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α_2-Macroglobulin gene expression during rat development studied by *in situ* hybridization

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The sites of α_2-macroglobulin mRNA synthesis during rat development have been localized by *in situ* hybridization using a rat α_2-macroglobulin cDNA probe. Fetal liver was found to be the major site of α_2-macroglobulin mRNA synthesis. In addition, α_2-macroglobulin mRNA was detected in brain, spinal cord and eye. α_2-Macroglobulin mRNA was quantitated by use of a sensitive RNAse protection assay. Maximal levels of α_2-macroglobulin mRNA were found in fetal livers shortly before birth. A rapid decline of α_2-macroglobulin mRNA occurred within 1 day after parturition. A similar time course, although at an ~20-fold lower level, was observed for α_2-macroglobulin mRNA in livers of pregnant rats. α_2-Macroglobulin mRNA could also be detected in placenta. The levels were comparable to those found in maternal livers.

Key words: gene expression/*in situ* hybridization/α_2-macroglobulin/pregnancy/rat development

Introduction

Many proteolytic reactions occur in blood and require control. Proteinase inhibitors which are synthesized in the liver and secreted into the blood play an important role in the regulation of proteinase activity. Particularly during inflammatory processes proteinases such as elastase, collagenase and cathepsin G are released by granulocytes and macrophages (Koj, 1974; Kushner, 1982; Koj *et al.*, 1982; Fritz and Jochum, 1984; Koj, 1985). Due to their capability to destroy connective tissues in many organs these proteinases represent a severe hazard for the organism. In the rat one of the major proteinase inhibitors, which is synthesized in response to acute inflammation, is α_2-macroglobulin (α_2M). It has a mol. wt of about 700 000 and consists of four probably identical subunits (Gordon, 1976; Okubo *et al.*, 1981; Nelles and Schnebli, 1982). α_2M inhibits the majority of proteinases regardless of their catalytic mechanism by means of proteinase trapping (Barrett and Starkey, 1973; Harpel, 1973; Starkey and Barrett, 1979; van Leuven, 1982). During acute inflammation the α_2M plasma levels increase from 0.01 mg/ml up to 2 mg/ml plasma (Okubo *et al.*, 1981; Schreiber and Howlett, 1983; Northemann *et al.*, 1983a). This increase in plasma is preceded by a corresponding increase in α_2M mRNA levels (Northemann *et al.*, 1983a,b; Hayashida *et al.*, 1985; Schreiber *et al.*, 1986) as well as an increased gene transcription in the liver (Northemann *et al.*, 1985). In addition to the fact that α_2M is strongly induced during acute inflammation, it is well known that α_2M increases in serum of pregnant and fetal rats (Weimer *et al.*, 1967; Hudig and Sell, 1979; Bell, 1979; Panrucker *et al.*, 1983a). In these studies, however, only α_2M protein levels could be measured and no conclusions on the site of α_2M synthesis within an organism could be drawn.

Recently we have isolated a rat α_2M cDNA clone (Northemann *et al.*, 1985), which we have used in this study for *in situ* hybridization to identify the sites of α_2M gene transcription during rat development.

Results

To verify the specificity of the *in situ* hybridization we have carried out the following experiments. Cryosections of livers from normal and turpentine-treated rats were hybridized to a ^{32}P-labelled α_2M cDNA insert excised by *Pst*I from the recombinant plasmid pα_2M1.

Figure 1 illustrates that the liver section from the turpentine-treated rat (b) exhibits a much stronger hybridization signal than the one from a control animal (a). This finding is in agreement with previous observations that during acute inflammation α_2M mRNA levels increase drastically (Northemann *et al.*, 1983a,b, 1985; Hayashida *et al.*, 1985; Schreiber *et al.*, 1986). Additional proof that RNA sequences are responsible for the hybridization signals was obtained from an RNase digestion experiment. Pretreatment of the cryosections with RNase A effectively abolished the hybridization signal (Figure 1c). As a further control we have used ^{32}P-labelled pBR322 for the hybridization. When this vector DNA, completely unrelated to α_2M was used, no hybridization was detected (Figure 1d–f).

