

## $\alpha_2$ -Macroglobulin gene expression during rat development studied by *in situ* hybridization

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The sites of  $\alpha_2$ -macroglobulin mRNA synthesis during rat development have been localized by *in situ* hybridization using a rat  $\alpha_2$ -macroglobulin cDNA probe. Fetal liver was found to be the major site of  $\alpha_2$ -macroglobulin mRNA synthesis. In addition,  $\alpha_2$ -macroglobulin mRNA was detected in brain, spinal cord and eye.  $\alpha_2$ -Macroglobulin mRNA was quantitated by use of a sensitive RNase protection assay. Maximal levels of  $\alpha_2$ -macroglobulin mRNA were found in fetal livers shortly before birth. A rapid decline of  $\alpha_2$ -macroglobulin mRNA occurred within 1 day after parturition. A similar time course, although at an ~20-fold lower level, was observed for  $\alpha_2$ -macroglobulin mRNA in livers of pregnant rats.  $\alpha_2$ -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/ $\alpha_2$ -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  $\alpha_2$ -macroglobulin ( $\alpha_2$ M). 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).  $\alpha_2$ M 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  $\alpha_2$ M 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  $\alpha_2$ M 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  $\alpha_2$ M is strongly induced during acute inflammation, it is well known that  $\alpha_2$ M 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  $\alpha_2$ M protein levels could be measured and no conclusions on the site of  $\alpha_2$ M synthesis within an organism could be drawn.

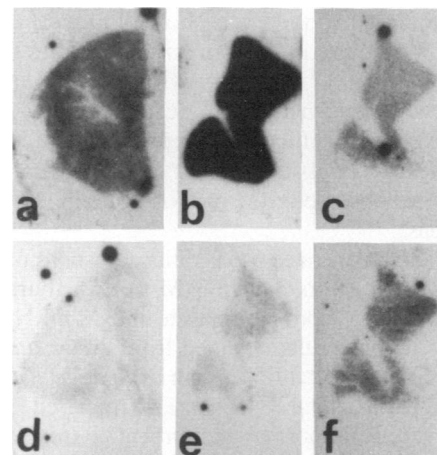
Recently we have isolated a rat  $\alpha_2$ M cDNA clone (Northemann *et al.*, 1985), which we have used in this study for *in situ* hybridization to identify the sites of  $\alpha_2$ M gene transcription during rat development.

