SEQUENCES OF INTEREST

Rat Corticosteroid Binding Globulin: Primary Structure and Messenger Ribonucleic Acid Levels in the Liver under Different Physiological Conditions

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Rat corticosteroid binding globulin (CBG) cDNAs were isolated from a λgt11 liver cDNA library. When rat hepatic mRNA was hybrid selected and translated *in vitro*, a major product reacted with antibodies against rat CBG and its M_r (~43,000) was consistent with a nonglycosylated, CBG precursor polypeptide. Two overlapping cDNAs produced a 1,432 nucleotide sequence with an open reading frame comprising 396 amino acids. This includes a potential signal peptide of 22 residues followed by the amino terminus of purified rat CBG. Rat CBG therefore contains 374 amino acids (M_r = 42,196), and has six consensus sites for *N*-glycosylation. There is 60% identity in the primary structures of rat and human CBG over 383 residues that comprise the human sequence. Furthermore, the single cysteine in rat CBG corresponds to one of two cysteines in human CBG, and this may be significant because a cysteine is located in the human CBG steroid binding site. Northern analysis of RNA from various rat tissues revealed an approximate 1.8 kilobase CBG mRNA only in the liver. Its relative abundance in a pregnant rat was only 30% higher than in an adult female; approximately 3-fold higher than in an adult male, and 25-fold higher than in the fetuses from the same animal. Southern analysis of rat genomic DNA suggests the presence of a single gene for CBG. (Molecular Endocrinology 3: 420–426, 1989)

INTRODUCTION

In vertebrate blood, corticosteroid binding globulin (CBG) serves as the main transport protein for glucocorticoids (1), and more than 90% of circulating corticosterone is bound to CBG in the adult rat (2). The protein has been purified from the blood of several mammalian species, and is generally characterized as an acidic glycoprotein with a molecular mass of 44–60 kilodaltons (kDa) (3). Although the steroid binding site of CBG displays a preference for glucocorticoids, its affinity for steroids is species specific and it may bind progesterone and testosterone with high affinity in some species (4). Recently, the primary structure of human CBG has been deduced by sequence analysis of a cDNA (5), and this revealed the presence and location of two cysteine residues; one of which is present in the steroid binding site (6, 7). We therefore wished to compare the cDNA-deduced primary structures of rat and human CBGs in the expectation that this would reveal information about biologically important regions.

Several studies have shown that CBG is produced by the rat liver (8, 9), and it is assumed that the major site of synthesis is the hepatocyte (10). However, the protein has been identified in a variety of glucocorticoid responsive tissues and cell types (11–13), and this may be attributed to either uptake from the blood or local synthesis. The latter is an important issue because it has been suggested that CBG may actively participate in the delivery of steroids to target cells by interacting directly with a plasma membrane receptor (14, 15). The availability of a rat CBG cDNA has allowed us to determine more precisely the tissue specificity of CBG synthesis, and to demonstrate that hepatic CBG mRNA levels reflect normal variations in plasma CBG concentrations in adult rats (16, 17). In contrast, relatively low levels of CBG mRNA in the fetal rat liver suggest that other factors may regulate plasma CBG levels *in utero*.

RESULTS AND DISCUSSION

Hybridization of 5×10^5 plaque forming units of a rat liver cDNA library with a human CBG cDNA at reduced

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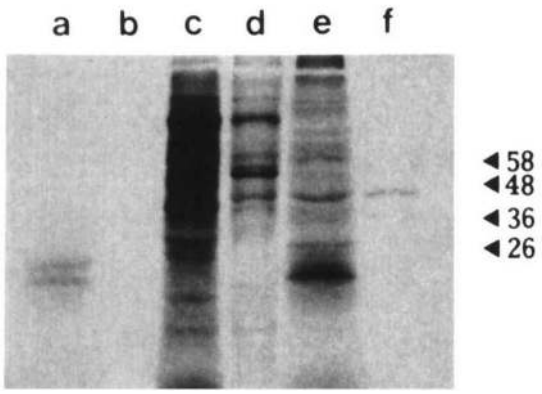

