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BIOSYNTHESIS OF HEPATIC CORTICOSTEROID-BINDING GLOBULIN: ONTOGENY AND EFFECT OF THYROID HORMONE

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Summary—The present study reports on the ontogeny and the effect of thyroid hormones on liver corticosteroid-binding globulin (CBG) biosynthesis, in relation to plasma CBG binding capacity in the rat. We show that $mRNA_{CBG}$ contents were high in liver of 18-day-old fetuses and decreased with age to reach almost undetectable levels by postnatal day 1. Interestingly, at the latter time point plasma CBG concentration remained elevated and disappeared thereafter from the circulation with a half-life of about 3 days. The messenger was localized in parenchymatous cells and not in hematopoietic ones, although the latter constitute the major cell population in fetal liver. It is not until after 10 days of age that $mRNA_{CBG}$ and plasma CBG levels increased in concert, with a sex-difference being noticed by postnatal day 30. Treatment of rats with 3,3',5-triiodo-L-thyronine (T_3), but not reverse T_3 (rT_3) (the predominant form present in fetal serum), enhanced CBG biosynthesis. These findings show that liver $mRNA_{CBG}$ contents undergo dramatic changes during ontogeny and suggest the existence of a differential regulation of the messenger in fetal and neonatal rats.

INTRODUCTION

Steroids are carried in plasma by albumin, as well as by specific binding glycoproteins (for review see [1]). One of the latter, namely corticosteroid-binding globulin (CBG) or transcortin, binds glucocorticoids with high affinity and appears to serve important functional roles. In addition to regulating the level of the unbound, biologically active, glucocorticoid moiety, the binder interacts with cell membrane receptors and, eventually, stimulates the formation of cyclic AMP (for review see [2]). This agrees well with previous evidence which suggested that CBG binds to plasma membrane fraction of pituitary tissue [3].

In a previous report [4], subsequently confirmed in a number of other studies [5–7], we showed that during ontogenesis in the rat, plasma transcortin underwent dramatic changes. While it was high in the fetus, the plasma CBG content fell rapidly after birth to reach almost undetectable levels. This resulted in an opposite increase of unbound corticosteroid, enhancing the negative feedback inhibition of CRF and ACTH secretions and providing a possible explanation for the so-called "stress nonresponse period" ([8] and for review [9]). In addition, we and others [4, 7] also showed that thyroxine treatment of neonates, similarly to mature rats [10], caused an increase in plasma transcortin binding capacity.

Because the liver is the major site of CBG synthesis [2] and the primary structure of rat CBG has actually been sequenced [11], we aimed at correlating plasma CBG concentrations with hepatic messenger RNA ($mRNA_{CBG}$) levels in both fetal and neonatal rats and, in addition, evaluating the role of the thyroid function in CBG biosynthesis.

EXPERIMENTAL

Animals and reagents

Female Wistar rats (200–250 g) were used. The time of conception in the pregnant females was assessed from sperm-positive vaginal smears examined on the morning following the mating night and was termed day 0. Fetuses and neonates were decapitated to collect the blood and the liver was then rapidly dissected out and frozen on dry ice. Plasma samples obtained after centrifugation and livers were kept frozen at −80°C until assayed. 3,3',5-Triiodo-L-thyronine (T_3) and reverse T_3 (rT_3) were purchased

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from Sigma Chimie (La Verpillière), while [α-^{32}P]deoxyadenosine triphosphate ([α-^{32}P]dATP; 3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and [4-^{14}C]corticosterone (55 mCi/mmol) were from New England Nuclear (Du Pont de Nemours, France). Terminal deoxynucleotidyl-transferase was from Boehringer Mannheim (Fed. Rep. Germany).

CBG, β-globin and actin probes

The CBG probe was a synthetic single stranded oligodeoxynucleotide, complementary to the rat CBG sequence and corresponding to the amino acids 29–42 [11]. It was obtained by means of the phosphoramidite method, followed by purification by reverse-phase high-performance liquid chromatography (IBMC, Strasbourg). The probe (50 ng) was labeled with 50 μCi [α-^{32}P]dATP and 50 U terminal deoxynucleotidyl-transferase, in a buffer composed of: 100 mM potassium cacodylate, 2 mM $CoCl_2$, 0.2 mM dithiothreitol, at pH 7.2. After being filtered through a Nensorb column (Du Pont de Nemours), the specific activity of the probe was calculated to be 5–10 × 10^3 Ci/mmol. The specificity of the probe was ascertained by the demonstration on Northern blot of a single radioactive band of about 1.8 kilobases (kb) in the liver extract, while no radioactivity was detected in extracts of either brain or pituitary tissues (Fig. 1). Identical results were obtained using tissues from either adult, fetal or neonatal rats (not shown).

