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Prothrombin Expression in the Adult and Fetal Rabbit Liver[1]

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ABSTRACT. Plasma prothrombin levels in newborn humans are lower than in adults. The same is true of many newborn and fetal mammals, including the rabbit. To determine if the lower levels are due to less expression of the protein, we have compared mRNA for prothrombin in fetal and adult rabbit liver. Northern blots were hybridized with a cDNA for rabbit prothrombin revealing a single mRNA of approximately 2 kb in both adult and fetal animals. mRNA specific for prothrombin was quantitated by slot blotting of RNA prepared from adults and fetuses aged 21 d to term (31 d). Prothrombin-specific mRNA in fetuses was greater than 50% of that in adults even when the fetal plasma prothrombin was only 15% of the adult level. This suggests that low plasma levels in the fetuses are not the result of less transcription. Examination of liver sections revealed that the predominant tissue in the fetus is hematopoietic, not hepatic. In the youngest fetuses, less than 20% of the liver consisted of hepatocytes, yet these fetuses expressed more than 50% of the adult level of prothrombin-specific mRNA. Thus, transcription of prothrombin mRNA may be proceeding at a greater rate in the fetal hepatocyte than in the adult, or hematopoietic cells may be expressing the protein. We conclude that in fetal rabbit liver, prothrombin is expressed at a high level relative to the hepatocyte content and that the cause of the low plasma levels is posttranscriptional. (*Pediatr Res* 30: 266–269, 1991)

On d 1 of life, the mean level of prothrombin in healthy, term infants who have received vitamin K is 48% of that in the adult (1). The level is lower in preterm infants, with a significant correlation with gestational age (2). Prothrombin levels rise slowly after delivery (1, 3), and the adult mean level is not reached until the 4th year of life (4). In infants who have received vitamin K, prothrombin and other K-dependent clotting factors are γ-carboxylated. The sp act of plasma prothrombin in these infants' plasma is the same as in adults (2, 5–8).

The reason for the lower levels of prothrombin in infants' plasma has not been elucidated. Possible reasons are diminished synthesis, differences in processing or secretion, or more rapid catabolism of the protein in the newborn circulation. To explore these possibilities, an animal model is necessary. Hathaway *et al.* (9) reported that in the rabbit on d 1 of life plasma prothrombin was one third of the adult level. We have confirmed that levels are low in the fetal rabbit and we have studied prothrombin mRNA expression in the fetus and adult. We report here a comparison between mRNA for prothrombin in the fetal and adult rabbit liver.

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MATERIALS AND METHODS

Collection of animal materials. New Zealand White rabbits were used throughout. Blood was drawn from the ear artery of adult females and anticoagulated, separated, and stored as described previously (10). At appropriate intervals after timed matings, pregnant rabbits were anesthetized with i.v. nembutal and the fetuses were delivered by cesarean section. Blood was drawn from the jugular vein into 3.8% sodium citrate one part to nine parts of blood. Plasma was obtained by centrifugation and stored at −70°C until assayed.

Livers were removed from anesthetized adult female rabbits, rapidly cut into approximately 1-cm cubes, frozen immediately in liquid nitrogen, and then stored at −70°C until use. Fetuses of anesthetized pregnant rabbits were delivered by cesarean section and the livers removed, frozen, and stored in a similar manner. Seven separate litters were studied, and litter sizes ranged from two to six fetuses. Liver and blood were not obtained from the same fetus. This was because it was not technically possible to collect an unclotted blood sample for prothrombin assay and remove the liver before the fetus died.

Preparation of rabbit liver sections. Livers were fixed in 4% paraformaldehyde and paraffin-embedded, and sections were cut and stained with hematoxylin and eosin.

Plasma prothrombin assay. Biologic assay was by a microtechnique described by Hurlet and Josso (11). RIA used an ^{125}I-labeled antibody to rabbit prothrombin raised in a guinea pig. The antibody was affinity-purified on Affi-gel (Bio-Rad Laboratories, Richmond, CA) and was monospecific as judged by Western blotting with rabbit plasma.