Fig. 1. Fluorograph of Rat Liver RNA Translation Products

Samples were resolved by SDS-polyacrylamide gel electrophoresis (12% acrylamide) fixed, and treated with EN^{3}HANCE before fluorography (18 h at −80 C). Lane a, Control *in vitro* translation (water); lane b, immunoadsorption of control translation with CBG antibody; lane c, *in vitro* translation of rat liver poly(A)$^+$ RNA; lane d, immunoadsorption of (c) with rat CBG antibody; lane e, *in vitro* translation of CBG cDNA hybrid-selected rat mRNA; lane f, immunoadsorption of (e) with rat CBG antibody. Protein standards (kDa) are shown on the right of the figure.

stringency, produced 14 positive clones. Upon induction with isopropyl-D-thiogalactoside, two of these clones produced β-galactosidase fusion proteins that reacted with an antibody against rat CBG. The same antibody was used to confirm the identity of CBG produced *in vitro*, and when rat liver poly(A)$^+$ RNA was translated with a rabbit reticulocyte lysate, the antibody recognized several minor products; one of which is approximately 43 kDa (Fig. 1). In addition, when a CBG cDNA was used to hybrid select hepatic RNA, before *in vitro* translation, it increased the relative abundance of the immunoreactive approximately 43 kDa protein. This is an appropriate size for a nonglycosylated CBG precursor, and therefore probably corresponds to a polypeptide originating from the correct initiation codon in the CBG mRNA. An approximate 36 kDa protein was the only other immunoreactive translation product of hybrid-selected mRNA, and may be the result of initiation of translation at the second methionine codon in the CBG mRNA. The identity of the cDNAs was further confirmed when the amino-terminal sequence of rat CBG (3) was located within the major open reading frame.

The cDNAs from clones containing the two largest inserts were isolated, and appropriate restriction fragments were analyzed to confirm the entire sequence in both directions. It was found that both cDNAs contained inversions at their 5′-ends, which are probably due to artifacts produced during library construction (18). However, when overlapping sequences were compared, a sequence of 1432 nucleotides was obtained (Fig. 2). Within this sequence, an initiation codon (located 44 nucleotides from the 5′-end) starts an open reading frame of 396 amino acids that terminates 196 nucleotides from the polyadenylated 3′-end. Like the human CBG cDNA (5), a second in-frame minicistron (predicted amino acid sequence: M-E-A-F-P-Q-S-S-Q-G-L-P-N-P-I-Y-F-STOP) is located between the CBG reading frame and a potential polyadenylation signal that is located 12 nucleotides before the polyadenylated 3′-end. However, the human and rat CBG cDNA 3′-noncoding nucleotide sequences are very different, and it is unlikely that these minicistrons have any biological significance.

The known amino-terminal sequence of rat CBG (3) was located 22 amino acids from the initiation codon for the CBG precursor. These first 22 amino acids, are predominantly hydrophobic and share a high degree of homology with the proposed signal peptide of human CBG (5), and therefore probably represent a leader sequence that is cleaved during translational processing (19). The mature form of rat CBG should therefore contain 374 amino acids with a predicted molecular mass of 42,196. This agrees with previous estimates if a carbohydrate content of approximately 28% is taken into consideration (20). The cDNA-deduced, amino acid composition of rat CBG also compares favorably with estimates obtained by direct analysis (21).

The open reading frame for rat CBG contains nine less amino acids than human CBG, and it was necessary to make allowances for this at three locations when their nucleotide and precursor polypeptide sequences (Fig. 3) were aligned for comparison. One of these occurs between the proposed signal peptide and the amino terminus of rat CBG; the first five residues of which differ when compared to human CBG. This is interesting in view of the considerable sequence homology (82%) between the human and rat CBG signal peptides. At the nucleotide level, rat and human CBG cDNAs are 71% identical over a 1254 nucleotide overlap that includes the open reading frame for their precursor polypeptides. The overall degree of homology between the mature forms of rat and human CBG is 60% over the 383 residues that comprise the human protein, and it is possible that differences in the size of the CBG polypeptide between species may be related to variations in the organization of their respective genes.