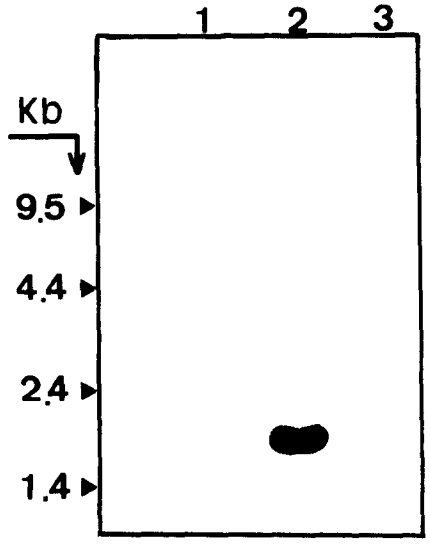

Fig. 1. Autoradiograph of a Northern blot analysis of mRNA coding for CBG. Total RNA (20 μg) isolated from forebrain (1), liver (2) and anterior pituitary gland (3). RNA molecular markers, in kb, are indicated. Liver $mRNA_{CBG}$ migrated as a species of about 1.8 kb.

The β-globin probe was a 2.3 kb fragment of the major mouse β-globin gene, subcloned from the gt-WES-MβG2 phage [12]. It contained the first two exons and the two introns of the gene and was found to hybridize with rat globin mRNA and poly-A RNA extracted from erythroblasts of rat fetal liver [13]. The actin probe was a cloned cDNA fragment of 1.2 kb [14], corresponding to the coding region of the human skeletal muscle α-actin gene. In our hands, it generated two distincts bands of about 2.2 and 1.6 kb, relative to β- and α-actin, respectively [15].

Both globin and actin probes were labeled with [α-^{32}P]CTP, with the multiprime DNA-labeling system (as indicated by Boehringer Manheim). The final specific activity of the probe, after gel filtration through Sephadex G50 columns, was 0.5–1.0 × 10^9 cpm/μg.

Preparation of hematopoietic- and parenchymatous-enriched cell populations from fetal liver

Enriched populations of hematopoietic and parenchymatous cells were prepared from fetal livers, according to [16]. In brief, 10–12 livers from 17-day-old fetuses were dissociated by hand in sterile and ice-cold phosphate-buffered saline (pH 7.4), using a plastic tube with a loosely-fitted pestle. The cell suspension was filtered through a 60 μm-mesh nylon screen and the filtrate centrifuged at 600 *g*/10 min to yield the hematopoietic cell fraction, which is composed of >95% erythropoietic cells and <5% leucopoietic cells and, importantly, which is not contaminated by hepatocytes [17]. The parenchymatous cell fraction (composed of mixture of hepatocytes and hematopoietic cells) was recovered from the nylon screen and pelleted by centrifugation in phosphate-buffered saline as indicated above.

Northern blot

Total RNA contents were extracted from whole liver and liver enriched populations of hematopoietic and parenchymatous cells according to [18], with an additional phenol–chloroform purification as described [19]. The RNA was recovered by ethanol precipitation and quantified by OD measurement at 260 nm.

Electrophoresis of total RNA was performed using a gel composed of 2% formaldehyde and 1.5% agarose, containing 0.7 μg/ml ethidium-bromide in 20 mM of 3(*N*-morpholino)-propansulfonic acid at pH 7.4 [20], for 3 h at 100 V. Known amounts (1–25 μg) of liver RNA were

run in parallel. After electrophoresis, the gels were washed in water and photographed under u.v. illumination using a Polaroid type 665 film. The gel was then processed to allow transfer to nitro-cellulose filter [21] and dried for at least 1 h at 80°C.

Hybridization conditions

Prehybridization (8 h) and hybridization (overnight) were carried out in 50% formamide, according to [22], at 30 and 42°C for CBG and globin/actin, respectively. The final stringencies of media were 0.5 SSC (1 SSC = 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4), 0.1 M sodium dodecyl sulfate at 45°C for the CBG probe and at 55°C for the globin and actin probes.

