

Developmental changes in the expression of genes involved in cholesterol biosynthesis and lipid transport in human and rat fetal and neonatal livers

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Cloned cDNAs encoding a number of enzymes involved in cholesterol biosynthesis as well as extracellular and intracellular lipid transport were used to compare the developmental maturation of these biologic functions in the fetal and neonatal rat and human liver. The results of RNA blot hybridization analyses indicate that steady-state levels of rat HMG-CoA synthase, HMG-CoA reductase and prenyl transferase mRNAs are highest in late fetal life and undergo precipitous (up to 80-fold) co-ordinate reductions immediately after parturition. These changes reflect the ability of the fetal rat liver to produce large quantities of cholesterol as well as the repression of this function during the suckling period in response to exogenous dietary cholesterol. Striking co-ordinate patterns of HMG-CoA synthase, reductase and prenyl-transferase mRNA accumulation were also observed in four extrahepatic rat tissues (brain, lung, intestine and kidney) during the perinatal period. The concentrations of all three mRNAs in the 8-week-old human fetal liver are similar to those observed throughout subsequent intrauterine development with less than 2-fold changes noted between the 8th through 25th weeks of gestation. Analysis of the levels of human apo AI, apo AII, apo B and liver fatty acid binding protein mRNAs during this period and in newborn liver specimens also indicated less than 2-3-fold changes. These observations suggest that the 8-week human liver has achieved a high degree of biochemical differentiation with respect to functions involved in lipid metabolism/transport which may be comparable to that present in 19-21 day fetal rat liver. Further analysis of human and rat fetal liver RNAs using cloned cDNAs should permit construction of a developmental time scale correlating hepatic biochemical differentiation to be constructed between these two mammalian species.

Introduction

Marked changes in the requirements for products derived from isoprene (e.g. cholesterol) occur during development. The enzyme which catalyzes the key, rate-limiting step in cholesterol biosynthesis—microsomal 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase)—undergoes large fluctuations during rat liver ontogeny [1,2]. Enzyme activity is high prior to birth, declines to low levels during the suckling period (postnatal days 1-13) and demonstrates a transient increase at weaning. Changes in its activity parallel

developmental alterations in the rate of incorporation of radiolabeled acetate into sterols [2]. Bruenger and Rilling [3] documented changes in the activities of two other cholesterologenic enzymes in the developing rat liver: squalene synthetase and prenyl transferase (or farnesyl/pyrophosphate synthetase). Prenyl transferase is one of five enzymes that participate in the conversion of mevalonate to squalene, the precursor of sterols. Both squalene synthetase and prenyl transferase display similar developmental activity profiles in rat liver: they fall following birth, rise to a peak during the mid to late suckling period (postnatal days 10-12), fall once more during the suckling-weaning transition reaching a nadir by postnatal day 20, only to rise one final time during weaning. The mechanisms which result in these alterations in enzyme activity may be quite complex as illustrated by the fact that the mevalonate-mediated

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decrease in HMG-CoA reductase levels observed in adult animals reflects decreases in gene transcription as well as increased rates of protein degradation [4,5].

Little is known about the developmental history of the activities of these enzymes in human fetal and neonatal liver or about the ontogeny of expression of genes involved in the transport and metabolic processing of lipids in this tissue. The rat liver provides a convenient reference for a comparative study of such developmental changes. For example in addition to the information about expression of cholesterologenic enzyme activities, recent analyses of apolipoprotein gene expression in the developing rat liver indicate that a complex pattern of activation occurs during late fetal and early neonatal life. Apolipoprotein AI and E mRNAs begin to accumulate in this tissue plus its embryonic homologue (the fetal yolk sac endoderm) between days 15–21 of the 21-day gestation period [6–9]. Remarkable increases in the levels of these mRNAs occur during the suckling period as the animals adapt to the high fat (principally triacylglycerol) diet of mothers milk [10,11]. By contrast, rat liver apo B mRNA levels reach a peak by the 18th fetal day that is not exceeded at any time during subsequent development [12]. Following birth, hepatic apo B mRNA concentrations progressively fall during the suckling and weaning periods [12]. This fall appears to be mediated by thyroxine [13]. A third pattern of activation is exhibited by the apo AIV gene which remains dormant until the suckling weaning transition (days 13–14) when the rat liver begins to export large amounts of triacylglycerol-rich lipoproteins [6].