It is, however, evident that several regions are well conserved between rat and human CBG, and this suggests they may be biologically important. The mature form of rat CBG, contains six potential *N*-glycosylation sites (22), two of which are in identical relative positions when compared to human CBG. They are also located within highly conserved areas, and it is likely that these two sites are used in both species. It is known that human CBG contains five N-linked carbohydrate chains (23), and one of these is located at Asn 9 (24). There is also a consensus site for *N*-glycosylation in a similar position (Asn 3) in the rat sequence, but according to direct sequence analysis (3), this is not used. In contrast to human CBG, the mature form of rat CBG contains only a single cysteine. This aligns with cysteine 228 in the human CBG sequence and is located in a conserved region, whereas the region surrounding cysteine 60 in human CBG is very different in the rat. It would therefore appear that cysteine 288 is probably the residue

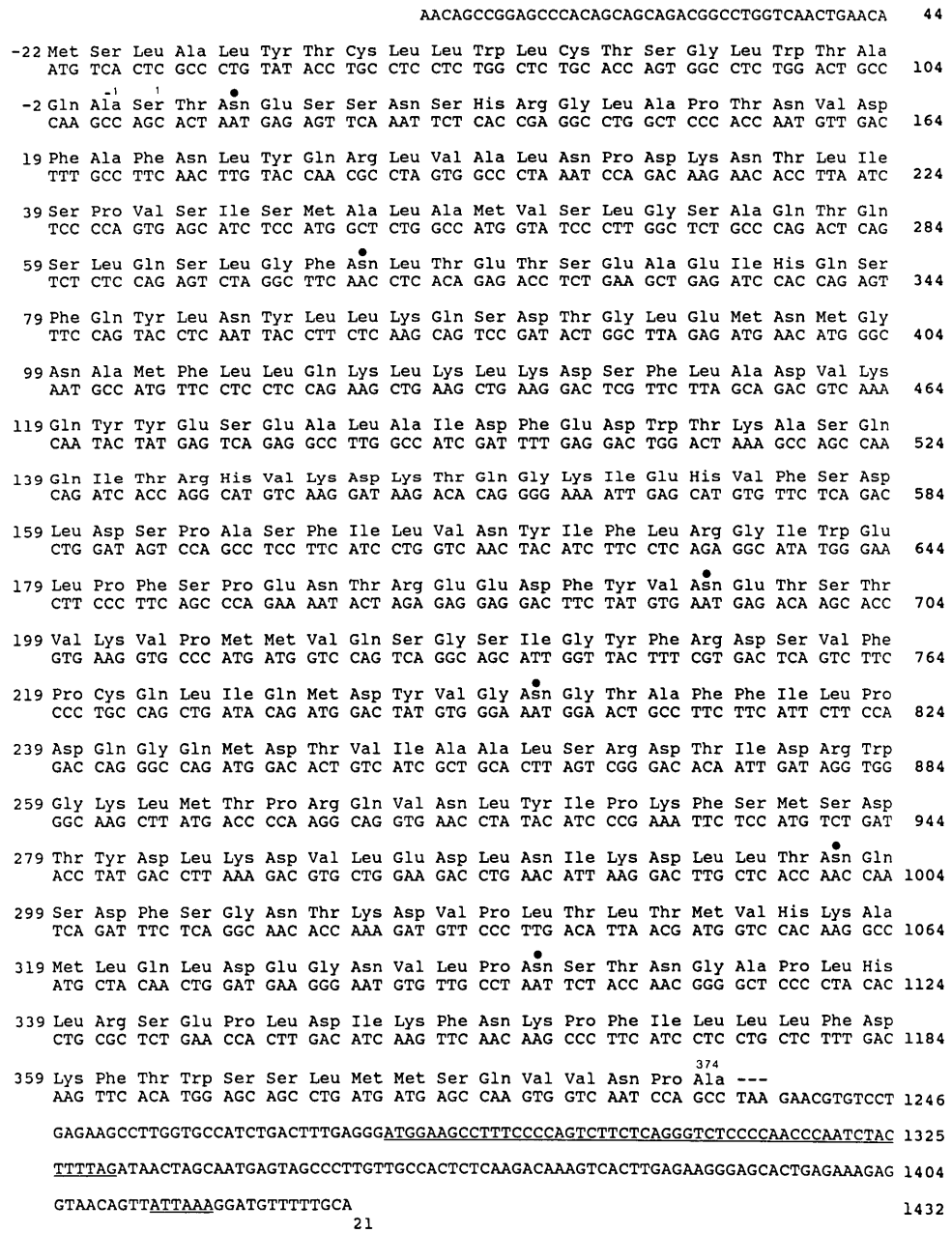

Fig. 2. Nucleotide Sequence of Rat Liver CBG cDNA, and Deduced Amino Acid Sequence of the Precursor CBG Polypeptide

The amino acids in the mature peptide are numbered 1 to 374, and those in the signal peptide are −22 to −1. There are six consensus sequences for *N*-glycosylation (●). A second in-frame minicistron and a potential polyadenylation signal are underlined.

previously identified in the human CBG steroid binding site (6, 7). Other regions are less well conserved between species; the most notable of which spans the 60 carboxy-terminal residues, where there is only 49% identity. This is interesting because it corresponds to the reactive region of the serine protease inhibitors (25) that are related to CBG (5).