Identical *in situ* hybridization conditions were used to localize

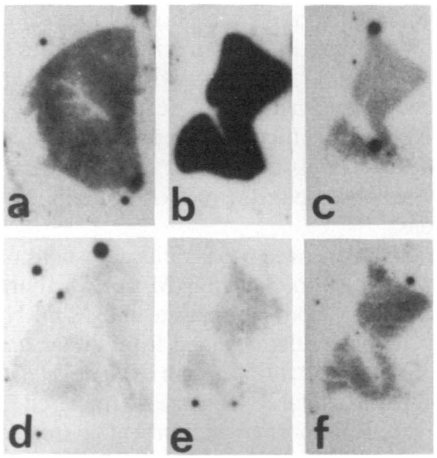

Fig. 1. *In situ* hybridization to liver sections from control and turpentine-treated rats. Liver sections were prepared from control (**a,d**) and inflamed animals (**b,c,e,f**). Acute inflammation was induced by the intramuscular injection of 0.8 ml turpentine per 200 g body weight and the livers were removed 14 h afterwards. ^{32}P-Labelled α_2M cDNA (**a–c**) or ^{32}P-labelled pBR322 vector DNA (**d–f**) was hybridized to the liver sections as detailed in Materials and methods. As a control, liver sections from turpentine-treated animals (**c,f**) were treated with 50 μg/ml RNase A for 90 min at 37°C prior to the hybridization.