### Results

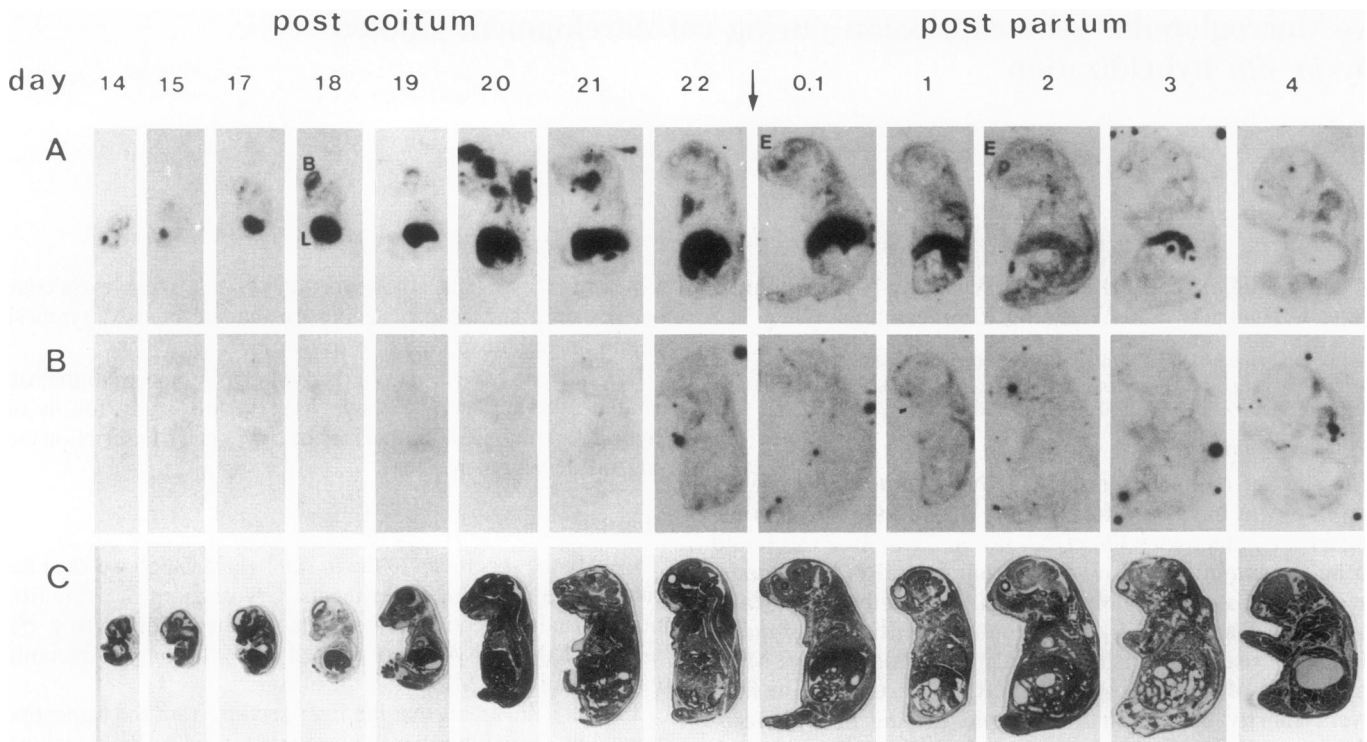
To verify the specificity of the *in situ* hybridization we have carried out the following experiments. Cryosections of livers from normal and turpentine-treated rats were hybridized to a <sup>32</sup>P-labelled  $\alpha_2$ M cDNA insert excised by *Pst*I from the recombinant plasmid p $\alpha_2$ M1.

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  $\alpha_2$ M 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. Pre-treatment of the cryosections with RNase A effectively abolished the hybridization signal (Figure 1c). As a further control we have used <sup>32</sup>P-labelled pBR322 for the hybridization. When this vector DNA, completely unrelated to  $\alpha_2$ M 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. <sup>32</sup>P-Labelled  $\alpha_2$ M cDNA (a–c) or <sup>32</sup>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  $\mu$ g/ml RNase A for 90 min at 37°C prior to the hybridization.



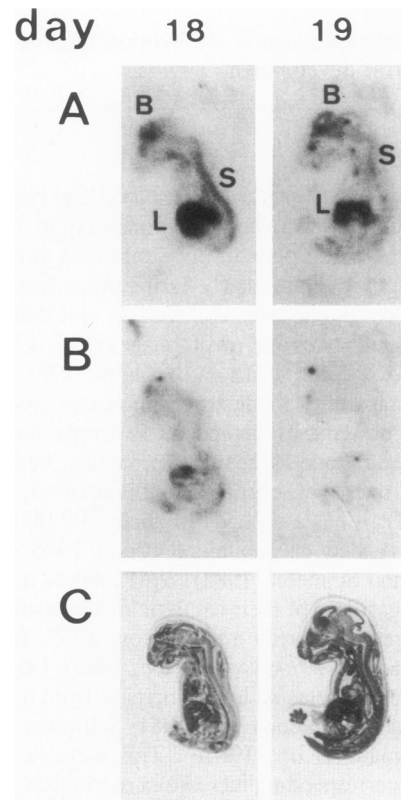
**Fig. 2.** *In situ* hybridization of  $\alpha_2M$  cDNA to whole body sections of fetal and newborn rats.  $^{32}P$ -labelled  $\alpha_2M$  cDNA (A) or  $^{32}P$ -labelled pBR322 vector DNA as control (B) was hybridized to saggital whole body sections from fetal and newborn rats at different stages 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.

$\alpha_2M$  mRNA in whole body sections of rats of various developmental stages. Figure 2A shows that the most prominent site of  $\alpha_2M$  mRNA synthesis is the liver. Hybridization with  $^{32}P$ -labelled pBR322 DNA did not give positive signals (Figure 2B).