RNA preparations. Total RNA was prepared by homogenization of frozen liver in a solution containing 4 M guanadinium thiocyanate, followed by extraction with phenol and with chloroform as described by Chomezynski and Sacchi (12). The RNA was precipitated with sodium acetate to remove glycogen (13). mRNA was selected from total RNA using oligo (Dt)-cellulose columns (14) or from a homogenate in guanadinium thiocyanate using the Fast Track Kit (Invitrogen, San Diego, CA). Diethylpyrocarbonate-treated solutions and glassware were used when appropriate in the preparation and storage of RNA.

cDNA probes. A cDNA for rabbit prothrombin was cloned from a λ-gt 11 rabbit liver cDNA library using a ^{32}P-labeled 2-kb cDNA for bovine prothrombin as probe (15). The cDNA is 1466 bp in length and the deduced amino acid sequence has 71% homology with human and bovine prothrombin. This identity is 80% in the portion of the molecule containing the enzyme thrombin. It extends from the equivalent of nucleotide 516 of bovine prothrombin through the coding region and 99 nucleotides into the 3′ nontranslated region.

A cDNA for rabbit β-globin PMB9-RcB1 was used as probe

(16). In contrast to β-globin in the human, β-globin in the rabbit is fully expressed in the fetus (17). Probes were labeled with ^{32}P by the random primer technique (18), using a kit manufactured by Pharmacia (Piscataway, NJ) according to the manufacturer's instructions.

Northern blots. Twenty-μg samples of total RNA were loaded onto denaturing 1% agarose gels (14). After electrophoresis, RNA was transferred onto Nytran membranes (Schleicher and Schuell, Keen, NH), dried at 80°C for 2 h, and stored at 4°C until hybridization. Between 1 and 3 μg mRNA were similarly electrophoresed and transferred.

Slot blotting. Slot blotting of total RNA and mRNA was performed using Nytran membranes and a manifold (Bethesda Research Labs, Gaithersburg, MD) according to the manufacturer's instructions.

Hybridization. Membranes were prehybridized at 42°C and hybridized overnight at the same temperature. After hybridization, membranes were washed extensively, the last wash being for 1 h in 0.1 × sodium chloride sodium citrate, 1% SDS at 65°C.

Autoradiography. Autoradiography was at −70°C using Kodak X-Omat AR film and an intensifying screen. RNA levels in the slot blots were quantified by densitometry.

All reagents were of molecular biology grade.

RESULTS

Prothrombin level in fetal rabbit plasma. Results in Table 1 demonstrate that prothrombin levels are reduced in fetal plasma compared with the adult. The values are expressed per mg plasma protein. This is to obviate dilutional differences as a result of the anticoagulant, which are unavoidable when collecting very small quantities of blood.

Sections of fetal and adult livers. Figure 1 illustrates the large amount of hematopoietic tissue that is present in the fetal liver. The gestational period in the rabbit averages 31 d. *Panel A* is from an adult rabbit, *panel B* from a 21-d fetus (the equivalent of the beginning of the 3rd trimester). The predominant cells with small dense nuclei are erythroid. A number of megakaryocytes are present. *Panel C* is a term fetus in which many erythroid precursors are still present and the vacuoles in the hepatocytes contain lipid. Similar vacuoles are also present in fetal hepatocytes in rodents and disappear during the postnatal period (19).

Expression of mRNA for prothrombin and β-globin in fetal and adult livers. Figure 2 is a Northern blot of mRNA from an adult rabbit (*lane A*) and from fetuses ranging in gestational age from 23 to 31 d (*lanes 1–3*). The filter was first hybridized with the cDNA for rabbit prothrombin and subjected to autoradiography. A single band of prothrombin mRNA of approximately 2 kb is seen in the adult and in the fetuses (*panel I*). After autoradiography, the filter was stripped by boiling and then hybridized with cDNA for rabbit β-globin (*panel II*). No mRNA for rabbit β-globin is detected in *lane A* (adult rabbit), but abundant mRNA is seen in *lanes 1–3* (fetuses). Northern blots of total RNA gave similar results.

Table 1. *Prothrombin levels in plasma of adult and fetal rabbits**

Gestational age (d)	Biologic assay (%)	Immunoassay (%)
25	10.5 (3)	15 (3)
28	31 ± 9.16 (9)	
29	49 ± 8.87 (4)	
Term (31 d)		44 (3)
Adult	102 ± 21.68 (5)	

* Results are the mean level of prothrombin compared with a pool from 10 adult rabbits. Figures in parentheses are the number of rabbits studied. SD is given and unpaired *t* test performed when four or more animals were studied. Levels in 28- and 29-d fetuses are significantly lower than in the adult animals ($p < 0.001$ and $p < 0.005$, respectively).