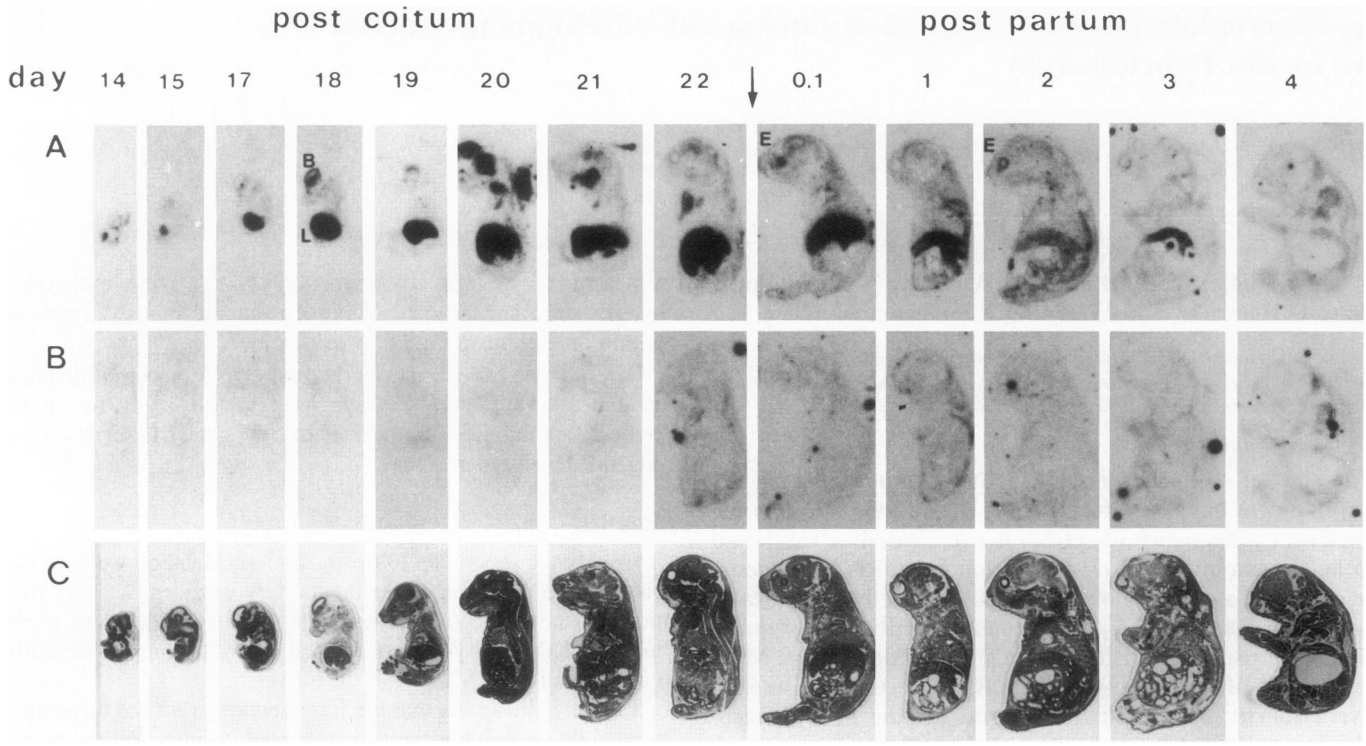

Fig. 2. *In situ* hybridization of α_2M cDNA to whole body sections of fetal and newborn rats. ^{32}P-Labelled α_2M cDNA (**A**) or ^{32}P-labelled pBR322 vector DNA as control (**B**) was hybridized to saggital whole body sections from fetal and newborn rats at different stages of development, which were subsequently stained with H&E (**C**). Autoradiography was for 5 days. The arrow separates the sections of fetal from newborn animals. B, Brain; E, eye; L, liver.

α_2M mRNA in whole body sections of rats of various developmental stages. Figure 2A shows that the most prominent site of α_2M mRNA synthesis is the liver. Hybridization with ^{32}P-labelled pBR322 DNA did not give positive signals (Figure 2B).

As early as 14 days after fertilization α_2M mRNA can be detected. α_2M mRNA concentrations increase during gestation and sharply decrease after birth. The increase in α_2M mRNA concentrations during gestation is more evident on an autoradiograph, which had been exposed for a shorter time (not shown). The longer time of exposure was chosen to reveal α_2M mRNA present in low amounts in other organs and tissues. On the sections obtained from 17- and 18-day-old fetuses and 0.1- and 2-day-old newborn rats α_2M mRNA can be detected in brain and eye, respectively. The difficulty in preparing cryosections in identical planes from the rats at different developmental stages may explain why we observed α_2M mRNA in brain and eye only in a few sections.

Figure 3a and b illustrates two additional sections of rat fetuses, where brain is shown to contain α_2M mRNA. Furthermore, it can be seen that spinal cord is also a site of α_2M mRNA synthesis.

To quantitate the results obtained from the *in situ* hybridization, we have isolated total RNA from livers of fetal and newborn rats. For comparison total RNA was also prepared from materal livers and livers from normal and turpentine-treated adult rats. To prevent an underestimation of the rather large and often partially degraded ~5000 nt-long α_2M mRNA (Northemann *et al.*, 1985; Hayashida *et al.*, 1985), we determined the amount of α_2M mRNA by quantitation of a 133-nt internal fragment using a complementary, single-stranded RNA probe as described recently (Melton *et al.*, 1984). This method also avoids the problems caused by unspecific hybridization of the probe with contaminating DNA, when RNA is quantitated by dot−blot analysis. This unspecific hybridization can be observed on Northern-blots where

