Messenger Ribonucleic Acid for Corticosteroid-Binding Globulin. Translation and Preliminary Characterization†

Martine Perrot-Applanat and Edwin Milgrom*

ABSTRACT: The characterization of the messenger RNA for corticosteroid-binding globulin (CBG) was undertaken to study the site(s) of synthesis and the regulation of this protein. CBG from pregnant guinea pig plasma was purified to homogeneity and a specific antiserum was obtained. Poly(A+) RNA was isolated from pregnant guinea pig liver and translated in a wheat germ extract in the presence of [^{3}H]leucine. A radioactive protein of molecular weight 37 000 was precipitated from this extract by the anti-CBG serum but not by the preimmune serum. This protein could be displaced from the antibody by pure unlabeled CBG in excess. The difference in molecular weight between the in vitro translation product and plasma CBG (molecular weight 52 000 in the same conditions) was probably due to the absence of carbohydrates in the former. When analyzed on sucrose gradients, messenger RNA for CBG sedimented at 16 S. In the livers of 40–60-day pregnant guinea pigs, the concentration of translatable CBG messenger RNA was three- to fourfold higher than that present in the livers of nonpregnant animals (~2–3% and 0.5–1%, respectively, of total messenger activity). The increased plasma concentration of CBG during pregnancy can thus probably be explained by an increased concentration of its messenger RNA in the liver.

Corticosteroid-binding globulin (CBG)[1] or transcortin is the specific plasma protein binding cortisol, corticosterone, and, in many species, progesterone. Since in most target cells only the unbound fraction of the hormone appears to be active (Sandberg & Slaunwhite, 1959; Westphal, 1971), variations in CBG concentration are of great importance in the modulation of glucocorticoid and progesterone hormone action. The concentration of CBG in plasma is regulated through various hormones in different species. However, in most species including human, CBG increases during pregnancy and after estrogen treatment (Sandberg & Slaunwhite, 1959; Slaunwhite & Sandberg, 1959; Rosenthal et al., 1969). The molecular mechanisms of these changes are unknown.

The site of synthesis of CBG has been supposed to be the liver (Guidollet & Louisot, 1969a,b). However, no definitive proof of this origin has been obtained. Recently, by in situ perfusion experiments, a corticosteroid-binding protein that might be CBG was shown to be synthesized in the liver (Weiser et al., 1979). CBG has also been found in various other organs, including liver (Koblinsky et al., 1972; Weiser et al., 1979), kidney (Feldman et al., 1973), brain and pituitary (Koch et al., 1976; De Kloet & McEwen, 1976), muscle (Mayer et al., 1975), lung (Giannopoulos, 1976), and uterus (Milgrom & Baulieu, 1970). This has led to the question of whether CBG was imported from the plasma or locally synthesized. The presence of this protein inside glucocorticoid or progesterone target cells might be of importance in the cellular mechanism of action of these hormones.

One possibility of experimentally studying these various problems is to characterize and quantitate CBG messenger RNA in different organs under different hormonal and developmental situations.

In this paper we report the purification to homogeneity of guinea pig CBG and the generation of a specific antiserum. This antiserum was used to characterize the in vitro synthesized CBG after translation of liver messenger RNA in a heterologous acellular system. The size and the control during pregnancy of the hepatic mRNA coding for CBG were also studied.

Materials and Methods

Steroids and Amino Acids. [1,2-^{3}H]Cortisol (85 Ci/mmol) and L-[^{3}H]leucine (43 Ci/mmol) were obtained from CEA (Saclay, France), and [1,2-^{3}H]corticosterone (26 Ci/mmol) was from New England Nuclear Corporation (Boston, MA). Chromatographically pure unlabeled cortisol and corticosterone were purchased from Roussel Uclaf (Romainville, France). Pure HACA, for monitoring the synthesis of HACA, was obtained from Ikapharm (Ramatgan, Israël).