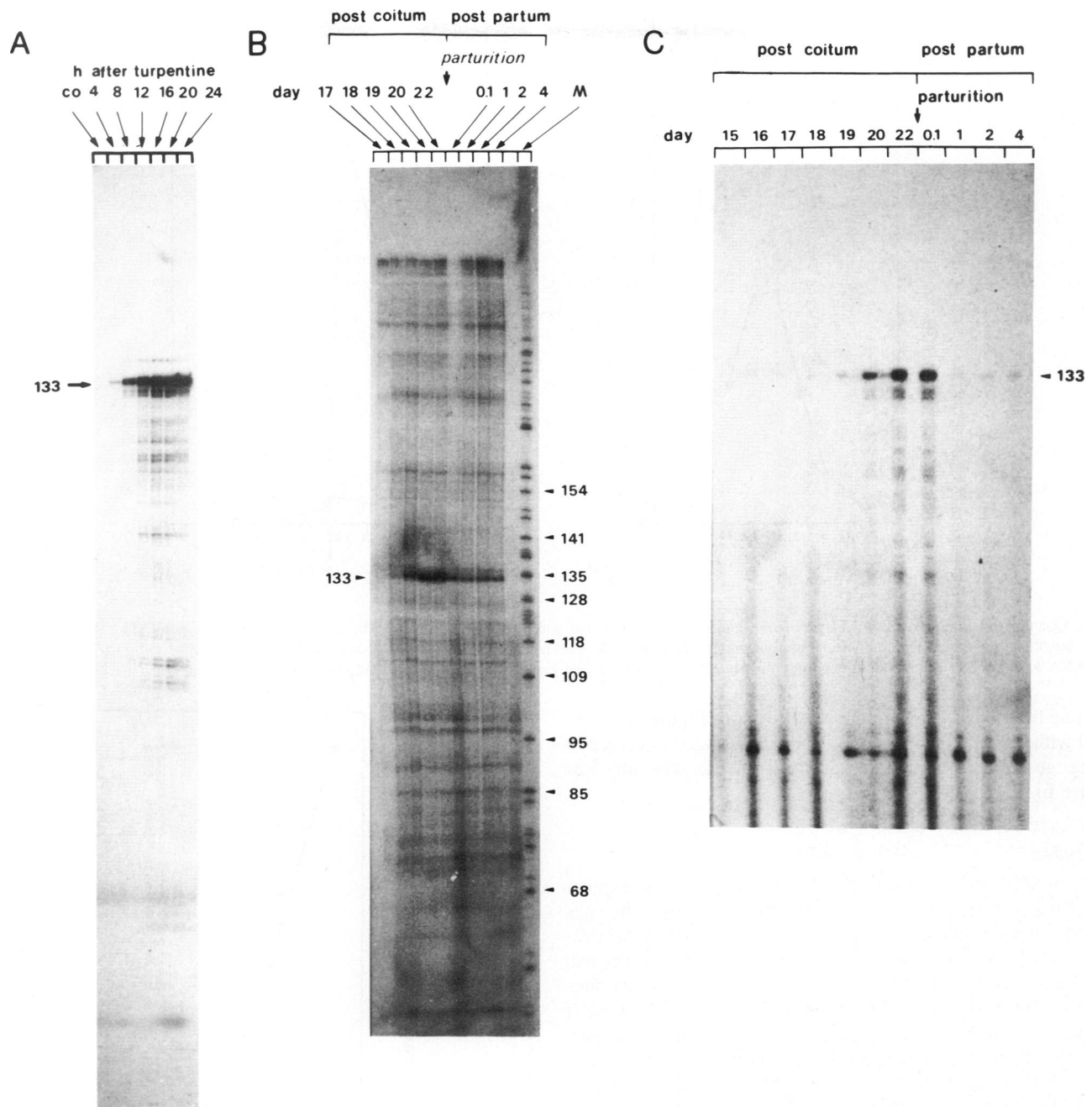
As early as 14 days after fertilization  $\alpha_2M$  mRNA can be detected.  $\alpha_2M$  mRNA concentrations increase during gestation and sharply decrease after birth. The increase in  $\alpha_2M$  mRNA concentrations during gestation is more evident on an autoradiograph, which had been exposed for a shorter time (not shown). The longer time of exposure was chosen to reveal  $\alpha_2M$  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  $\alpha_2M$  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  $\alpha_2M$  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  $\alpha_2M$  mRNA. Furthermore, it can be seen that spinal cord is also a site of  $\alpha_2M$  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 maternal 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  $\alpha_2M$  mRNA (Northemann *et al.*, 1985; Hayashida *et al.*, 1985), we determined the amount of  $\alpha_2M$  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-blot where