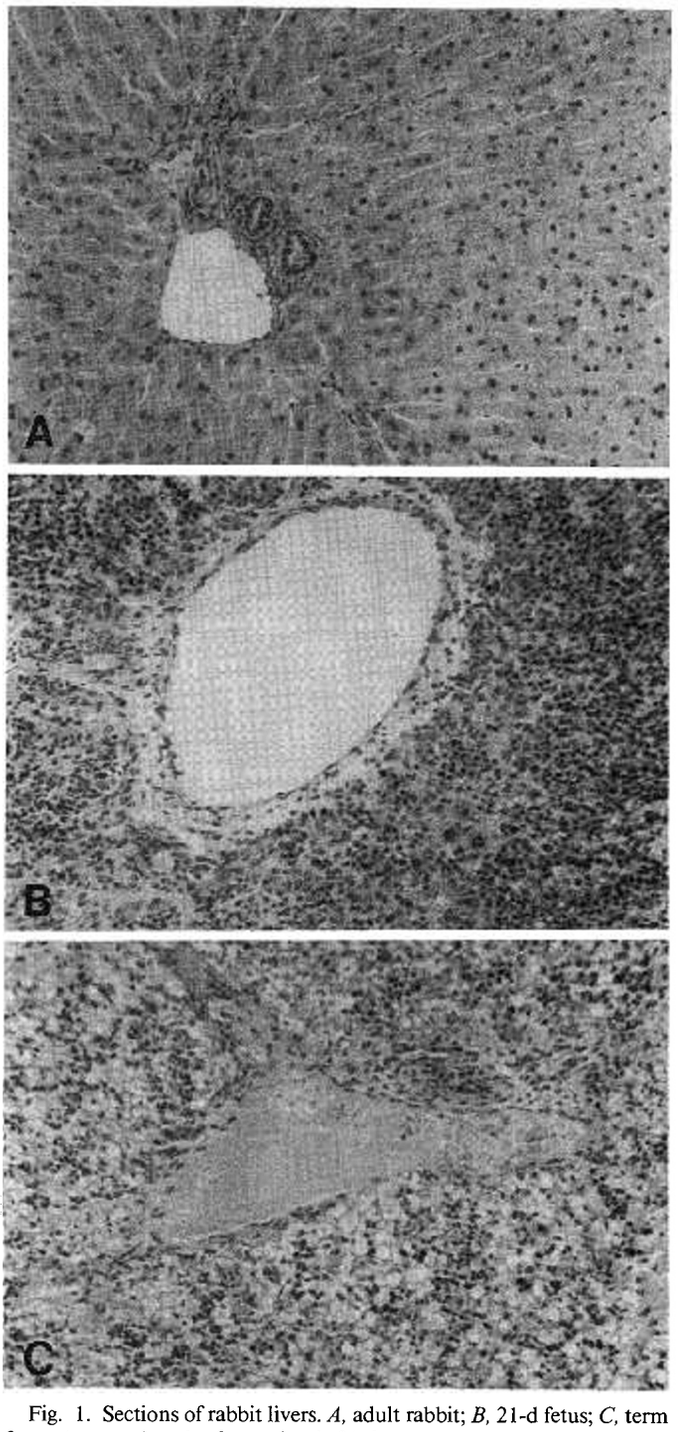

Fig. 1. Sections of rabbit livers. *A*, adult rabbit; *B*, 21-d fetus; *C*, term fetus. Average length of gestation is 31 d.

Slot blots. mRNA from the liver of one adult rabbit was loaded in increasing amounts ranging from 125 ng to 1 μg per slot and the filter was hybridized with rabbit prothrombin cDNA. This was to determine a range in which quantitative differences in prothrombin mRNA could be appreciated. The autoradiograph is shown in Figure 3 with ng mRNA loaded plotted against the area under the densitometer.

