Decidua and placentae were active in the synthesis of a small cysteine-rich, heat-stable protein which comigrated with authentic carboxymethylated MT. These results establish that MT mRNA is actively translated in decidua and placentae. However, the relative rate of synthesis of MT remained high in the D14 placenta despite the decline in MT mRNA levels in total RNA at this stage.

Analysis of regulation of decidual and placental MT gene expression

The effect of heavy metals, which are potent inducers of MT gene expression in adult tissues (Hamer, 1986; Palmiter, 1987), on decidual and placental MT gene expression was examined. Animals were injected on D8 with 50 μmol kg⁻¹ body weight of Cd which caused developmental abnormalities (neural tube and limb bud defects and severe growth retardation) in all of the embryos. Co-injection of 100 μmol Zn kg⁻¹ completely prevented the teratogenic and embryotoxic effects of Cd (data not shown) as has previously been documented (Warner et al. 1984; see Dencker et al. 1983). Northern blot hybridization demonstrated that decidual MT mRNA levels on D8 were only slightly elevated in response to metals, regardless of whether the metal was Cd and/or Zn (Fig. 3A). This likely reflects the already high levels of MT mRNAs in decidua. However, these results suggest that it is unlikely that zinc prevents the teratogenic effects of cadmium by short-term changes in decidual MT-I gene expression.

In contrast to the D8 deciduum, placental (D14) MT-I gene expression was markedly enhanced by Cd (Fig. 3B), and the maximal response was obtained following injection of a subtoxic dose (25 μmol Cd kg⁻¹). Zinc also enhanced placental MT mRNA levels (data not shown). These results establish that the
Fig. 5. Localization of MT-I mRNA in mouse decidua using in situ hybridization. Pregnant mice, on the indicated days of gestation, were anesthetized, and tissues were fixed by perfusion with 4% paraformaldehyde in PBS. Samples were paraffin embedded and serially sectioned at 7 μm. MT-I mRNA was hybridized in situ for 5 h at 42°C with a 35S-labeled MT-I cRNA probe and RNase A-resistant hybrids were detected following 4 days of autoradiography using Kodak NTB-2 liquid emulsion. Slides were poststained lightly in hematoxylin. (A) Bright-field and dark-field photomicrographs (magnification is 40×) of a section of the D4 uterus. Abbreviations are as follows: LE, luminal epithelium; GE, glandular epithelium; CM, circular muscle; LM, longitudinal muscle; S, stroma. (B) Shown here are dark-field photomicrographs of sections of deciduum which were hybridized on the same microscope slide. Magnification is 40×, except for day 6B which is 100×. PDZ; primary decidual zone; SDZ; secondary decidual zone. (C) Dark-field photomicrographs (magnification is 40×) of sections of the D7 deciduum after in situ hybridization with antisense or sense strand MT probes.
B. DECIDUA

Day 5

Day 6A

Day 6B

Day 8
Day 12

**Fig. 6. Localization of MT-I mRNA in mouse placentae by in situ hybridization.** Placentae, on the indicated days of gestation, were analyzed by in situ hybridization as described in the legend to Fig. 5. Autoradiography was for 4 days (sections were on the same slide), and slides were poststained lightly with hematoxylin. Shown here are dark-field photomicrographs (40x magnification) of longitudinal sections near the outer edge through the midregion of placentae. Day 12A, MT-I mRNA localization; GC, giant trophoblast cells; ST, spongiotrophoblast cells. Day 12B, placental lactogen-II mRNA localization. Day 14; MT-I mRNA localization; ST, spongiotrophoblast; LT, labyrinthine trophoblast; zinc-treated, placenta taken 4h following injection of 100 μmol zinc kg⁻¹ body weight.

Low levels of MT mRNA in the D14 placenta are not the result of an inability of the placental MT genes to respond to metals and suggest that placental MT levels may respond to changes in maternal Zn levels. In order to examine the involvement of the embryo in the regulation of decidual MT gene expression, the decidual reaction was induced by injection of sesame oil into the utero–tubular junction of pseudopregnant mice on D4. The experimentally induced deciduomata tissue was harvested on D8 and RNA was analyzed by northern blot hybridization (Fig. 4). Levels of MT mRNA in deciduomata were nearly identical to those in the normal D8 deciduum (Fig. 4, lane 1 and 2) and injection of 100 μmol Zn kg⁻¹ lead to only a slight increase in MT mRNA in deciduomata (Fig. 4, lane 3). Therefore, it is apparent that decidual MT gene expression is not dependent on the presence of the embryo or some embryo-derived factor.