Buffers. A 0.05 M sodium phosphate, pH 7.4, and 0.2 M NaCl buffer (phosphate buffer) was used in CBG purification and in equilibrium dialysis studies. A 0.001 M sodium phosphate, pH 6.8, buffer was used for chromatography on hydroxylapatite. A 0.01 M sodium phosphate, pH 7.4, and 0.15 M NaCl buffer (phosphate-buffered saline or PBS) was used in immunological studies. All solutions used for RNA extraction and purification were autoclaved. Nonautoclavable equipment was thoroughly rinsed prior to use with 0.2% (v/v) diethyl pyrocarbonate.

Plasma and Tissue Preparation. Forty- to sixty-day pregnant Hartley guinea pigs were anesthetized with ether, and blood was collected into heparinized tubes from the blood vessels of the neck. A pool was prepared by combining plasma from 20 pregnant guinea pigs and kept at −20 °C.

Livers from animals at 40–60 days of gestation and from nonpregnant females were excised, perfused with ice-cold saline, and stored frozen in liquid nitrogen.

Determination of CBG Concentration. The concentration of CBG was measured by 40-h equilibrium dialysis in phos-

† From the Groupe de Recherches sur la Biochimie Endocrinienne et la Reproduction (INSERM, U.135), Faculté de Médecine, Paris-Sud, 94270 Bicêtre, France. *Received June 15, 1979; revised manuscript received September 7, 1979.* This work was supported by the INSERM (CRL No. 79.5.223.4), the CNRS, the DGRST, and the U.E.R. Kremlin-Bicêtre.

[1] Abbreviations used: CBG, corticosteroid-binding globulin; HACA, 11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

0006-2960/79/0418-5732$01.00/0

phate buffer at 4 °C and in the presence of 5×10^{-9} to 10^{-6} M [^{3}H]cortisol. The concentration of specific binding sites was obtained by the Scatchard (1949) plot.

Purification of CBG. Preparation of HACA–Sepharose. HACA–Sepharose was prepared according to Le Gaillard et al. (1974). A total of 10 mmol of corticosterone and 75 μCi of [1,2-^{3}H]corticosterone were oxidized with periodic acid to HACA in 60% yield. The purity of the HACA was verified by silica gel thin-layer chromatography (chloroform–ethanol–water, 80:19:1 v/v). Amino-Sepharose was prepared as described by Cuatrecasas (1970) using 25 g of cyanogen bromide (Prolabo, Paris) and 200 mmol of dipropylenetriamine (Fluka, Buchs, Switzerland) per 100 mL of packed Sepharose 4B (Pharmacia, Uppsala, Sweden). A total of 2.5 mmol of HACA and 15 μCi of [1,2-^{3}H]HACA in 30 mL of dioxane were stirred with an equimolar amount of dicyclohexylcarbodiimide (Prolabo, Paris) and 25 mL of amino-Sepharose at room temperature and for 24 h. After the mixture was washed, the amount of HACA coupled to amino-Sepharose was determined by counting the radioactivity of the gel and averaged 1 μmol of HACA per mL of packed gel.

Preparation of plasma for affinity chromatography. After thawing, the plasma from pregnant guinea pigs was centrifuged for 15 min at 1200*g* and incubated with an equal volume of a suspension of 1.7% charcoal and 0.17% dextran in phosphate buffer. The suspension was shaken at 25 °C for 60 min and centrifuged for 10 min at 1200*g*. The supernatant was diluted fivefold with phosphate buffer. In these conditions, the plasma was stripped of 75% of endogenous cortisol (not shown).

Chromatography on HACA–Sepharose. The method reported by Le Gaillard et al. (1974) for the isolation of human CBG was used with the following modifications. A total of 90 mL of the diluted plasma was added to 5.5 mL of HACA–Sepharose and stirred for 60 min at room temperature and then for 4 h at 4 °C. The supernatant was removed by centrifugation, and HACA–Sepharose was washed once with 20 mL of phosphate buffer. The gel was transferred into a column and washed with the same buffer at 4 °C until the optical density at 280 nm fell below 0.1. One column volume of the phosphate buffer containing 1 mM cortisol and tritiated cortisol (10.7 μCi/mmol) was added, and the gel was incubated for 4 h at 4 °C. Elution was performed by adding another column volume of the phosphate–cortisol buffer. A second identical elution was performed after a supplementary incubation of 12 h at 4 °C. The two eluates were pooled and concentrated by ultrafiltration through Selectron cones (Schleicher & Schuell) against 0.001 M sodium phosphate buffer, pH 6.8. The preparation was chromatographed through a Sephadex G-25 (coarse) column (15 × 200 mm) in the same 0.001 M phosphate buffer.