Figure 4 is a representative experiment in which the levels of prothrombin mRNA in adults and fetuses are compared. One hundred ng mRNA per slot were loaded from each of four different adult rabbits (*slots 1–4*) and four fetuses ranging in gestational age from 21 to 31 d (*slots 5–8*) and hybridized with

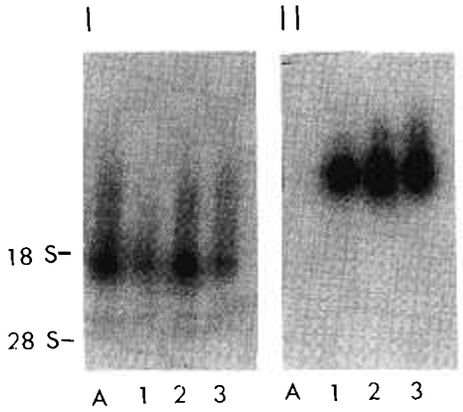

Fig. 2. Northern blot analysis of mRNA from rabbit liver. Three μg of mRNA were loaded into each lane. *Lane A* is mRNA from an adult rabbit; *lanes 1, 2,* and *3* are from 23-, 29-, and 31-d fetuses, respectively. In *panel I,* the membrane has been hybridized with cDNA for rabbit prothrombin. *Panel II* is the same membrane stripped and rehybridized with cDNA for rabbit β-globin.

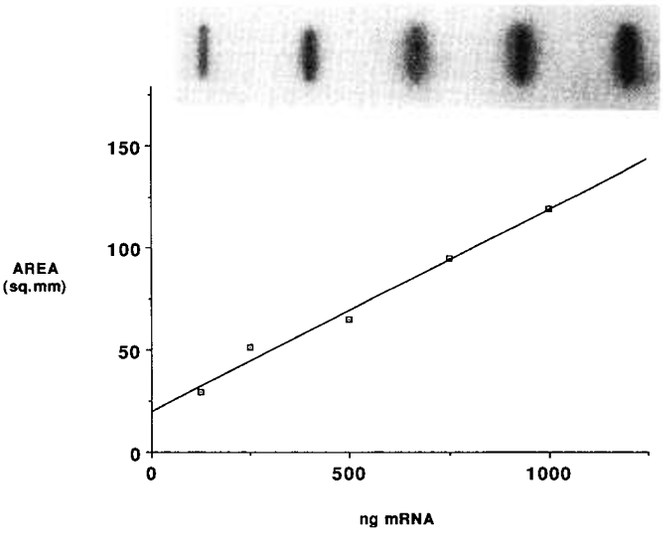

Fig. 3. Slot blot of mRNA from the liver of one adult rabbit to determine the range in which quantitative differences in mRNA could be appreciated. The autoradiogram is of increasing quantities from left to right (125 ng to 1 μg) of mRNA hybridized with rabbit prothrombin cDNA. The graph is the area under the densitometer reading plotted against the quantity of mRNA loaded for each slot. $r = 0.9930$.

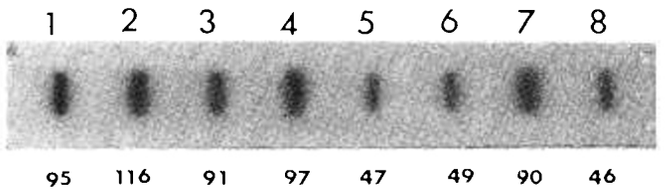

Fig. 4. Slot blot of a representative experiment comparing mRNA in four different adult rabbits (*slots 1–4*), and four fetuses aged 21, 23, 29, and 31 d (*slots 5–8*). One hundred ng of mRNA were loaded in each slot and hybridized with cDNA for prothrombin. The mean densitometer reading for the four adults was designated 100%, and each slot was then expressed as a percentage of this. These percentages are shown beneath the slots.

cDNA for prothrombin. The mean densitometer reading for the four adults was designated 100%, and the densitometer reading for each slot expressed as a percentage of this mean is shown below the autoradiograph. This experiment was carried out on four separate occasions, and the results are presented in Table 2.

Table 2. *Prothrombin mRNA in fetal and adult livers compared by slot blot**

Gestational age (d)	mRNA
21	61 ± 20.07
23	60.5 ± 15.97
29	99 ± 32.25
31	53.2 ± 19.95
Adult	100.0 ± 21.01

* Values are mean ± SD of four experiments using liver from fetuses aged 21 d to term and liver from four adults. One hundred ng mRNA per slot was loaded. In each experiment, the mean reading for the four adults was designated 100% and the densitometer reading for each sample was expressed as a percentage of this. Twenty-one-, 23-, and 31-d fetuses had significantly less prothrombin mRNA than adults ($p < 0.01$, $p < 0.005$, $p < 0.001$, respectively). Twenty-nine-d fetuses were not significantly different from adults. There was no significant difference between any of the groups of fetuses (unpaired t test).