We have begun a comparative analysis of the accumulation of mRNAs encoding proteins involved in lipid metabolism in the fetal and neonatal human and rat liver. A panel of cloned cDNAs encoding apolipoproteins AI, AII, and B, an intracellular fatty acid binding protein as well as HMG-CoA reductase, HMG-CoA synthase and prenyl transferase were used to characterize the state of enzymatic differentiation of the human fetal liver from weeks 8 to 25 of development and in the newborn. The results indicate that these mRNAs appear at an early phase of human fetal life (by week 8) and undergo only minimal (less than 3-fold changes) in their concentration in total liver RNA during the rest of intrauterine as well as early postnatal life. This early expression of lipid metabolic capability contrasts with the more marked changes in mRNA levels observed in the perinatal rat liver.

Materials and Methods

Preparation of RNA from rat and human liver

Timed-pregnant, neonatal and young adult Sprague-Dawley rats were obtained from Sasco (St. Louis, MO). Weaned animals were maintained on a standard chow

diet ad libitum and a fixed 12 h (6:00 a.m. to 6:00 p.m.) light cycle. Animals ($n = 10$ –40 for each time point) were killed between 1200 and 1400 h and their livers, lungs, brains, kidneys and small intestines immediately frozen in liquid nitrogen. Human fetal liver samples from first and second trimester aborted fetuses were obtained by Schwartz et al. [14] and maintained at -90°C for 15–20 years. Fetal age was estimated from crown-rump lengths using nomograms developed by Tanimura et al. [15]. Breitfeld and Schwartz [16] and Michaelson and Orkin [17] have used these human fetal liver samples previously to successfully prepare RNA for in vitro translation. Additional human liver samples were obtained from a preterm newborn and a full term newborn, both of whom died of acute respiratory failure. These were stored at -90°C for about 15 years [14]. A single adult liver specimen was procured from a male organ donor who died of trauma and had no history or clinical evidence of hepatic dysfunction [18].

Total cellular RNA was extracted from frozen pulverized tissues using the guanidine thiocyanate/cesium chloride procedure [19]. RNA integrity was assessed by denaturing, methylmercury agarose gel electrophoresis [20].

RNA blot hybridization studies

Dot blots were prepared by applying four amounts of each tissue RNA sample (0.5, 1, 2, and 3 μg) to nitrocellulose filters as described in a previous publication [6]. Yeast tRNA was added to each tissue RNA sample prior to denaturation so that the total RNA input per dot was always 3 μg . Blots containing sample of rat liver, intestine, kidney, brain and lung RNA were probed with ^{32}P -labeled, double-stranded cDNAs encoding hamster HMG-CoA reductase [22], rat HMG-CoA synthase [23], and rat prenyl transferase [24]. Dot blots of human liver RNA samples were probed with ^{32}P -labeled cDNAs specifying human preproapo A [25], human preproapo AII [26], human preapo B [27], human liver fatty acid binding protein [28], human α -fetoprotein [29], human HMG-CoA reductase [30], rat prenyl transferase and rat HMG-CoA synthase. Conditions selected for filter hybridization and washing are listed in Ref. 12. These stringencies were equivalent to those used by others to produce specific interaction between these cDNAs and their respective mRNAs. The relative abundance of each mRNA in the tissue RNA preparations was determined by quantitative scanning laser densitometry of filter autoradiographs using a LKB XL Ultrascan densitometer. Only signals in the linear range of film sensitivity were utilized for calculations of relative mRNA concentration.