Chromatography on hydroxylapatite was performed as previously described (Schneider & Slaunwhite, 1971). The protein solution (which contained 0.2 mg of CBG per mL) was equilibrated for 2 h at 4 °C with 4 μM cortisol to stabilize CBG. It was applied to a column of hydroxylapatite (HT Biorad, Richmond, CA) (200 × 10 mm) previously equilibrated in 0.001 M sodium phosphate buffer. Elution was accomplished at 4 °C with sodium phosphate buffer of increasing concentrations (0.005–0.05 M). CBG was present in the 0.005 M eluate.

Immunological Methods. Three-month-old male New Zealand rabbits were immunized according to the method of Vaitukaitis et al. (1971), modified as previously described (Perrot & Milgrom, 1978). A total of 75 μg of pure CBG were injected to each rabbit. At 5.5 and 6.5 week intervals, booster injections were given. Eight days after the injections, blood was collected.

Anti-CBG activity of rabbit sera was tested by double immunodiffusion (Ouchterlony, 1958) and immunoelectrophoresis [Grabar & Williams (1953), modified by Scheidegger (1955)] using 1.2% agarose (Indubiose A_{37}, Industrie Biologique Francaise, Paris) in 0.025 M sodium barbital buffer, pH 8.6. Specificity of the anti-CBG serum was ascertained by two-dimensional immunoelectrophoresis (Laurell, 1965) in 1.5% agarose gel (10 × 8.5 cm) in the same barbital buffer. The two consecutive electrophoreses were run at 250 V for 3 h and 80 V for 15 h, respectively, and at 4 °C. The plates were stained with Coomassie brillant blue G_{250} (Uriel, 1960).

The anti-CBG serum was fractionated by ammonium sulfate precipitation (40% saturation) for the experiments on immunoprecipitation of CBG.

Protein Assay. The proteins were measured according to Lowry et al. (1951), using bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis. Electrophoresis in nondenaturing conditions was carried out in 7.5% polyacrylamide gels containing Tris–glycine buffer (pH 8.9) (Orenstein, 1964; Davis, 1964). The gels were stained with Coomassie brillant blue G_{250}. The sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide electrophoresis method of Weber & Osborn (1969) was used to analyze the immunoprecipitates from the mRNA translation experiments. The immunoprecipitates were dissolved in 40 μL of 10 mM sodium phosphate buffer, pH 7.0, containing 1% $NaDodSO_4$, 10% glycerol (v/v), 1% 2-mercaptoethanol, and 0.01% bromophenol blue. The solution was heated for 1 min at 100 °C and 10 min at 60 °C and kept overnight at room temperature. Electrophoresis in 10% polyacrylamide gels containing 0.1% $NaDodSO_4$ was performed for 5.5 h at 8 mA/gel. The gels were frozen, cut into 1.25-mm slices, solubilized in 1 ml of NCS (Amersham/Searle) for 3 h at 50 °C, and counted for radioactivity. Radioactivity incorporated into CBG was expressed as the sum of the counts per minute, after correction of the background (nonspecific precipitation), in the CBG peak. Proteins of known molecular weight were electrophoresed in parallel.