**Fig. 3.** Whole body *in situ* hybridization demonstrating  $\alpha_2M$  mRNA synthesis in fetal brain and spinal cord.  $^{32}P$ -labelled  $\alpha_2M$ 1 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.



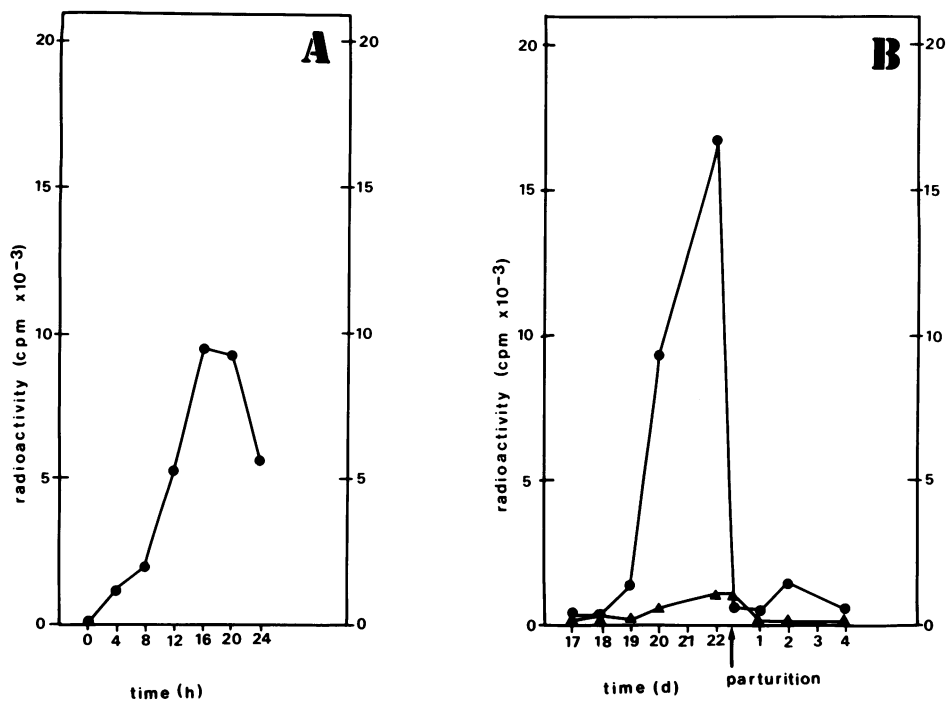
**Fig. 4.** RNase protection assay of total RNA from livers of fetal, newborn, maternal and turpentine-treated rats. 50  $\mu$ g (A) or 10  $\mu$ g (B,C) of total RNA isolated from livers of turpentine-treated (A), fetal and newborn (B) and maternal rats (C) were hybridized to a uniformly labelled [ $^{32}$ P]RNA transcribed with SP6-RNA polymerase from a 145-bp *EcoRI/PstI*  $\alpha_2$ M 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  $\alpha_2$ M 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  $\alpha_2$ M mRNA was found 16–18 h after turpentine injection. This finding is in agreement with previous results, where  $\alpha_2$ M 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  $\alpha_2$ M 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  $\alpha_2$ M mRNA concentrations with a maximum around birth was measured in maternal liver, although the absolute amount of  $\alpha_2$ M mRNA was lower by one order of magnitude (Figure 4C and triangles in Figure 5B). In addition,  $\alpha_2$ M mRNA was also found in total RNA of fetal membranes and placenta. The  $\alpha_2$ M mRNA expression in placenta showed



**Fig. 5.** Quantitation of  $\alpha_2M$  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  $\mu$ g of total RNA was blotted versus hours after turpentine injection (A) or days of gestation (B).  $\bullet$ , Fetal and newborn;  $\blacktriangle$ , maternal.