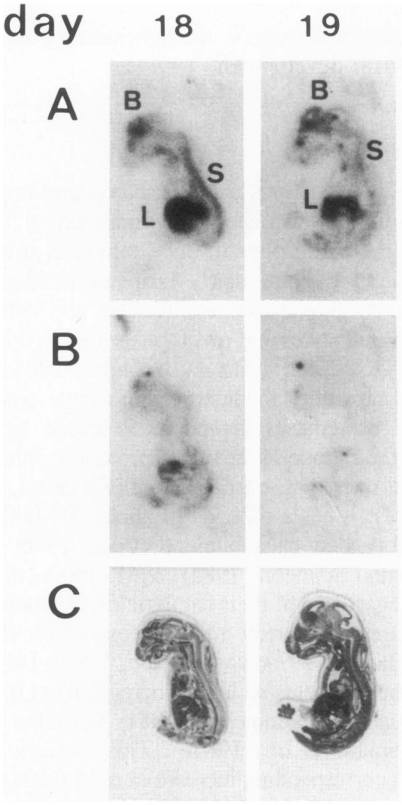

Fig. 3. Whole body *in situ* hybridization demonstrating α_2M mRNA synthesis in fetal brain and spinal cord. ^{32}P-labelled pα_2M1 plasmid DNA (**A**) or ^{32}P-labelled pBR322 plasmid DNA as control (**B**) was hybridized to saggital whole body sections from rat fetuses from 18 and 19 days of gestation. The sections were exposed to X-ray film for 2 and 5 days, respectively. (**C**) H&E-stained sections. B, Brain; L, liver; S, spinal cord.