Extraction and Chromatography of RNA. Total cellular RNA was extracted from livers by a phenol–$NaDodSO_4$ solution as previously described (Rosen et al., 1975). Frozen livers (40 g) were broken into small pieces, transferred into a Waring Blendor, and homogenized for 1 min in 150 mL of buffer A (25 mM EDTA, 75 mM NaCl, and 0.5% $NaDodSO_4$, pH 9.0) and 150 mL of phenol previously saturated with buffer A. Extraction was performed at 4 °C for 30 min. The aqueous and the phenolic phases were reextracted with respectively an equal volume of buffer-saturated phenol (pH 9.0) and buffer A. The two aqueous phases were combined and made 0.2 M in NaCl. An equal volume of cold ethanol was added, and DNA was then carefully removed by spooling onto a glass rod. The remaining RNA was precipitated by a second volume of cold ethanol. The pellet containing RNA was dissolved in cold buffer B (10 mM Tris-HCl, pH 7.5, 0.2 mM $MgCl_2$, and 0.5% $NaDodSO_4$) and reprecipitated with ethanol. Low molecular weight RNA and contaminating DNA were removed by washing the pellet twice with 2 M LiCl and 10 mM EDTA, pH 7.0. The pellet was redissolved in buffer B and ethanol-precipitated. This was repeated 3 times. The final pellet was dissolved in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 200 mM NaCl, and 0.5% $NaDodSO_4$.

Poly(U)–Sepharose 4B was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Poly(A)-containing RNA was

isolated by using poly(U)–Sepharose 4B chromatography, as previously described (Atger & Milgrom, 1977). The poly(A+) RNA was eluted with 1 mM potassium phosphate, 0.02% $NaDodSO_4$, and 90% deionized formamide. It was ethanol-precipitated, washed 3 times with 80% ethanol, and dissolved in distilled water.

Translation of Messenger RNA in the Wheat Germ Extract. The wheat germ extract was prepared according to Roberts & Paterson (1973) or Marcu & Dudock (1974).

The conditions for protein synthesis were optimized for Mg^{2+}, K^+, and mRNA concentrations in each wheat germ extract. A standard translation mixture contained one-fifth volume of wheat germ extract, 60 μg/mL poly(A+) RNA, 20 mM Hepes (pH 7.5), 100 mM $Mg(C_2H_3O_2)_2$, 4 mM $K(C_2H_3O_2)$, 2 mM ATP, 0.2 mM GTP, 20 μM each amino acid minus leucine, 10 μCi of [^{3}H]leucine, 8 mM phosphoenolpyruvate, and 100 mM dithiothreitol. Following a 60-min incubation at 30 °C, ribosomes were removed by centrifugation (60 min at 4 °C and 105000*g*). The incorporation of [^{3}H]-leucine into proteins was measured on 5-μL aliquots by precipitation at 90 °C in 10% trichloroacetic acid containing 1 mg/mL leucine. The incorporation of [^{3}H]leucine into CBG was determined by specific immunoprecipitation.

Specific Immunoprecipitation of the CBG Synthesized in Vitro. A double-antibody technique similar to that described by Atger & Milgrom (1977) was used. The immunoprecipitation incubation contained 60 μL of the radioactive peptides synthesized in vitro, 5 μL of anti-CBG globulin fraction (1.5 mg/mL), and 5.8 μL of a detergent solution (120 mM sodium phosphate, pH 7.2, 168 mM NaCl, 6% Triton X-100, 6% deoxycholate, and 120 mM unlabeled leucine). Following incubation for 48 h at 4 °C, 36 μL of goat immunoglobulin fraction prepared from antirabbit γ globulin serum (6 mg/mL) and 5 μL of the detergent solution diluted threefold were added. A second incubation was performed for 30 min at 25 °C and 16 h at 4 °C. The precipitates were collected, washed 3 times with the detergent solution diluted 12-fold, and solubilized for counting radioactivity (Atger & Milgrom, 1977) or for analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. To evaluate the nonspecific contribution, parallel immunoprecipitations were carried out in the same conditions except that the anti-CBG γ globulin fraction was replaced by a rabbit preimmune γ globulin fraction used at the same concentration.