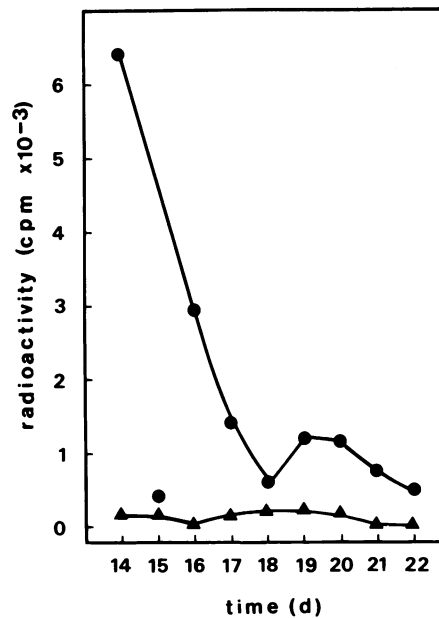
a decline from day 14 to the time of parturition (Figure 6). Compared with the amounts of  $\alpha_2M$  mRNA determined in placentas during gestation the amounts in fetal membranes are low (Figure 6).

**Discussion**

*In situ* hybridization is a sensitive method to study the expression of genes during development. We have examined the sites of  $\alpha_2M$  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  $\alpha_2M$  mRNA synthesis. These would be missed otherwise if total RNA from the various organs and tissues is analysed, particularly if the  $\alpha_2M$  gene expression is restricted to small defined areas of an organ. In the case of  $\alpha_2M$  the liver is the major producing site of  $\alpha_2M$  mRNA. Nevertheless, we have also found  $\alpha_2M$  mRNA in brain, spinal cord, eye, placenta and fetal membranes.

The detection of  $\alpha_2M$  mRNA in neuronal tissues may explain the presence of  $\alpha_2M$  in cerebrospinal fluid of rat fetuses as described by Panrucker *et al.* (1983b). In order to identify the type of cells responsible for the  $\alpha_2M$  mRNA synthesis in brain, the *in situ* hybridization should be carried out with a  $^3H$ - or  $^{35}S$ -labelled probe to achieve a higher resolution. Possibly, astrocytes represent candidates for  $\alpha_2M$  mRNA synthesis in brain, since recently human astrocytes in culture were shown to produce  $\alpha_2M$  (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  $\alpha_2M$ . In normal adult rat liver there is essentially no  $\alpha_2M$  mRNA synthesis. As shown in the present study the  $\alpha_2M$  gene is turned on in liver during rat development and pregnancy. Maximal  $\alpha_2M$  mRNA levels are reached around parturition both in fetal and



**Fig. 6.** Quantitation of  $\alpha_2M$  mRNA in placentas and fetal membranes during development. As described in the legends to Figures 4 and 5 the protected 133-nt fragment of  $\alpha_2M$  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  $\alpha_2M$  mRNA.  $\bullet$ , Placenta;  $\blacktriangle$ , fetal membranes.

maternal livers. The  $\alpha_2M$  mRNA levels in fetal livers are about twice as high as those measured in livers of rats suffering from acute inflammation. The increases in  $\alpha_2M$  mRNA concentrations in liver during gestation have also been observed on the level of  $\alpha_2M$  protein both in livers and serum of fetuses and pregnant rats (Panrucker *et al.*, 1983a). It is interesting to note that the

$\alpha_2$ M mRNA levels in fetal and maternal liver decline more rapidly than the  $\alpha_2$ M protein in liver and serum. Whereas  $\alpha_2$ M mRNA concentrations decrease to normal levels within 1 day after birth, the  $\alpha_2$ M protein levels in serum are still elevated even 24 days after birth (Panrucker *et al.*, 1983a).