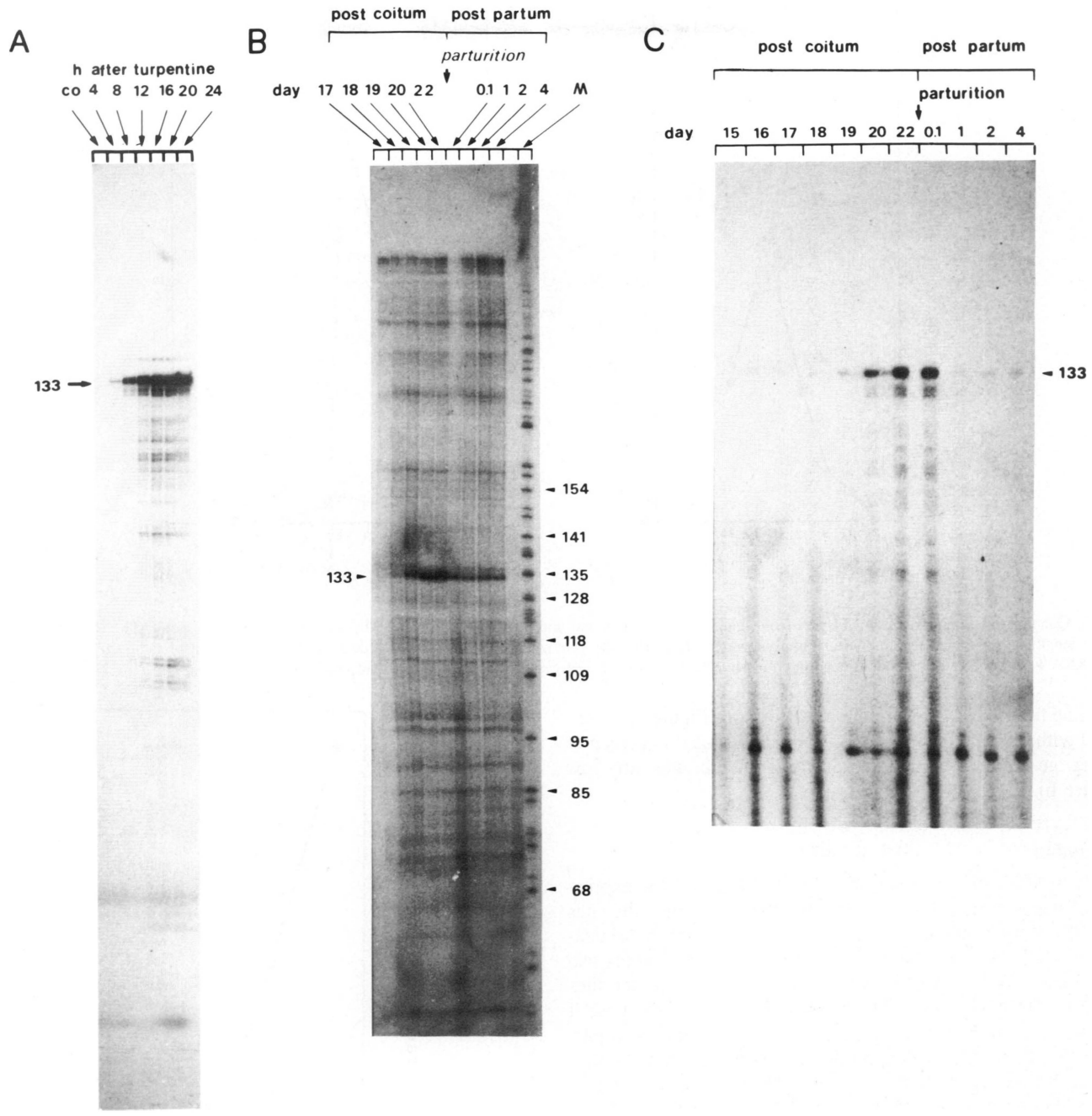

Fig. 4. RNase protection assay of total RNA from livers of fetal, newborn, maternal and turpentine-treated rats. 50 μg (**A**) or 10 μg (**B,C**) of total RNA isolated from livers of turpentine-treated (**A**), fetal and newborn (**B**) and maternal rats (**C**) were hybridized to a uniformly labelled [^{32}P]RNA transcribed with SP6-RNA polymerase from a 145-bp *Eco*RI/*Pst*I α_2M cDNA fragment inserted into the pSP64 vector (see Materials and methods). After digestion with RNase the protected RNA/RNA-hybrids were separated on denaturing polyacrylamide gels. The gels were exposed to X-ray films overnight (**A**) and (**B**), or for 4 days (**C**). M, DNA markers with sizes in nucleotides.

high mol. wt DNA is separated from RNA (not shown).

Figure 4A shows the expected 133-nt-long RNA fragment protected from the action of RNase. Its amount increases from barely detectable to high levels during acute inflammation. For quantitation of this increase in α_2M mRNA the radioactivity in the gel slices was determined containing the RNA fragment with a length of 133 nucleotides (Figure 5A). A maximal level of α_2M mRNA was found 16–18 h after turpentine injection. This finding is in agreement with previous results, where α_2M mRNA levels have been determined by cell-free translation (Northemann *et al.*, 1983a,b; Hayashida *et al.*, 1985) and by dot–blot hybridization (Hayashida *et al.*, 1985; Schreiber *et al.*, 1986). When total RNA from fetal liver was analysed, a rapid increase in α_2M mRNA concentrations from day 17 of gestation to the time of birth was found. The increase was followed by a sharp decline after parturition (Figures 4B and 5B).

A similar time-course of α_2M mRNA concentrations with a maximum around birth was measured in maternal liver, although the absolute amount of α_2M mRNA was lower by one order of magnitude (Figure 4C and triangles in Figure 5B). In addition, α_2M mRNA was also found in total RNA of fetal membranes and placenta. The α_2M mRNA expression in placenta showed