A Southern blot of rat genomic DNA is presented in Fig. 4. Restriction endonucleases which do not cleave the rat CBG cDNA used as probe, yield either one band (>23 kilobase) by Southern analysis (*Eco*RI and *Kpn*I), or two large bands (>23 and ~21.5 kilobase) in the case of *Bam*HI. On the other hand, *Hin*dIII, which cuts the cDNA approximately 500 bases from its 3′-end, produces an approximate 6.8 kilobase fragment which hybridizes strongly and a larger fragment (>23 kilobase) which hybridizes weakly. Similarly, *Pvu*II cuts the cDNA approximately in half, and produces two fragments (~8.7 and ~4.6 kilobase) which both hybridize intensely. On the basis of this information, it appears that rat CBG is probably encoded by a single gene that is approximately 15 kilobase pairs in length.

We have previously identified CBG mRNA in the rhesus monkey liver, testis, and kidney, and have cloned a CBG cDNA from a human lung library (5). The availability of a rat CBG cDNA has now allowed us to perform a more comprehensive study of CBG mRNA distribution. When a Northern blot of RNA extracts from

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          -20       -10         1         10        20        30        40
HUMAN   MPLLLYTCLLWLPTSGLWTVQAMDPNAAYVNMSNHHRGLASANVDFAFSLYKHLVALSPKKN
RAT     -S-A--------C------A--[::::::]STNES--S-----PT------N--QR----N-D--

          50        60        70        80        90        100       110
IFISPVSISMALAMLSLGTCGHTRAQLLQGLGFNLTERSETEIHQGFQHLHQLFAKSDTSLEMTMGNALF
TL------------V---SAQ[:]-[::]-S--S----T--A----S--Y-NY-LKQ---G---N----M-

         120       130       140       150       160       170       180
LDGSLELLESFSADIKHYYESEVLAMNFQDWATASRQINSYVKNKTQGKIVDLFSGLDSPAILVLVNYIF
-LQK-K-KD--L--V-Q-----A--ID-E--TK--Q--TRH--D------EHV--D-----SFI------

         190       200       210       220       230       240       250
FKGTWTQPFDLASTREENFYVDETTVVKVPMMLQSSTISYLHDSELPCQLVQMNYVGNGTVFFILPDKGK
LR-I-EL--SPEN----D---N--ST------V--GS-G-FR--VF----I--D-----A------Q-Q
                                   *