The dramatic increase in  $\alpha_2$ M mRNA and subsequently  $\alpha_2$ M protein of fetal and maternal liver shortly before birth may be of great physiological importance. The proteinase inhibitor  $\alpha_2$ M 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  $\alpha_2$ M is a proteinase inhibitor of an extremely wide specificity, it represents a very suitable inhibitor. The fact that  $\alpha_2$ M mRNA levels in placenta decrease during the end of gestation seems to be in good agreement with the proposed role of  $\alpha_2$ M in pregnancy.

It is interesting that  $\alpha$ -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  $\alpha_2$ M 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  $-70^\circ\text{C}$ . 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- $\mu\text{m}$ -thick sagittal sections on a Reichert-Jung Model 2700 Frigocut cryomicrotome at a temperature of  $-8$  to  $-12^\circ\text{C}$ . 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  $-70^\circ\text{C}$  till used for *in situ* hybridization. For *in situ* hybridization, the sections were prehybridized at  $37^\circ\text{C}$  for 12–24 h in 50% deionized formamide,  $5 \times \text{SSPE}$  ( $1 \times \text{SSPE} = 0.2 \text{ M NaCl}$ ,  $10 \text{ mM Na-phosphate}$ ,  $\text{pH } 7.4$ ,  $1 \text{ mM Na-EDTA}$ ),  $2.5 \times \text{Denhardt's solution}$  [ $1 \times \text{Denhardt's solution} = 0.02\%$  (w/v each) Ficoll, polyvinyl pyrrolidone, bovine serum albumin (fraction V)] containing  $100 \mu\text{g/ml}$  sonicated and heat-denatured calf thymus DNA. Hybridization was performed at  $37^\circ\text{C}$  for 60–68 h in 1/3 volume of the same solution used for prehybridization, containing  $2-4 \times 10^6$  c.p.m. of  $^{32}\text{P}$ -labelled nick-translated DNA probe with a specific activity of  $1-2 \times 10^8$  c.p.m./ $\mu\text{g}$  of DNA. The plasmid  $\text{p}\alpha_2\text{M1}$  containing a 657-bp fragment of rat  $\alpha_2\text{M}$  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  $^{32}\text{P}$ -labelled pBR322 vector DNA alone. After hybridization, the sections were washed with slight agitation three times for 30 min each with  $2 \times \text{SSPE}$  at  $37^\circ\text{C}$  and at  $55^\circ\text{C}$  in  $2 \times \text{SSPE}$  and in  $0.1 \times \text{SSPE}$ . The wet sections were covered with Saran wrap and exposed for 1–5 days at  $-70^\circ\text{C}$  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 Ultrascan laser densitometer in connection with a LKB 2200 recording integrator. RNA concentrations were corrected for their thus determined DNA content.

**Quantitation of  $\alpha_2$ -macroglobulin mRNA by RNase protection.** To quantitate  $\alpha_2$ M mRNA by an RNase protection assay (Melton *et al.*, 1984) a 145-bp *EcoRI/PstI* fragment of  $\text{p}\alpha_2\text{M1}$ , corresponding to an internal region of  $\alpha_2$ M 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 *PvuII*, transcription with SP6-RNA polymerase (Boehringer Mannheim, Mannheim, FRG) in the presence of [ $\alpha$ - $^{32}\text{P}$ ]UTP (Amersham, Braunschweig, FRG) and an RNase inhibitor, isolated from human placenta (Blackburn, 1979), yielded an RNA probe complementary to  $\alpha_2$ M 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  $\mu\text{g}$  of total cellular RNA. After digestion with RNase A and  $\text{T}_1$ , 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  $-80^\circ\text{C}$  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  $45^\circ\text{C}$  overnight and their radioactivity was quantitated after addition of 100  $\mu\text{l}$  of glacial acetic acid and 5 ml of Rotiscint 22 (Roth, Karlsruhe, FRG) by liquid scintillation counting.

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