Northern blots of total cellular RNA were produced following electrophoretic fractionation through agarose gels containing formaldehyde [31].

Results and Discussion

Accumulation of HMG-CoA synthase, HMG-CoA reductase and prenyl transferase mRNAs in the developing rat liver

Cloned cDNAs encoding HMG-CoA synthase, HMG-CoA reductase, and prenyl transferase were used to probe dot blots of total cellular RNA prepared from rat livers harvested during fetal days 16–21, the suckling period (defined as the first 13 postnatal days, Ref. 32), the weaning phase (days 14–28) and from animals proceeding through sexual maturation (postnatal days 35–70). Material was collected from 10 to 40 male and female animals representing 1–4 litters at each developmental stage studied. The results of our dot blot hybridization analyses are presented in Fig. 1 and show

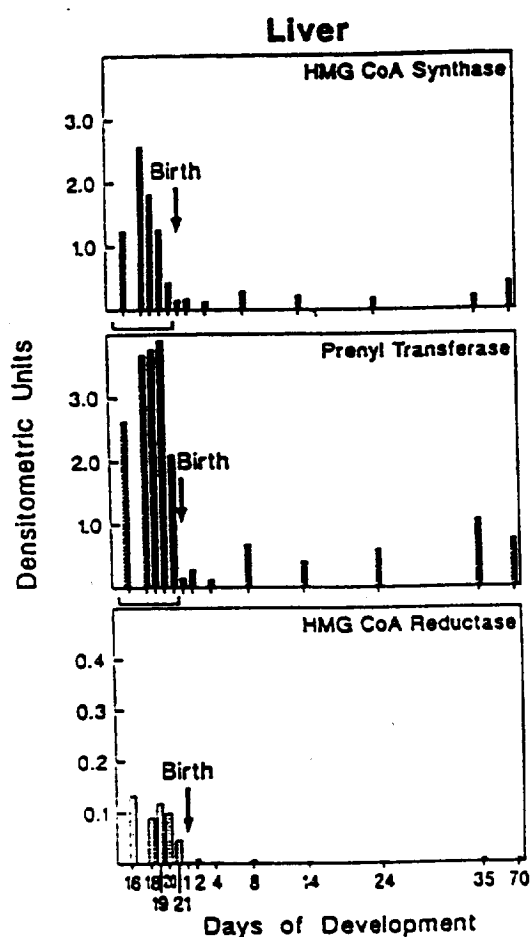


Fig. 1. Developmental changes in rat liver HMG-CoA Synthase, HMG-CoA Reductase and Prenyl Transferase mRNA levels. Total cellular RNA was prepared from pooled rat livers ($n = 10-40$ animals per time point). Cloned rat cDNAs encoding the three enzymes were used to probe dot blots containing four concentrations of each RNA. The relative concentration of each mRNA was calculated based on scanning laser densitometry of filter autoradiographs and expressed in arbitrary densitometric units. Note that the specific activities of the probes were not identical and therefore no comparisons can be made about the relative levels of each mRNA at a particular stage of liver development.

a remarkable similarity in the patterns of change of each mRNA during rat fetal and neonatal development. High levels are noted during late gestation. These fall abruptly within 24 h after parturition reaching concentrations that are as much as 80-fold lower than those peak levels encountered prior to birth (e.g., see the middle panel of Fig. 1 which shows changes in prenyl transferase mRNA concentration). A transient 2–4-fold rise in HMG-CoA synthase and prenyl transferase but not HMG-CoA reductase mRNA accumulation occurs during the mid-suckling period (day 8) followed by yet another rise during early adulthood. It is important to note that this pattern of change is not a general phenomenon in the developing rat liver. For example, when the developmental profiles of apo AI, apo AIV and L-FABP mRNAs were studied using these RNAs, quite different ontologic changes were noted (see, for example, Ref. 6).