         260       270       280       290       300       310       320
MNTVIAALSRDTINRWSAGLTSSQVDLYIPKVTISGVYDLGDVLEEMGIADLFTNQANFSRITQDAQLKS
-D-----------D--GKLM-PR--N-----FSM-DT---K----DLN-K--L---SD--GN-K-VP-TL

         330       340       350       360       370       380
SKVVHKAVLQLNEEGVDTAGSTGVTLNLTSKPIILRFNQPFIIMIFDHFTWSSLFLARVMNPV
TM[:]----M---D-GN-LPNSTN-AP-H-R-E-LDIK--K---LLL--K------MMSQ-V--A
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Fig. 3. Comparison of the Amino Acid Sequences (Single Letter Code) of Rat and Human CBG Precursor Polypeptides

Hyphens (-) indicate identical amino acids. Potential *N*-glycosylation sites are indicated (●). The cysteine residue common to both rat and human CBG is also identified (*). *Stippled blocks* indicate where gaps in the rat CBG sequence were required to align the two sequences.

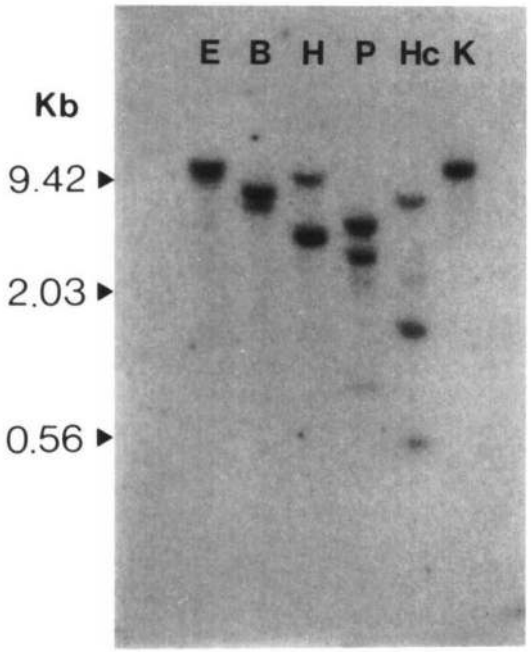

Fig. 4. Southern Blot of Rat Placental Genomic DNA

Kodak XAR film was exposed to the blot for 6 days at −80 C with a Dupont Cronex HI-PLUS XH intensifying screen. Restriction enzymes used: E, *Eco*RI; B, *Bam*HI; H, *Hind*III; P, *Pvu*II; Hc, *Hin*cII; K, *Kpn*I. DNA molecular size standards are shown on the *left* of the figure.

various tissues was probed with radiolabeled, rat CBG cDNA, an approximate 1.8 kilobase CBG mRNA was detected only in the liver (Fig. 5). We have also analyzed poly(A)$^+$ RNA from various tissues in an attempt to increase the sensitivity obtained by northern blotting, but were still unable to detect CBG mRNA in tissues other than the liver (data not shown). However, the possibility cannot be excluded that CBG mRNA is present in a small population of specific cells within a given tissue, and this question will only be resolved by a combination of immunocytochemistry and *in situ* hybridization with high specific activity, CBG cRNA probes.

It is clear from the Northern blot that the relative abundance of hepatic CBG mRNA varies with the physiological status of the animal. In order to quantify this difference more accurately, the liver RNA extracts were also analyzed for CBG mRNA content by a solution hybridization assay (Table 1). This revealed that the relative amount of CBG mRNA was 30% higher in the 21-day pregnant rat, when compared to the nonpregnant female. A sex difference was also observed, and the CBG mRNA content of the adult male liver was 45% of that found in the nonpregnant female liver. The most remarkable difference was that the CBG mRNA level in the 21 day fetal liver was only approximately 4% of that found in the maternal liver.