Sucrose Gradient Ultracentrifugation of RNA. Analysis of mRNA on 15–30% linear sucrose gradients containing 0.5 mM EDTA, 0.2% $NaDodSO_4$, and 5 mM Tris-HCl, pH 7.5, was performed as described by Atger & Milgrom (1977). RNA samples were heated at 65 °C for 5 min, chilled, and applied in 0.4-mL aliquots per tube at a concentration of 0.65–0.85 mg/mL. Centrifugation was performed for 18 h at 40000 rpm, in a Beckman SW41 rotor at 20 °C. Fractions were collected and their absorbance at 260 nm was measured. Following ethanol precipitation, the RNA pellets were rinsed with 80% ethanol, dried, and redissolved in 100 μL of water. Aliquots (40 μL) were translated in the wheat germ extract.

Results

To characterize CBG messenger RNA, it was necessary to obtain a monospecific anti-CBG serum and thus to purify this protein to homogeneity.

Purification of Guinea Pig CBG. Serum from pregnant animals was used since it contains a four- to sixfold higher concentration of CBG (2×10^{-5} M) compared to that of nonpregnant females. The affinity chromatography step enriched the protein 65-fold with a 60% yield. However, when analyzed by polyacrylamide gel electrophoresis or immunoelectrophoresis, the preparation still contained small amounts of contaminating proteins and especially γ globulins (not shown). After hydroxylapatite chromatography (yield 48%), the CBG preparation appeared to be homogeneous by both polyacrylamide gel electrophoresis (Figure 1, top left) and immunoelectrophoresis (Figure 1, top right). The purified CBG had one binding site for cortisol per molecule of protein (19.5×10^{-6} site/mg of protein).

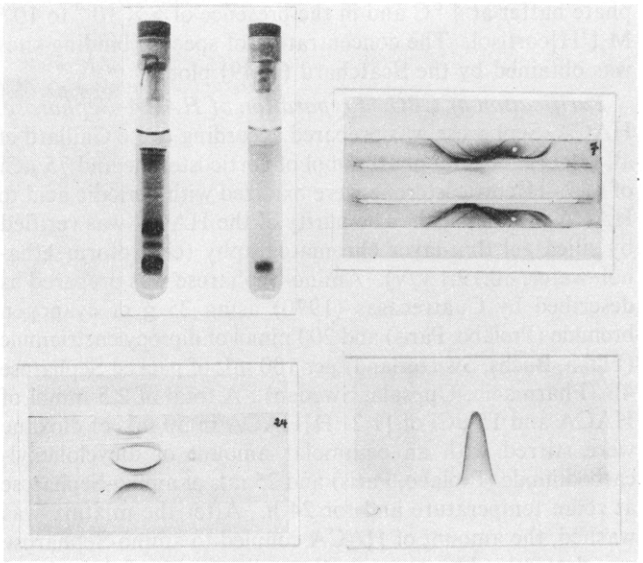

FIGURE 1: Purification of CBG from pregnant guinea pig plasma and characterization of anti-CBG serum. Top: Purification of CBG. Left: electrophoresis (Orenstein, 1964; Davis, 1964) in 7.5% polyacrylamide gel. (Left) Pregnant guinea pig plasma (200 μg of protein); (right) purified CBG (150 μg). [^{3}H]Cortisol (40 nM) binding was demonstrated to coincide with the protein band (not shown). Right: immunoelectrophoresis. (Central well) Purified CBG (2 μL of a solution containing 0.5 mg/mL); (upper and lower wells) pregnant guinea pig plasma (2 μL, diluted fivefold). Electrophoresis was carried out at 150 V for 3 h. Rabbit antiserum (100 μL) elicited against guinea pig plasma (Microbiological Associates, Bethesda, MD) was applied in both troughs. Bottom: Anti-CBG serum. Left: immunoelectrophoresis. Central well was filled with pure CBG (0.5 mg/mL). Upper and lower wells were filled with pregnant guinea pig plasma (2 μL, diluted fivefold). Electrophoresis of the samples was carried out at 150 V for 3 h. Anti-CBG serum (100 μL) was added in both troughs and immunodiffusion was carried out for 24 h. Right: two-dimensional immunoelectrophoresis. Pregnant guinea pig plasma (10 μL, diluted 10-fold) was electrophoresed on a 1.5% agarose gel (anode to the right). A second electrophoresis was carried out upward in a gel containing the anti-CBG serum (12.5 μL/mL of agarose solution).