The developmental profile of rat liver HMG-CoA reductase and prenyl transferase mRNAs parallel the changes in the activities of these enzymes which have been previously documented by several groups [3,33–35]. The corresponding activities of HMG-CoA synthase have not been reported. The rat fetus obtains only about 10% of the sterol required for growth and development from its mother [36]. Therefore, it is not surprising that very high levels of these mRNAs are observed in rat fetal liver during the period surveyed. The highest levels of apo B mRNA occur during the same phase of rat liver development (i.e., in late gestation) only to be followed by an abrupt, early postnatal drop [12]. This provision for expressing the principal apolipoprotein involved in cholesterol transport at a time of maximal endogenous production emphasizes the co-ordinate nature of these developmental changes in the fetal rat liver.

The precise signals for the rapid postnatal fall in the capacity of the livers of suckling rats to synthesize cholesterol are not known. It may represent an adaptive response to adequate supplies of exogenous cholesterol delivered via mother's milk [3,33,37]. Based on the RNA blot hybridization data, it appears that the mechanism involves at least in part a reduction in the steady-state levels of mRNAs encoding these key enzymes in cholesterol biosynthesis. The data do not allow us to say to what extent such alterations reflect a change in gene transcription or mRNA stability.

The changes observed in the levels of prenyl transferase mRNA during the late suckling through weaning phases can be directly correlated with changes in the levels of this enzyme activity [3]. By the 14th postnatal day, HMG-CoA reductase activity in rat liver has fallen to nearly undetectable levels [36]. However, during this second postnatal week, rises in prenyl transferase occur. This rise coincides with that exhibited by another enzyme involved in the mevalonate to squalene pathway—