Cell type-specific MT gene expression in decidua and placenta

*In situ* hybridization was used to examine the cellular distribution of MT-I mRNA during decidual and placental development (Figs 5 and 6). Initial experiments showed that MT-I mRNA is specifically localized in the visceral yolk sac endoderm, as previously reported (Andrews et al. 1984), and in the parenchymal cells of the fetal liver as expected (data not shown). The intensity of the autoradiographic signal increased dramatically in those cells following metal injection, and in the adult, detection of MT-I mRNA in liver and pancreas was dependent on pretreatment with metal (data not shown). These results, and the use of other cRNA probes (see below), provide positive controls for the specificity of the *in situ* hybridization technique. Lack of hybridization in specific cells and tissues or with sense strand MT probes provide appropriate negative controls for this technique.
MT-I mRNA levels, detected by Northern blot hybridization, were very low in the uterus from D1 to 4, but increased soon after implantation (data not shown). MT-I mRNA was localized on D4 in the uterine luminal epithelium, but at apparently very low levels (Fig. 5A). However, on D5, MT-I mRNA was specifically elevated in the primary decidual zone at the site of blastocyst implantation, while the deeper uterine stroma and myometrium were negative (Fig. 5B). MT-I mRNA was down-regulated in the primary decidua (Fig. 5B; DAY 6B), and expressed at high levels specifically in the newly forming secondary decidual zone on D6. By D8, MT-I mRNA was located in the peripheral secondary decidual zone (Fig. 5B). No hybridization was detected using the sense strand MT probe (Fig. 5C).

Although Northern blot analysis showed that levels of MT mRNAs declined during formation of the placenta (Fig. 1), in situ hybridization demonstrated that spongiotrophoblasts, which are located in the outer placenta, constitutively express high levels of this mRNA (Fig. 6). In contrast, the trophoblast giant cells (outer placenta), and labyrinthine trophoblasts (inner placenta) contained little MT-I mRNA. However, some MT-mRNA-positive cells were detected in the trophoblast giant cell region and these represent decidual cells or spongiotrophoblasts. The trophoblast giant cells, however, contained placental lactogen-II mRNA (Duckworth et al. 1986) (Fig. 6, DAY 12B), and these cells a major source of synthesis of placental lactogens (Lee et al. 1988). These results also confirm the reliability of the in situ hybridization technique. The demarcation between the high level of MT-I mRNA in the spongiotrophoblasts, and the low level of this mRNA in the labyrinthine trophoblasts on D14 is striking (Fig. 6; DAY 14, control). Although the visceral yolk sac attaches to the central portion of the labyrinthine placenta, and the visceral endoderm contains high levels of MT mRNA, only the outermost regions of the placenta are shown here (Fig. 6). The relative difference in MT-I gene expression between the spongiotrophoblasts and the labyrinthine trophoblasts was maintained following injection of metal. Metal ions induced MT-I mRNA to extremely high levels in the spongiotrophoblasts, and increased the basal levels found in the labyrinthine trophoblasts (Fig. 6; DAY 14, zinc-treated). This pattern of placental MT-I mRNA distribution was also detected following injection of Cd, and Cd plus Zn (data not shown). These results establish that MT-I gene expression is regulated in a cell- and temporal-specific manner during formation and maturation of the mouse deciduum and placenta.

Discussion

In the present study, expression of the mouse MT genes in decidua and placenta was examined, and it was found that MT is expressed in a cell-specific and temporally regulated manner during pregnancy. The results strengthen the concept that MT plays a role in embryonic development by establishing that, from the time of implantation to late in gestation, the embryo is surrounded by cells that actively express the MT-I gene: first, the decidium, and then the spongiotrophoblasts of the placenta, and the visceral yolk sac endoderm. These cell types are each positioned between the maternal and embryonic environments.