In many respects, the ontogeny (2) and hormonal regulation (26, 27) of plasma CBG levels in rats resembles the situation in humans (28), but there are differences. For instance, in humans estrogens are assumed to be responsible for an approximately 2-fold, pregnancy-associated increase in plasma CBG levels (28). This is not observed in rats (17), and is reflected in only a small difference in the relative abundance of hepatic CBG mRNA levels in the pregnant and nonpregnant female. Unlike humans, there is also a sex difference in plasma CBG levels in adult rats (16) which has been attributed to a difference in hepatic CBG mRNA levels, measured indirectly by *in vitro* translation (29), and our

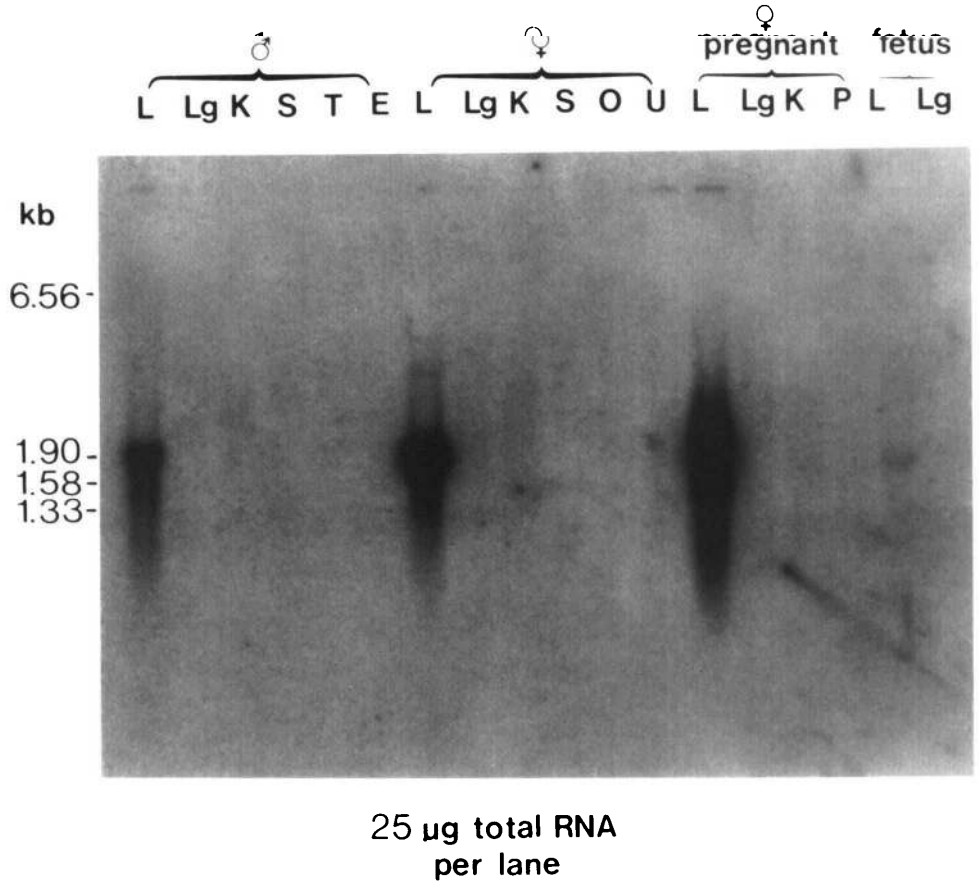

Fig. 5. Northern Blot of RNA Extracts from Various Rat Tissues under Different Physiological Conditions

The autoradiograph (18 h at −80 C), was obtained using Kodak XAR film and a Dupont Cronex HI-PLUS XH intensifying screen. Denatured DNA molecular size standards are shown on the *left* of the figure: L, liver; Lg, lung; K, kidney; S, spleen; T, testis; E, epididymis; O, ovary; U, uterus; P, placenta.

Table 1. Comparison of Hepatic CBG mRNA Levels under Various Physiological Conditions Determined by Solution Hybridization Assay

Physiological Status	CBG cRNA Bound[a] (cpm/µg RNA)	Relative CBG mRNA Levels
Pregnant Female (21 day)[b]	625	1.00
Adult Female	482	0.77
Adult Male	216	0.35
Fetus (21 day)[b]	25	0.04

[a] Specific binding (counts per min) was determined after subtraction of nonspecific values (counts per min) obtained with 25 µg tRNA instead of hepatic RNA extracts. All values were within the linear portion of a dose response curve.