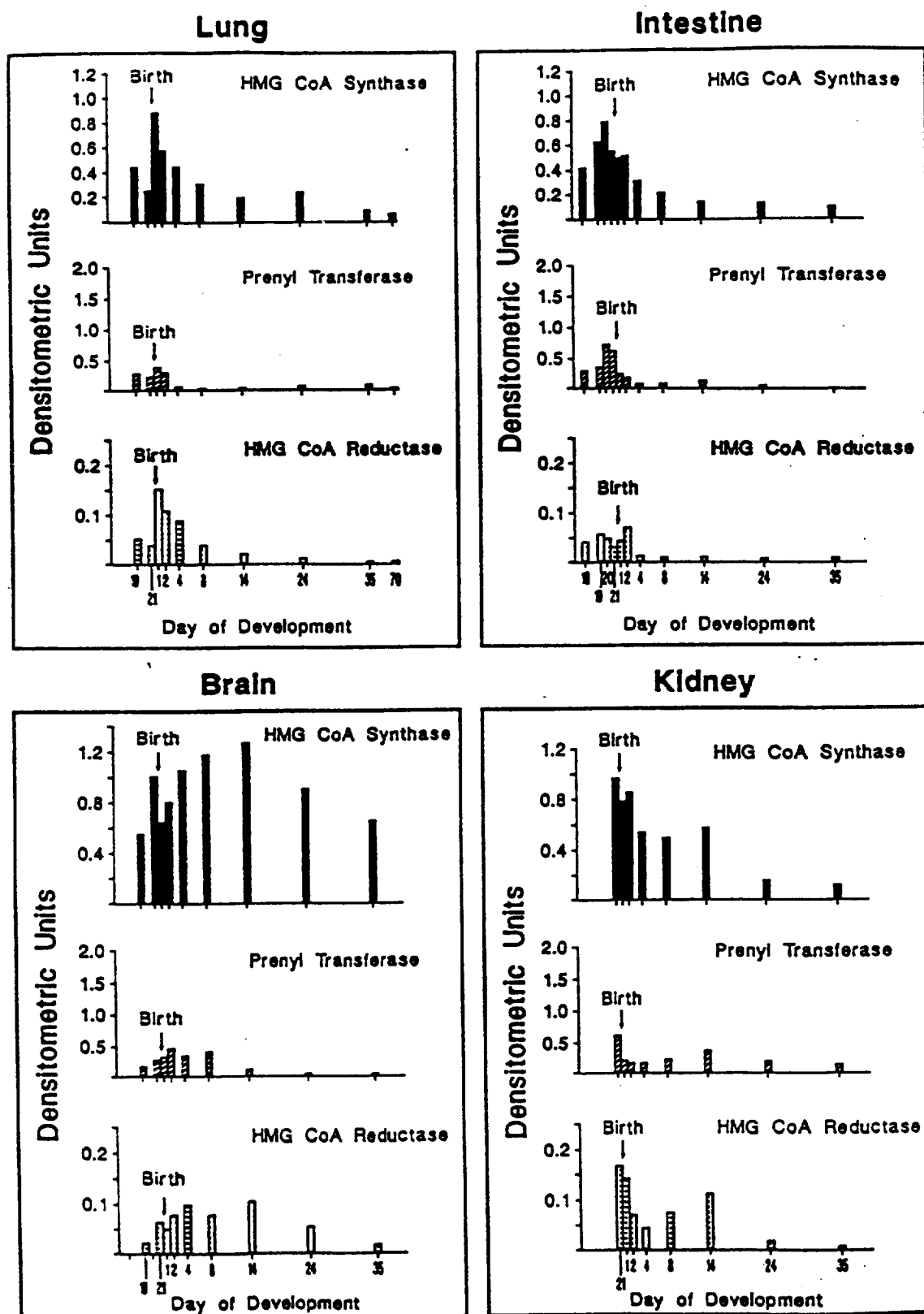


Fig. 2. Coordinate patterns of accumulation of HMG-CoA synthase, prenyl transferase, and HMG-CoA reductase mRNAs during lung, intestinal, brain and kidney development. Total cellular RNA was isolated from pooled tissues harvested from 10-40 rats at each day of fetal and postnatal life noted on the x-axis. Relative mRNA concentrations were determined by scanning dot blot autoradiographs and expressed in arbitrary densitometric units. The only comparison which is permitted by this form of data expression is that which involves the same mRNA within a given tissue.

squalene synthetase. These changes in prenyl transferase and squalene synthetase activity do not correspond to any known change in cholesterol synthesis. Bruenger and Rilling [3] noted that since the primary metabolic destination of isoprenoid precursors is cholesterol, changes in the activities of these enzymes may reflect as yet unknown developmental alterations in the metabolic targeting of mevalonate to other compounds (e.g., dolichol and its derivatives, ubiquinones, or isopentenyl tRNAs [38] and prenylated proteins [39]). Although information about the activity profile of HMG-CoA synthase during rat liver development has not been documented in the literature, based on the mRNA data presented in Fig. 1, its developmental profile would be predicted to more closely resemble that of prenyl transferase than HMG-CoA reductase. Moreover, the blot hybridization studies demonstrate for the first time that there are co-ordinate developmental changes in the levels of these three mRNAs encoding enzymes involved in cholesterol biosynthesis.

Expression of the HMG-CoA synthase, HMG-CoA reductase and prenyl transferase genes in extrahepatic tissues during rat development

The remarkable similarity in the accumulation 'profiles' of these three mRNAs observed during the perinatal period of rat liver differentiation was not unique to this organ. This is illustrated in Fig. 2 which summarizes results obtained from probing dot blots of lung, small intestinal, brain and kidney RNAs prepared from 10–40 animals at each of as many as ten different stages of development. Two obvious conclusions can be made after inspection of the data. First, the timing and direction of developmental variation in relative mRNA concentration was virtually identical for each species within each tissue. Second, as in the liver, the highest concentration of each mRNA during lung, intestinal and kidney development was encountered in the late and early postpartum period with subsequent declines occurring in the suckling and/or weaning phases. The notable exception was brain where a progressive postnatal rise in the concentration of each mRNA occurred that generally reached a peak in the mid to late suckling period. Thus expression of the rat HMG-CoA synthase, HMG-CoA reductase and prenyl transferase genes appears to be elaborately programmed to respond in a similar temporal fashion both within and between different tissues from late fetal life through the end of weaning.