Measurement of $mRNA_{CBG}$

Quantitation of $mRNA_{CBG}$ was performed according to [23], with minor modifications. In brief, after hybridization with the radioactive probe, the nitro-cellulose membranes were exposed to Kodak XAR-5 film at −70°C, between two intensifying screens. Autoradiograms were scanned densitometrically using a digitized image-analysis system (RAG-200 Biocom, France). The signal densities of the unknown samples were expressed as arbitrary units (A.U.), relative to the linear response curve generated by increasing concentrations of liver total RNA. Furthermore, the densities relative to 28S RNA were measured by scanning the negative Polaroid 665 film, as described above; which allowed us to compensate for any differences in the amounts of RNA (15 μg) that were loaded onto the gel.

Measurement of plasma CBG binding capacity

A modification of the method described by Labrie [10] was used. Briefly, 0.15 ml plasma samples were labeled by incubation at 4°C overnight with various concentrations of unlabeled and [^{14}C]corticosterone. Bound and unbound steroid moieties were separated by filtrating 0.1 ml plasma aliquots through micro-columns (0.6 × 15 cm) of Sephadex G-25, equilibrated and eluted at 4°C with 0.1 M phosphate buffer, at pH 7.4.

RESULTS

Developmental pattern of hepatic $mRNA_{CBG}$

As depicted in Figs 1 and 2, hepatic CBG mRNA migrated as a radioactive band of ap-

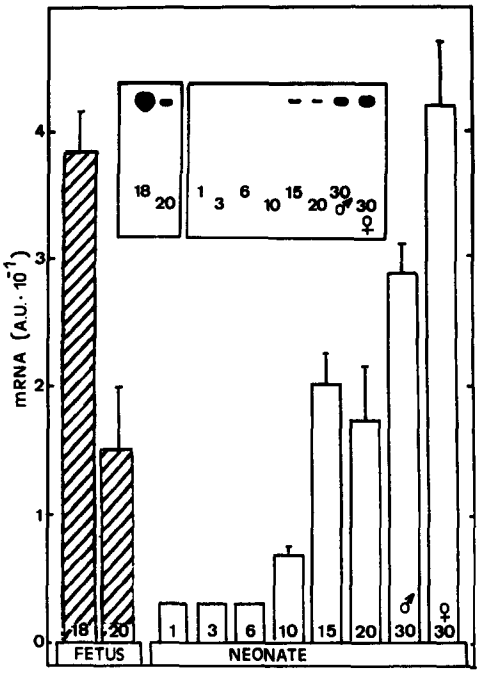

Fig. 2. Developmental pattern of liver $mRNA_{CBG}$ levels. The bars are the means ± SEM of 3 determinations, each with extracts from either 4 fetal livers or 1 neonatal liver from separate litters. The figures in the bars refer to developmental age in days. Values for 1-, 3- and 6-day-old pups were below the limit of detection. The inset shows a representative autoradiograph of a Northern blot for liver RNA extracts of rats at the given developmental ages.

prox. 1.8 kb. There were dramatic changes in $mRNA_{CBG}$ during development, with high levels being present in liver of fetuses on days 18 and 20, which dropped after birth to reach almost undetectable levels of postnatal day 1. It increased gradually thereafter from postnatal days 10 to 30, at which time point a sex-difference in mRNA contents became apparent. No significant differences in both liver mRNA and plasma CBG levels were found at earlier developmental stages.

Because in fetal livers the hematopoietic cell population greatly outnumbers that of hepatocytes [24], we meant to characterize the cell type(s) which actually synthesize(s) CBG. By examining enriched populations of hematopoietic and parenchymatous cells for their abilities to accumulate mRNA for CBG and β-globin, we showed (Fig. 3) that the homogeneous hematopoietic cell fraction (virtually free of hepatocytes; [17]) failed to accumulate $mRNA_{CBG}$; while the parenchymatous cell fraction (which was shown to contain hepatocytes; [17]), did express the CBG gene. In contrast, all cell fractions reacted positively for β-globin (Fig. 3).