Preparation and Characterization of Anti-CBG Serum. Pure CBG was used to immunize rabbits. The antisera were tested by double immunodiffusion against pregnant guinea pig plasma and pure CBG. The antisera obtained after two booster injections had the higher titer, yielding a precipitin line with the pregnant guinea pig plasma at a dilution of 128-fold (not shown). A single precipitin line in these experiments was observed between the antiserum and pure CBG or pregnant guinea pig plasma. No precipitin line was detected with rabbit preimmune serum or human plasma. These results suggested that the antiserum was specific for guinea pig CBG. This point was further verified by immunoelectrophoresis (Figure 1, bottom left). A single precipitin line was observed in the α-globulin region between the antiserum and pure CBG or guinea pig plasma. An identical result was observed by the more sensitive two-dimensional electrophoresis (Laurell, 1965) (Figure 2, bottom right). When the tandem two-dimensional electrophoresis (Kroll, 1968) was used with pure CBG and

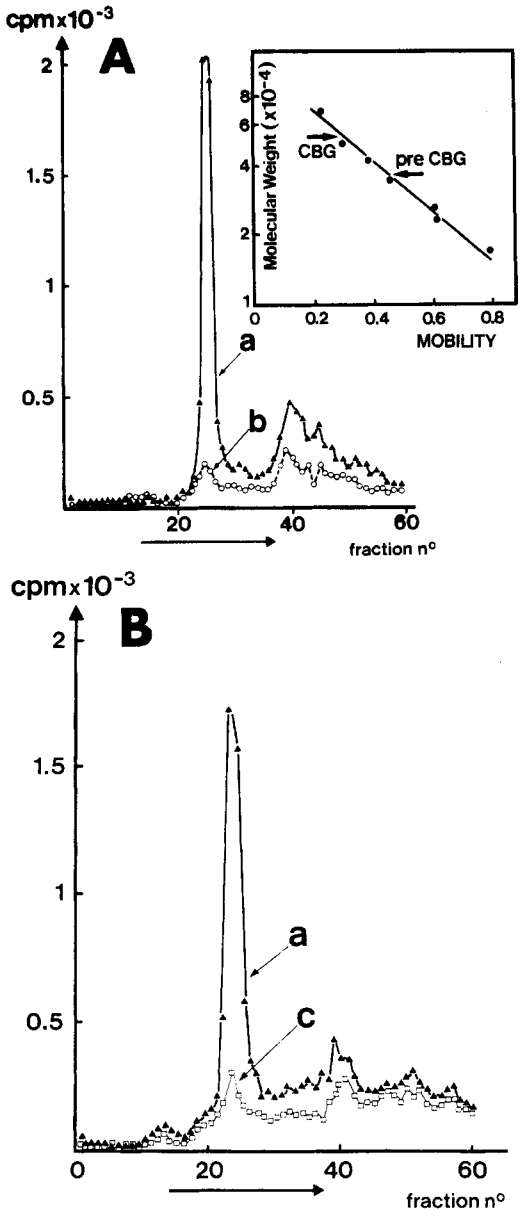

FIGURE 2: Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis of peptides synthesized in the wheat germ extract and precipitated by anti-CBG serum. Electrophoresis was performed according to Weber & Osborn (1969) and as described under Materials and Methods. (a) Translation products [100 μL in (A) and 60 μL in (B)] were immunoprecipitated with anti-CBG serum. (b) Translation products (100 μL) were precipitated with preimmune serum. (c) Translation products (60 μL) were immunoprecipitated with anti-CBG serum in the presence of 20 μg of pure CBG. (A) and (B) show two different experiments. Direction of migration is from left to right. The insert shows the molecular weight of pure CBG and that of the immunoprecipitated radioactive protein compared with bovine serum albumin (M_r 67 000), rabbit γ globulins (M_r 50 000 and 23 500), ovalbumin (M_r 43 000), pepsin (M_r 35 000), chymotrypsinogen (M_r 25 700), and myoglobin (M_r 17 200).

pregnant guinea pig plasma, the two precipitin peaks were coalescent, showing complete immunological identity (not shown). The antiserum thus appeared to be highly specific for guinea pig CBG and could be used for the characterization of its messenger RNA.