Accumulation of mRNAs encoding cholesterol biosynthetic enzymes and lipid transport proteins in fetal, neonatal and adult human liver

Fig. 3 provides the results of our analysis of developmental changes in the concentration of the three cholesterol biosynthetic enzyme mRNAs in human liver.

A cloned human HMG-CoA reductase cDNA plus cDNAs encoding rat HMG-CoA synthase and rat prenyl transferase were used for these studies. Since the last two represent heterologous cDNAs, a preliminary experiment was performed. Northern blots of RNA prepared from a human hepatocellular carcinoma cell line (Hep G2) were probed with the rat cDNAs employing the same hybridization stringencies listed in Ref. 12 but the final wash temperature was reduced from 55°C to 45°C. The results indicated that each rat probe reacted with a unique human mRNA species—3.1 and 2.2 kb HMG-CoA synthase mRNA and a 1.2 kb prenyl transferase mRNA (data not shown). These sizes are comparable to those previously reported for the corresponding rat mRNAs [23,40]. Dot blots of total cellular RNA isolated from 8.5–25-week fetal livers ($n = 1-3$ individuals per time point) plus two newborns and one adult without evidence of liver dysfunction were then prepared and probed with the three cDNAs using hybridization and washing conditions established above. As in the case of the rat liver RNA dot blots, multiple concentrations (0.5–2 µg) of each human liver RNA sample were included in the dot blots (see panel A of Fig. 3).

Inspection of Fig. 3B reveals a relatively monotonous developmental profile for all three mRNAs. By 8 weeks of gestation these mRNAs have achieved steady-state levels which do not change more than 2-fold during the remaining 17 weeks of fetal life that were surveyed and less than 3–5-fold when compared to the two newborn and single adult RNA preparations. When there was an opportunity to compare more than one sample at a given stage of human fetal development, remarkably little individual variation was noted in the relative level of a given mRNA.

This less than 2-fold change in cholesterologenic mRNA levels during fetal life is not a general phenomenon. It contrasts with results obtained when these same RNA preparations were probed with other cDNAs encoding proteins not involved in lipid metabolism. For example, a control experiment which examined the levels of α -fetoprotein mRNA disclosed the approx. 10-fold reduction expected between first trimester liver and liver harvested at the beginning of the last trimester (see panels A and B of Fig. 3) [41]. In addition, a recent study revealed marked changes in the levels of epsilon, gamma and theta globin mRNAs as well as ceruloplasmin mRNA in the 10–25-week human fetal liver RNA samples [42]. Moreover, the patterns of change of these five different mRNAs were quite distinct from one another.

There is little information about the activities of these cholesterol biosynthetic enzymes during human fetal development. However, the relatively monotonous HMG-CoA reductase, HMG-CoA synthase and prenyl transferase mRNA levels are compatible with observa-