[b] Pooled fetal liver samples taken from the pregnant female.

results confirm this. This appears to be caused by neonatal, androgen-dependent imprinting in male animals (30), and which therefore presumably occurs at the level of hepatic CBG synthesis.

At term, fetal plasma CBG levels are also much lower than maternal levels in both species, but increase during neonatal life (2, 27). In the rat, fetal plasma CBG levels decrease approximately 5-fold between day 18 of gestation and term (17), and it has been reported that fetal rat hepatocytes removed at day 15 of gestation produce more CBG than those removed at day 18 (31). Although a decrease in fetal hepatic CBG mRNA synthesis could account for the 3- to 4-fold difference in maternal and fetal plasma CBG levels at term (17, 32, 33), we observed a remarkable 25-fold difference in their hepatic CBG mRNA levels. Thus, the relative changes in plasma CBG and hepatic CBG mRNA levels in the fetus do not appear to be parallel. This may be due to differences in their relative clearance rates, but some contribution to fetal plasma CBG from the maternal compartment cannot be excluded. The availability of a cDNA for rat CBG will now enable us to address this and other questions related to the ontogeny and hormonal regulation of CBG synthesis in more detail.

MATERIALS AND METHODS

Materials

Restriction endonucleases, sequencing reagents, T_4 DNA ligase, RNase A, agarose and oligo(dT)-cellulose were from Pharmacia (Piscataway, NJ). Yeast tRNA and RNase T_1 were from Boehringer Mannheim (Indianapolis, IN). Cloning vectors were obtained from Pharmacia (M13 Bacteriophage) and Stratagene Cloning Systems (pBluescript phagemids, La Jolla, CA). Nitrocellulose filters (BA85) and *O*-aminophenylthioether (APT)-paper were from Schleicher & Schuell (Keene, NH). Rabbit reticulocyte lysate, *in vitro* translation reagents and a

Riboprobe Gemini system were obtained from Promega Biotec (Madison, WI). Radiolabeled nucleotides ([^{32}P]dCTP, [^{35}S] dATP, and [^{35}S]UTP), [^{35}S]methionine, and EN^{3}HANCE were purchased from New England Nuclear (Boston, MA). Kaolin-conjugated, donkey anti-rabbit immunoglobulin was a gift from Farmos Diagnostica (Turku, Finland). The hybridization solution used for blotting experiments contained 5× Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% BSA), 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured salmon sperm DNA. Tissue samples for RNA analyses were obtained from Wistar rats, and were used immediately or stored at −80 C until analyzed.

Complementary DNA Cloning

A nick-translated, ^{32}P-labeled human CBG cDNA (about 10^8 dpm/μg) was used at reduced stringency (hybridization solution with 40% formamide at 37 C) to screen a λgt11 rat liver cDNA library (Clontech Laboratories Inc.). Phage DNA was isolated (34), and their cDNA inserts (excised with *Eco*RI) were subcloned into pBR322 for restriction analysis. Positive clones were also rescreened with an antibody (provided by Dr. R. W. Kuhn, UCSF, San Francisco, CA) against rat CBG using the method of Young and Davis (35), except that peroxidase-labeled protein A was used to detect antibody-antigen complexes in the presence of the chromogenic substrate, 4-chloro-1-naphthol.