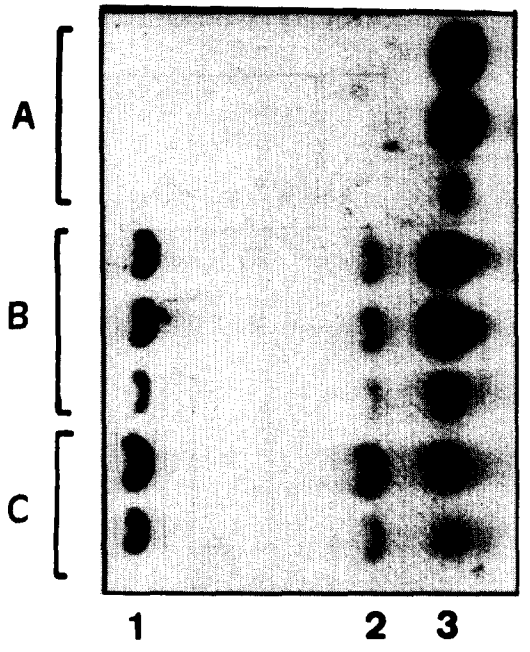

Fig. 3. Autoradiogram of Northern blot analysis of mRNAs for CBG and β-globin in liver extracts from 16-day-old fetuses. Enriched populations of hematopoietic cells (A) and parenchymatous cells (B), compared with whole liver extract (C). Samples were first probed with the CBG probe (1, 2) and then with the β-actin probe (3) without removing the CBG signal. CBG and β-globin mRNAs migrated respectively as, approx. 1.8 kb and 0.6–0.7 kb entities. The amounts of applied RNA samples were (from top to bottom): 4.5, 8 and 2 μg for A; 6, 6 and 3.5 μg for B; 7.5 and 9 μg for C.

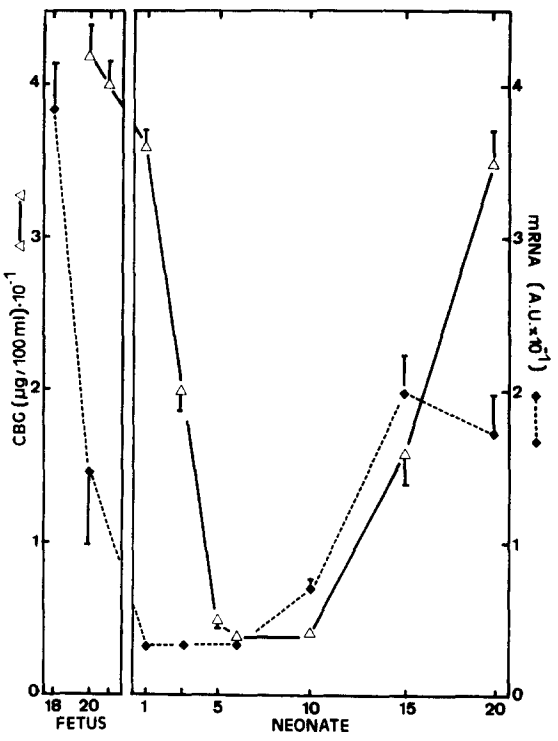

Fig. 4. Developmental patterns of plasma CBG binding capacity and liver $mRNA_{CBG}$ levels. Values are means ± SEM of 6 determinations for plasma and 3 determinations for liver (values derived from data in Fig. 2).

Dissociation between plasma CBG levels and liver $mRNA_{CBG}$ contents in neonatal rats

It is clear from data depicted in Fig. 4 that plasma CBG concentration was high in 1-day-old neonates, while the $mRNA_{CBG}$ concentration had already reached a low level at that time point. There was actually a delay in plasma CBG binding capacity, which disappeared from the circulation with a calculated half-life of about 3 days.

After postnatal day 10, hepatic $mRNA_{CBG}$ levels and plasma CBG concentrations increased in parallel, with the binder's biosynthesis preceding its presence in plasma at day 10 and leveling off at day 15.

Effect of administration of T_3 and rT_3 on hepatic CBG biosynthesis in neonatal rats

Because, on the one hand, thyroid hormones are known to enhance CBG binding capacity in plasma and, on the other, only rT_3 seems to be present at high concentrations in the fetal rat [25], we tested both T_3 and rT_3 for their abilities to alter CBG biosynthesis. As shown in Fig. 5, injection of T_3 resulted in an approx. 10–15-fold increment in CBG mRNA levels, relative to the lower limit of messenger detection observed in control samples. The stimulatory action of T_3 appeared to be specific, because the expression of the actin gene was unchanged by hormone treatment. Administration of rT_3, however, was without detectable effect.

DISCUSSION

There is clearcut evidence that plasma CBG binding capacity decreases sharply after birth in rats [4–7], allowing an opposite increase in the levels of the unbound, biologically active, glucocorticoid moiety. This would enhance the negative feedback inhibition of the activity of the brain–pituitary–adrenal axis during the so-called "stress nonresponsive period" [8, 9] and also participate in other important physiological functions, such as the regulation of intestinal lactase and sucrase production rates [26], the maturation and differentiation, as well as surfactant and phospholipid production of the lung [27]. Furthermore, lack of binding of CBG to cell membrane receptor, which may be coupled to adenylate cyclase [2], may be