The functions of MT during development are speculative. However, hepatic MT is high in the fetus and newborn of all mammals (reviewed by Webb, 1987). This has led to the suggestion that a mechanism for control of Zn and Cu metabolism exists in the liver during late gestation. Zn is an essential metal required by many important enzymes involved in DNA replication, transcription and translation. Deficiency of Zn leads to teratogenic effects on skeletal, brain, heart and eye morphogenesis (Rogers and Hurley, 1987), and reduced DNA synthesis in the embryo (Eckert and Hurley, 1977; Record and Dreosti, 1979). The teratogenic period in the mouse is early during morphogenesis (D8 to D10), just before or during the early phases of organogenesis. The elevated expression of MT in the decidua reported here (Figs I and 5) suggests a role in Zn and Cu homeostasis during the early post implantation period. This function could subsequently be provided by the placental spongiotrophoblast cells and visceral endoderm. It has been shown that during late gestation the transfer of Zn across the placenta increases (Andersen et al. 1983). Our earlier work suggested that MT mRNA was at very low levels in placenta, amnion and brain (Andrews et al. 1984). However, the Northern blot analysis of total RNA from placenta was misleading, and, as reported here, high levels of MT gene expression occur in the placental spongiotrophoblasts. This suggests the importance of studying the tissue specificity of MT gene expression using in situ hybridization. With regard to placental MT, others have reported MT-like proteins in human (Waalkes et al. 1984), and rat placenta (Charles-Shannon et al. 1981), and Cd induces MT protein in mouse and rat placenta (see Webb, 1979).

The elevated cell-type-specific expression of MT in decidua and placenta provides a plausible explanation of the mechanism by which Cd is rapidly sequestered in these structures. Studies of metal disposition in the embryo and fetus demonstrate that Cd is almost totally prevented from reaching the embryo (Dencker et al. 1983), and is accumulated in the placenta-yolk sac. We have found that the level of MT mRNA is very low in the embryo (D9 and 11), and that teratogenic doses of Cd may increase that level somewhat (data not shown). More experiments are required in order to clearly delineate direct metal effects on the embryo and the potential involvement of MT in the barrier function of the deciduum and placenta.

What regulates decidual and placental MT gene expression is unknown. Maternal systemic effects have been suggested to influence hepatic MT expression in the fetus. Glucocorticoids increase in the maternal circulation most dramatically after D10 gestation (Barlow et al. 1974), and appear to enhance both maternal and...
and fetal hepatic MT gene expression in the mouse (Quaife et al. 1986). Maternal dietary Zn deficiency causes reduced hepatic MT mRNA levels in the newborn rat (Andrews et al. 1987b) and mouse (Vruwink et al. 1988). Elevated expression of decidual MT (DS) seems unlikely to involve either elevated Zn or glucocorticoids in the maternal serum because maternal hepatic MT mRNA levels are low at this stage of gestation (Quaife et al. 1986; Andrews, G. K., unpublished observation). The finding that MT mRNA is low in the preimplantation uterus also suggests that ovarian steroids are not directly involved in regulation of this gene. During this period (D1 to 4) there are substantial changes in uterine gene expression in response to changing levels and types of ovarian steroids (Huet-Hudson et al. 1989). It is conceivable that some local effect may be mediating expression of deciduoid and placental MT. Analysis of MT gene expression in artificially induced deciduomata suggests that deciduoid MT expression does not require the presence of the embryo or some embryo-derived factor. An alternate possibility is that macrophages, which are present around the implantation chamber at the time of implantation in the rat (Tachi et al. 1981), may release monokines which, in turn, induce MT gene expression (see Palmiter, 1987). Clearly this matter remains to be resolved and other equally plausible explanations can be invoked.

In summary, it is apparent that cell-specific expression of the MT genes occurs throughout gestation in the mouse. In particular, this study demonstrates, for the first time, that those cells that are interposed between the fetal and maternal environments (decidua, placental spongiotrophoblasts, visceral yolk sac endoderm) display heightened expression of MT. This suggests that MT may play an important role in the establishment and maintenance of normal pregnancy.

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