Hybrid Selection

APT-paper was activated as previously described (36). Plasmid (pBR322) containing rat CBG cDNA was bound to the activated paper, and used to hybrid-select CBG mRNA from approximately 1 mg liver RNA that had been isolated by the lithium chloride-urea method (37). The hybrid-selected RNA was then translated in a rabbit reticulocyte lysate system supplemented with [^{35}S]methionine. A rabbit antibody against rat CBG was incubated (16 h at 4 C) with the *in vitro* translation products, and the immunocomplexes were separated by adsorption (1 h at 20 C) with kaolin-conjugated, donkey anti-rabbit immunoglobulins. After centrifugation (5 min at 5000 × *g*), the pellet was washed extensively in PBS containing 1% Triton X-100, 1 mM phenylmethyl-sulfonylfluoride and 0.01% gelatin. Total and immunoadsorbed translation products were separated by SDS-polyacrylamide gel electrophoresis. The gel was fixed in 10% acetic acid, 5% methanol (vol/vol); treated with EN^{3}HANCE; dried under vacuum (2 h at 60 C), and fluorographed (18 h at −80 C) with Kodak XAR film and a Dupont Cronex HI-PLUS XH intensifying screen.

Sequence Analysis

Appropriate restriction enzyme fragments of two rat CBG cDNAs were subcloned into M13mp18 and M13mp19 or pBluescript SK(+) and KS(+) for the production of single-stranded templates which were sequenced using the dideoxy chain-termination method (38). The cDNA-deduced amino acid sequences of rat and human CBG were compared using the optimizing algorithm (PRTALN) of Wilbur and Lipman (39).

Southern Blot Analysis

Genomic DNA was extracted from rat placenta (40). It was digested with restriction endonucleases, resolved on a 0.6% agarose gel, and transferred to nitrocellulose by capillary blotting (41). The Southern blot was baked (2 h at 80 C), prehybridized in the presence of 50% formamide at 42 C, and hybridized with random-primed, ^{32}P-labeled rat CBG cDNA (about 10^9 cpm/μg) under the same conditions. It was then washed to high stringency [0.1× SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate), 42 C] and autoradiographed for 6 days at −80 C.

RNA Analyses

Total RNA was extracted from various rat tissues using lithium chloride-urea (37). Before Northern blot analysis, approximately 25 μg RNA from each tissue was subjected to 1% agarose gel electrophoresis in the presence of formaldehyde (42), and was stained with ethidium bromide to assess the quality and relative quantity of RNA. Only intact RNA, as judged by the integrity of the 28S and 18S ribosomal bands, was used for analysis. Approximately 25 μg RNA from each tissue were then resolved by electrophoresis in the same way and transferred to a nylon membrane (GeneScreen Plus) by capillary blotting (43). The blot was baked (2 h at 80 C), prehybridized in the presence of 50% formamide at 42 C, and hybridized with ^{32}P-labeled rat CBG cDNA (about 10^8 dpm/μg) under the same conditions. It was then washed to high stringency (0.1× SSC, 68 C), and autoradiographed for 18 h at −80 C.

Liver RNA extracts were further analyzed for CBG mRNA content by solution hybridization. In brief, a cRNA probe was synthesized (44) in the presence of [^{35}S]UTP (~1300 Ci/mmol), and incubated (16 h at 70 C) with 25 μg hepatic RNA or 25 μg tRNA (to measure nonspecific binding) in 100 μl 20 mM Tris, pH 7.5, containing 0.6 M NaCl, 10 mM EDTA, and 0.2% SDS. Subsequently, 1 ml RNA digestion buffer (10 mM Tris, pH 7.5, containing 0.3 M NaCl, 5 mM EDTA, 75 μg/ml denatured salmon sperm DNA, 25 μg/ml RNase A, and 200 U/ml RNase T_1) was added, and the incubation was continued for 30 min at 37 C. Remaining RNA/RNA hybrids were precipitated with 370 μl ice-cold 30% trichloroacetic acid, and washed with 1 ml 5% trichloroacetic acid/0.01% Triton X-100. The pellets were resuspended in 100 μl 20 mM NaOH and the radioactivity was measured in the presence of 4 ml Aqueous Counting Scintillant (Amersham, Arlington Heights, IL).

Acknowledgments

We thank Bobbie Lucas and Gail Howard for typing the manuscript.

Received August 22, 1988. Accepted October 15, 1988

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This work was supported by Grant MA-9123 from the Medical Research Council of Canada and a grant from the Ontario Lung Association.

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