DISCUSSION

We have studied mRNA for prothrombin in livers of adult and fetal rabbits. Our data suggest that the lower level of plasma prothrombin in the fetus is not due to reduced expression; when Tables 1 and 2 are compared, it can be seen that the level of prothrombin in the plasma of the fetal rabbits is less than the level of mRNA when both are expressed as a percentage of the adult levels.

One may hypothesize that the low plasma levels result from a posttranscriptional event that is less efficient in the fetal liver, such as translation or intracellular processing or secretion. Hassan *et al.* (20) studied factor X in 5- to 10-wk human embryos and fetuses and found a similar disparity between mRNA and plasma levels. The plasma level was 20 to 40% of that in the adult, although the mRNA for this protein was 100% of that in the adult. These workers measured factor X in the liver and found more in the fetus than in the adult, suggesting that the protein accumulates in the liver, resulting in lower plasma levels.

Kisker *et al.* (21) have studied development of prothrombin in sheep. Using RNA transcripts of a human prothrombin cDNA, they compared mRNA levels in 3rd-trimester fetal lambs, newborn lambs, and an adult sheep. They demonstrated a gradual rise in prothrombin mRNA during the 3rd trimester that paralleled the rise in plasma prothrombin. The difference between their findings and ours may reflect species differences.

The SD for plasma prothrombin (Table 1) reflects a wide range of levels at each time point. A similar range is seen in human adults and children (1, 3, 4) and in fetal and adult sheep (21). There is a similar wide range of prothrombin mRNA levels in the rabbit (Table 2), but data is not available for mRNA levels in the human and the sheep. Hassan *et al.* (20) studied only one adult liver and one fetal liver for each time point, whereas Kisker *et al.* (21) studied one or two animals at each time point.

In the rabbit fetuses, prothrombin-specific mRNA levels were unexpectedly high, considering the paucity of hepatic and the abundance of hematopoietic cells in the livers (Fig. 1). RNA prepared from fetal liver is derived from both hepatocytes and hematopoietic cells, the majority of which are erythroid. These are presumed to be synthesizing globin, not prothrombin. Figure 2 demonstrates that globin mRNA is not produced by the adult liver but is abundant in fetal liver. The amount of globin mRNA in the fetal liver did not decrease between 21 d and term, as judged by Northern blot (Fig. 2) and slot blot (data not shown).

Possible explanations for the relatively high level of prothrombin mRNA are that in the fetal hepatocytes transcription of prothrombin is proceeding at a greater rate than in the adult, or that it is also being transcribed in the hematopoietic cells. There are examples in development to support both of these possibilities. A number of proteins are expressed at different levels in the

adult and fetal liver, including α-fetoprotein and albumin (22, 23), lecithin cholesterol amino transferase (24), haptoglobin (25) tyrosine oxoglutarate aminotransferase, and phosphoenolpyruvate carboxykinase (26). Examples of mRNA that are liver-specific in the adult being expressed in other tissues in the fetus are tyrosine oxoglutarate aminotransferase and phosphoenolpyruvate carboxykinase, which are expressed equally in the liver, brain, heart, and lung of the fetal mouse (26), and albumin and α-fetoprotein, which are expressed at low levels in fetal and neonatal kidney (27).

The human embryos and fetuses studied by Hassan *et al.* (20) were at a stage of human development when the liver is the sole blood-forming organ and hematopoietic cells predominate (28). As already stated, factor X mRNA was 100% of the adult level but mRNA for other liver-specific proteins measured by these workers were 50% of the adult level by 10 wk of gestation. This data also suggests a greater transcriptional rate for certain proteins in the fetal hepatocyte or participation of hematopoietic tissue.

In conclusion, comparison of plasma prothrombin levels and mRNA levels in the fetal rabbit suggests that the low plasma levels are due to a posttranscriptional event. mRNA for prothrombin in fetal rabbits is higher than would be expected if transcription were occurring at the same rate as in the adult and were confined to the hepatocyte. These observations raise interesting questions regarding the synthesis and processing of proteins during development.

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