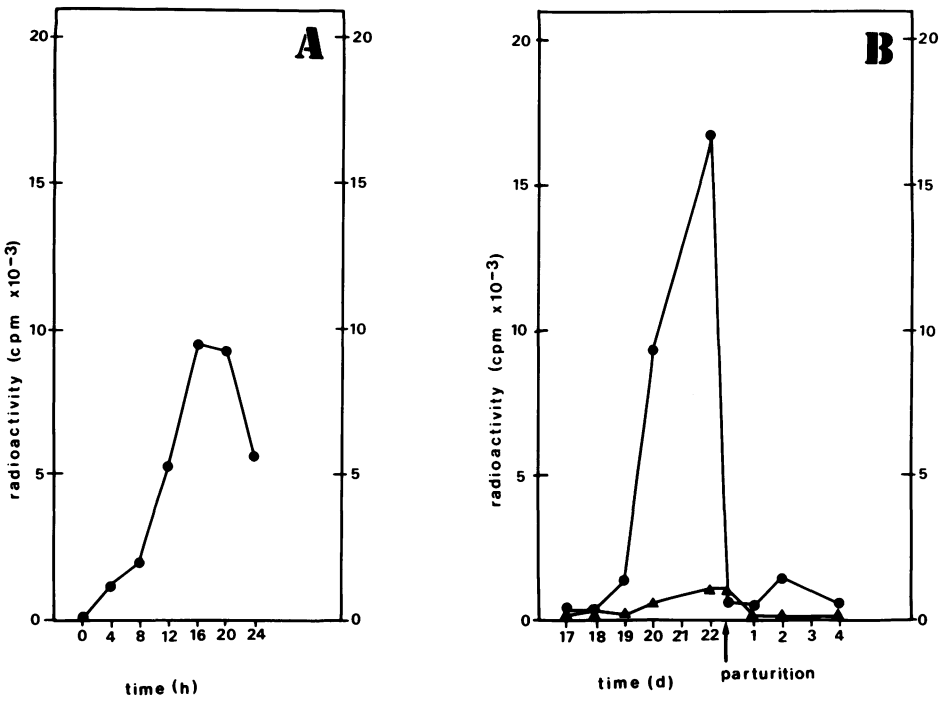

Fig. 5. Quantitation of α_2M mRNA in livers from fetal, newborn, maternal and turpentine-treated rats by an RNase protection assay. The RNA fragment of 133 nt length of the experiment shown in Figure 4 was excised from the gels and its radioactivity was determined. The amount of radioactivity per 10 μg of total RNA was blotted versus hours after turpentine injection (**A**) or days of gestation (**B**). ●, Fetal and newborn; ▲, maternal.

a decline from day 14 to the time of parturition (Figure 6). Compared with the amounts of α_2M mRNA determined in placentas during gestation the amounts in fetal membranes are low (Figure 6).

Discussion

In situ hybridization is a sensitive method to study the expression of genes during development. We have examined the sites of α_2M mRNA synthesis in fetal and neonatal rats by hybridization of whole body cryosections. The *in situ* hybridization also turned out to be a powerful tool for the detection of minor sites of α_2M mRNA synthesis. These would be missed otherwise if total RNA from the various organs and tissues is analysed, particularly if the α_2M gene expression is restricted to small defined areas of an organ. In the case of α_2M the liver is the major producing site of α_2M mRNA. Nevertheless, we have also found α_2M mRNA in brain, spinal cord, eye, placenta and fetal membranes.

The detection of α_2M mRNA in neuronal tissues may explain the presence of α_2M in cerebrospinal fluid of rat fetuses as described by Panrucker *et al.* (1983b). In order to identify the type of cells responsible for the α_2M mRNA synthesis in brain, the *in situ* hybridization should be carried out with a ^{3}H- or ^{35}S-labelled probe to achieve a higher resolution. Possibly, astrocytes represent candidates for α_2M mRNA synthesis in brain, since recently human astrocytes in culture were shown to produce α_2M (J.Bauer, personal communication).

Thus far, besides liver cells of inflamed animals only macrophages (Hovi *et al.*, 1977) and lung fibroblasts (Mosher and Wing, 1976) have been found to be capable of synthesizing α_2M. In normal adult rat liver there is essentially no α_2M mRNA synthesis. As shown in the present study the α_2M gene is turned on in liver during rat development and pregnancy. Maximal α_2M mRNA levels are reached around parturition both in fetal and