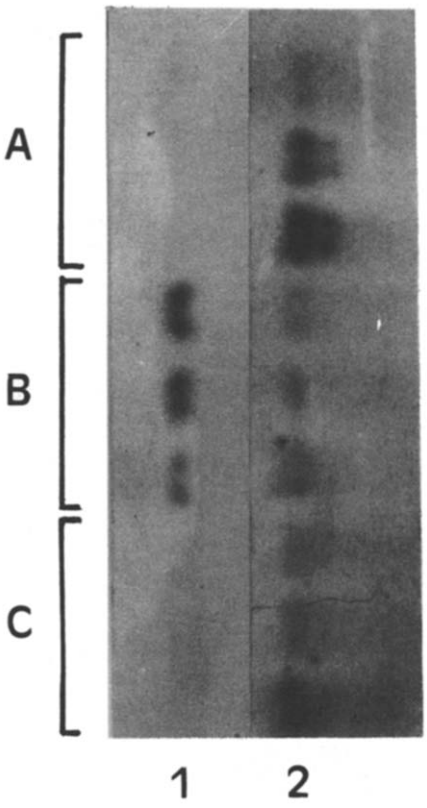

Fig. 5. Effect of thyroid hormone treatments on liver mRNA contents of CBG and actin in 7-day-old neonatal rats. Pups from 3 litters received daily s.c. injections of 1 μg of either T_3 (B) or rT_3 (C) or vehicle (A), from postnatal days 1 to 6. Samples of liver extracts (15 μg) were probed for CBG (lane 1) and, after being treated to remove the signal, for actin (lane 2).

assumed to decrease the overall production of cyclic AMP in target tissues.

We show here that plasma CBG content in neonates disappeared slowly from the circulation with a half-life of about 3 days and was preceded by a much more dramatic fall in hepatic CBG mRNA level. The latter, which was revealed on Northern blot autoradiograms as an approx. 1.8 kb entity, actually corresponds to a state of equilibrium between both synthesis and degradation of the messenger. The $mRNA_{CBG}$ was present at high concentration in the liver of fetuses and, strikingly, was almost undetectable in tissues of neonates as soon as 1 day after birth, indicating that plasma CBG in the neonate was of fetal origin. These observations agree well with previous evidence that high levels of a 1.8 kb CBG mRNA were present in fetal liver, while the messenger's expression was low in livers of 1-week-old rats, as was the plasma concentration of the binder at this stage of development [28].

Given that in fetal livers $mRNA_{CBG}$ is present in high concentration and that the hematopoietic cell population outnumbers that of the hepatocytes [24], we attempted to identify the very cell type(s) that is (are) involved in expression of the CBG gene. We showed that the parenchymatous-enriched cell population expressed $mRNA_{CBG}$, while the homogeneous hematopoietic cell population proved to be negative in this regard. It is clear, however, that additional data based on *in situ* hybridization will be required to ascertain that conclusion. Anyhow, it is a striking observation that fetal hepatocytes, which lack the main phenotypic features of the differentiated hepatocytes, do express $mRNA_{CBG}$ at such a high level.

Plasma CBG binding capacity is regulated, at least in the adult rat, mainly by glucocorticoids and thyroid hormones, which exert inhibitory and stimulatory effects, respectively; the stimulatory action of estrogens being partly mediated via thyroid hormones [10]. During development in the rat the stimulatory component of CBG biosynthesis, namely the thyroid function, shows striking differences in activity: TRH mRNA production in the hypothalamus do not increase in response to hypothyroidism until after 7 days of age [29]; hepatic generation of T_3 is impaired in the fetus and the hormone is present in serum mainly as rT_3 [25], while in the neonate serum T_4 and T_3 concentrations increase after birth to reach adult levels by day 20 [29, 30]. Given these findings, we examined the effects of both T_3 and rT_3 on CBG mRNA in liver of neonatal rats and showed that only T_3 was able to enhance the level of the messenger. This suggests that in the fetus, in which rT_3 is the predominant form of circulating thyroid hormone, liver $mRNA_{CBG}$ may be regulated differentially compared to neonates and adults. In fact, after propylthiouracil treatment of pregnant rats, which impairs both maternal and fetal thyroid function, plasma CBG levels [31], as well as hepatic $mRNA_{CBG}$ content (unpublished results) were unchanged in the fetus.

Our study demonstrates the marked changes in hepatic CBG mRNA levels which occur during ontogenesis, although it needs to be assessed whether they reflected differences in transcription and/or degradation of the messenger. Our data also suggest the existence of a differential regulation process of $mRNA_{CBG}$ biosynthesis in fetal and neonatal livers, which is currently being investigated.

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