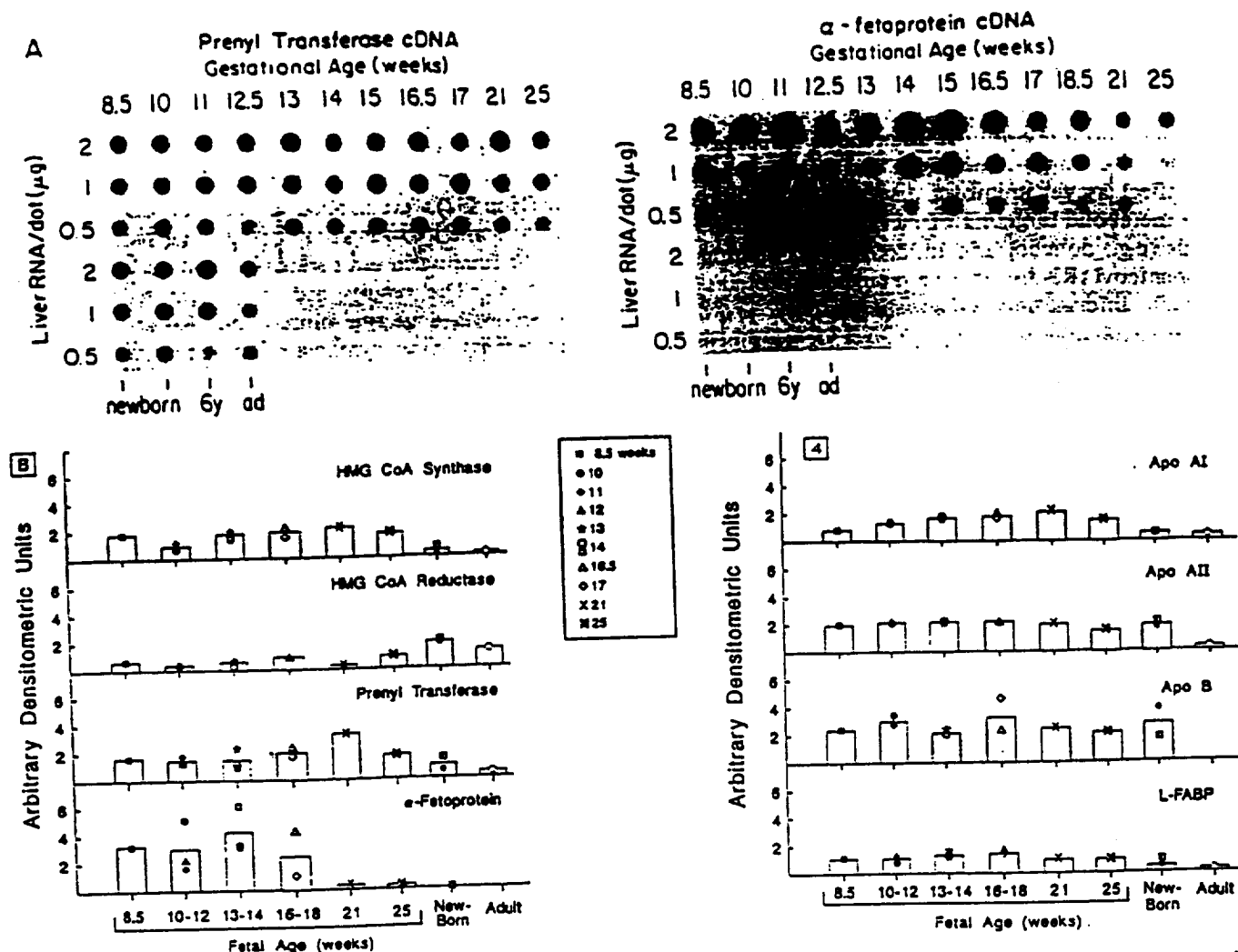


Fig. 3. Developmental changes in the concentration of mRNAs encoding cholesterologenic enzymes in samples of human fetal liver. cDNAs encoding human HMG-CoA reductase plus rat HMG-CoA synthase, rat prenyl transferase (plus human α -fetoprotein) were used to probe dot blots containing several concentrations (0.5–2 μ g) of each human liver total cellular RNA sample. Fetal age is based on crown-rump lengths [15]. Note that the two 14-week samples came from separate fetuses (see the key defining the symbol ascribed to each RNA preparation). Panel A displays representative dot blots probed with 32 P-labeled prenyl transferase and α -fetoprotein cDNAs. Panel B presents developmental profiles of mRNAs encoding the three cholesterol biosynthetic enzymes and α -fetoprotein. As in Fig. 1 arbitrary densitometric units were used to express mRNA levels. No conclusions can be made about the relative concentrations of each of the four mRNAs in a liver RNA sample prepared at a particular developmental stage.