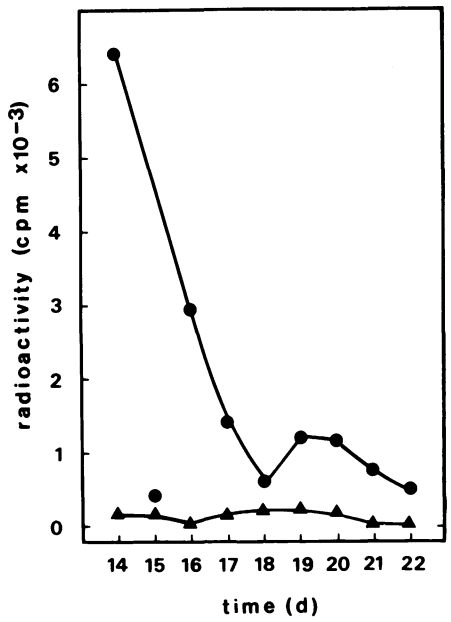

Fig. 6. Quantitation of α_2M mRNA in placentas and fetal membranes during development. As described in the legends to Figures 4 and 5 the protected 133-nt fragment of α_2M mRNA from placentas and fetal membranes was quantitated. In order to compare the data of different experiments, total RNA from livers of turpentine-treated rats was included in the RNase protection assay as an internal standard for α_2M mRNA. ●, Placenta; ▲, fetal membranes.

maternal livers. The α_2M mRNA levels in fetal livers are about twice as high as those measured in livers of rats suffering from acute inflammation. The increases in α_2M mRNA concentrations in liver during gestation have also been observed on the level of α_2M protein both in livers and serum of fetuses and pregnant rats (Panrucker *et al.*, 1983a). It is interesting to note that the

α_2M mRNA levels in fetal and maternal liver decline more rapidly than the α_2M protein in liver and serum. Whereas α_2M mRNA concentrations decrease to normal levels within 1 day after birth, the α_2M protein levels in serum are still elevated even 24 days after birth (Panrucker *et al.*, 1983a).

The dramatic increase in α_2M mRNA and subsequently α_2M protein of fetal and maternal liver shortly before birth may be of great physiological importance. The proteinase inhibitor α_2M may be involved in the protection of newborn and mother against hazardous proteolytic enzymes released from the detaching placenta, which is known to contain large amounts of proteinases of different specificities (Unger and Struck, 1977). Since α_2M is a proteinase inhibitor of an extremely wide specificity, it represents a very suitable inhibitor. The fact that α_2M mRNA levels in placenta decrease during the end of gestation seems to be in good agreement with the proposed role of α_2M in pregnancy.

It is interesting that α-macroglobulins, which are induced during pregnancy, have also been described in humans (Sutcliffe *et al.*, 1980; Sand *et al.*, 1985). Unfortunately, the function of these pregnancy-associated macroglobulins is unknown. Therefore, the rat system may turn out to be a valuable model for the elucidation of the role of α_2M during pregnancy.

Materials and methods

Rat fetuses and tissues

For mating, male and female BDII rats (Druckrey, 1971) were kept together for 24 h. This day was designated as day 0 of gestation. From day 14 onward at daily intervals rats were anesthetized by chloroform and killed by cervical dislocation. The uteri were immediately removed and cooled in phosphate buffered saline (PBS) on ice. The fetuses were dissected free of fetal membranes and maternal tissues and then frozen in 2.5% carboxymethyl cellulose solution (Serva, Heidelberg, FRG) by immersion in dry ice/ethanol (Southern *et al.*, 1984). The resulting blocks were stored at −70°C. Newborn rats were killed by chloroform and embedded as described for the fetuses. Fetal or adult tissues used for RNA isolation and *in situ* hybridization were rapidly removed, flash frozen and stored in liquid nitrogen.

In situ hybridization

Preparation of sections of fetuses, tissues and *in situ* hybridization were performed with slight modifications as described by Southern *et al.* (1984). Briefly, the embedded animals were cut into 40-μm-thick saggital sections on a Reichert-Jung Model 2700 Frigocut cryomicrotome at a temperature of −8 to −12°C. The sequential sections were collected on waterproof, transparent tape, rapidly transferred to a desiccator, precooled on dry ice and freeze dried *in vacuo* for 6−7 h. Then the sections were fixed in 4% paraformaldehyde in PBS, rinsed twice in PBS, air-dried and stored at −70°C till used for *in situ* hybridization. For *in situ* hybridization, the sections were prehybridized at 37°C for 12−24 h in 50% deionized formamide, 5 × SSPE (1 × SSPE = 0.2 M NaCl, 10 mM Na-phosphate, pH 7.4, 1 mM Na-EDTA), 2.5 × Denhardt's solution [1 × Denhardt's solution = 0.02% (w/v each) Ficoll, polyvinyl pyrrolidone, bovine serum albumin (fraction V)] containing 100 μg/ml sonicated and heat-denatured calf thymus DNA. Hybridization was performed at 37°C for 60−68 h in 1/3 volume of the same solution used for prehybridization, containing 2−4 × 10^6 c.p.m. of ^{32}P-labelled nick-translated DNA probe with a specific activity of 1−2 × 10^8 c.p.m./μg of DNA. The plasmid pα$_2$M1 containing a 657-bp fragment of rat α_2M cDNA (Northemann *et al.*, 1985) was isolated as described (Maniatis *et al.*, 1982) and used either directly or after excision and gel purification of the cDNA insert. As a negative control, adjacent tissue sections were hybridized with ^{32}P-labelled pBR322 vector DNA alone. After hybridization, the sections were washed with slight agitation three times for 30 min each with 2 × SSPE at 37°C and at 55°C in 2 × SSPE and in 0.1 × SSPE. The wet sections were covered with Saran wrap and exposed for 1−5 days at −70°C to Kodak X-Omat AR X-ray film using an intensifying screen. In order to identify the labelled tissues, the sections used for *in situ* hybridization were stained with H&E and photographed still wet in order to avoid shrinking of the sections.

RNA preparation and analysis

Isolation of RNA. Total RNA was isolated from fetal or adult tissues using the guanidinium thiocyanate/CsCl method of Fiddes and Goodman (1979) as described previously (Zimmermann *et al.*, 1983). The quality of the RNA preparations was assayed by electrophoresis of the RNAs on denaturing methylmercury hydroxide agarose gels (Bailey and Davidson, 1976). The high mol. wt DNA, occasionally contaminating the RNA preparations, was quantitated by scanning the negative of a polaroid print taken from the ethidium bromide-stained agarose gel under u.v. transillumination, using a LKB 2202 Ultroscan laser densitometer in connection with a LKB 2200 recording integrator. RNA concentrations were corrected for their thus determined DNA content.

Quantitation of α_2-macroglobulin mRNA by RNase protection. To quantitate α_2M mRNA by an RNase protection assay (Melton *et al.*, 1984) a 145-bp *Eco*RI/*Pst*I fragment of pα$_2$M1, corresponding to an internal region of α_2M mRNA (Northemann *et al.*, 1985), was subcloned into pSP64, which was a generous gift of Dr G.Ringold, Stanford, USA. After linearization of the plasmid with *Pvu*II, transcription with SP6-RNA polymerase (Boehringer Mannheim, Mannheim, FRG) in the presence of [α-^{32}P]UTP (Amersham, Braunschweig, FRG) and an RNase inhibitor, isolated from human placenta (Blackburn, 1979), yielded an RNA probe complementary to α_2M mRNA. After transcription, the DNA template was digested with RNase-free DNase (Merck, Darmstadt, FRG). The RNA was then hybridized overnight in a 20- to 50-fold excess to 10−50 μg of total cellular RNA. After digestion with RNase A and T_1, the protected RNA fragment was separated from degradation products by electrophoresis on 8% polyacrylamide/8 M urea sequencing gels (acrylamide:bisacrylamide = 19:1). The protected fragments were localized by exposure of the gels to Kodak X-Omat AR film at −80°C for 0.5−7 days and cut out of the gel. The gel slices were treated with Protosol (NEN, Dreieichenhain, FRG)/water (9:1) at 45°C overnight and their radioactivity was quantitated after addition of 100 μl of glacial acetic acid and 5 ml of Rotiscint 22 (Roth, Karlsruhe, FRG) by liquid scintillation counting.

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