Fig. 4. Developmental profile of human liver mRNAs specifying proteins involved in lipid transport. Blots prepared with the same fetal, neonatal and adult human liver RNA samples as those used to generate the data shown in Fig. 3, were probed with 32 P-labeled cloned cDNA specifying human apolipoproteins apo AI, apo AII, apo B, and liver fatty acid binding protein (L-FABP).

tions made by Carr and Simpson [43] that only slight alterations occur in the rate of cholesterol synthesis in the developing human fetal liver. (This rate was estimated to be approx. 9 mg/day during the 18th week of gestation.)

When the levels of other mRNAs encoding proteins involved in lipid transport were examined in human fetal liver, similar 'flat' developmental profiles were observed (Fig. 4). mRNAs specifying the two principal human HDL apolipoproteins, AI and AII, plus the

principal protein component of LDL, apo B, achieve steady-state concentrations by 8 weeks of gestation that are similar to those documented during weeks 10–25 and in term infants. Variations were less than 2-fold in all cases. Moreover, the mRNA encoding liver fatty acid binding protein (L-FABP), a small cytoplasmic protein believed to participate in the uptake and/or metabolic processing of exogenous fatty acids (reviewed in Ref. 44), displays a similar lack of change in its concentration during this period (Fig. 4). Together these data

suggest an early biochemical maturation of the human fetal liver with respect to its capacity for cholesterol synthesis and lipid transport.

Differentiation of the human and rat fetal liver

There are very few studies which have provided information about the biochemical maturity of human liver from midgestation to term, so it is very difficult to put our data concerning expression of genes involved in lipid metabolism in any sort of comparative metabolic context at the present time. Greengard has conducted an extensive analysis of the literature dealing with enzymatic differentiation of human and rat liver [45]. Although the data for human are both limited in the number of enzymes examined and the scope of the analyses through fetal life, based on published reports concerning approx. 30 enzymes it appears that most liver enzymes exhibit significant quantitative differences in their activities between the second trimester and adulthood. For example, enzymes involved in synthesis of DNA, pentoses, nonessential amino acids, as well as glycolysis have activities in the actively growing fetal liver which are different from those in the adult tissue. In the few examples where developmental profiles are available in fetal and newborn human and rat liver, it appears that enzyme activities change in the same direction in both mammalian species [45] although the timing of such changes has not been well enough characterized to provide a comparative time scale for human and rat liver differentiation. Comparison of our observations concerning human apo A1, apo B and L-FABP mRNA levels in total human fetal liver RNA with previously published studies in rat liver [6,12,46] suggests that the 8-12-week human fetal liver is at least as well 'differentiated' as the 19-21-day fetal rat liver with respect to expression of these mRNAs. Further studies, based on RNA hybridization techniques, should prove valuable in establishing this comparative developmental time scale for a variety of differentiated functions.

One important caveat needs to be mentioned concerning interpretation of these data. Total cellular RNA prepared from fetal rat and human liver by definition includes mRNAs from all cellular constituents in the liver. These cellular populations undergo remarkable changes during development. A limited number of quantitative morphometric studies of human fetal liver indicate that hepatic parenchymal cells account for approx. 50-70% of its cellular population between the 8th to 28th weeks of gestation with erythroid cells representing 40-50% of fetal liver nuclei and granulocytic precursors less than 10% [47]. In the fetal rat liver, approx. 50% of liver cells are involved in hematopoiesis just prior to parturition with this number rapidly decreasing to less than 5% by the end of the suckling period [48]. The consequences of these shifts in cell

populations are obviously important when considering regulation of gene expression in this tissue. For example, the relatively constant levels of mRNAs encoding proteins involved in lipid metabolism that were documented in total human fetal liver RNA during gestation may 'mask' relatively dramatic changes in their concentrations within a given cell type and/or changes in their expression in different cell types. Therefore, it will be very important to extend these analyses using *in situ* hybridization and immunocytochemical techniques to examine changes in the cell specific expression of these genes in the fetal human and rat liver.

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