Translation of Messenger RNA from Pregnant Guinea Pig Liver. Since optimal K^+, Mg^{2+}, and RNA concentrations for the translation of different messengers have been shown to vary, these parameters were determined with poly(A+) RNA from pregnant guinea pig liver after chromatography on poly(U)–Sepharose. Maximal incorporation of [^{3}H]leucine into polypeptides was obtained at 110 mM K^+, 4–5 mM Mg^{2+}, and 60 μg of RNA per mL of incubation mixture by using the wheat germ extract prepared according to Roberts & Paterson (1973).

The products synthesized in the wheat germ extract were analyzed by immunoprecipitation. mRNA chromatographed twice on poly(U)–Sepharose was used for these studies. Preliminary experiments were designed to optimize the immunoprecipitation. It was found that 120 μg of monospecific anti-CBG immunoglobulins was necessary to immunoprecipitate the [^{3}H]leucine-labeled CBG synthesized in 1 mL of the translation incubation. The immunoprecipitate was submitted to polyacrylamide gel electrophoresis in denaturing conditions (Figure 2). A peak of radioactivity was observed having a molecular weight of 37 000. In the same conditions, pure CBG had a molecular weight of 52 000. The immunological similarity between the radioactive protein and CBG was further established on the following evidence. When preimmune serum was used instead of anti-CBG serum, there was no precipitation of the 37 000-dalton protein (Figure 2A). This protein could also be displaced from its antibody by pure native CBG (Figure 2B).

Immunoprecipitable radioactive CBG represented 2–3% of total protein synthesis in the wheat germ system using poly(A+) RNA after two successive runs on poly(U)–Sepharose. Various amounts of fast migrating polypeptides were observed in these experiments. One discrete band had a molecular weight of 19 000. A part of these peptides corresponded to nonspecific precipitation but some were also specifically immunoprecipitated (see in parts A and B of Figure 2 the difference between specific and nonspecific immunoprecipitation). Their presence might have been due either to incomplete polypeptide chain synthesis (Tse & Taylor, 1977) or to translation of endonuclease-cleaved mRNA (Hunter et al., 1977).

Size of CBG Messenger RNA. Poly(A+) RNA chromatographed twice on poly(U)–Sepharose was centrifuged through a sucrose gradient. RNA in each fraction was used to direct protein synthesis in the wheat germ extract. Radioactivity precipitated by the anti-CBG serum was measured. In the peak region, the immunoprecipitates were also analyzed by polyacrylamide gel electrophoresis. As shown in Figure 3, the CBG messenger RNA activity sedimented in the 16S region.

Increased Concentration of CBG Messenger RNA during Pregnancy. Poly(A+) RNA was isolated from livers of either pregnant or nonpregnant guinea pigs with similar recoveries (~2% after two successive runs on poly(U)–Sepharose). After translation and immunoprecipitation, followed by $NaDodSO_4$–polyacrylamide gel electrophoresis, a three- to fourfold difference in [^{3}H]leucine incorporation into CBG was observed (Figure 4) while total hepatic protein synthesis remained unchanged.

Messenger activity for CBG represented 0.5–1% of total translatable messenger in nonpregnant animals (three experiments) and 2–3% in pregnant guinea pigs (11 experiments).

Discussion

The choice of the guinea pig to characterize the messenger RNA for CBG was justified by the fact that in this species CBG attains extremely high concentrations during pregnancy (10^{-5} to 4.5×10^{-5} M) (Rosenthal et al., 1969; Milgrom et al., 1970; this study), thus suggesting that messenger concentrations might also be high. Another interesting feature is the presence in pregnant guinea pig plasma of a second

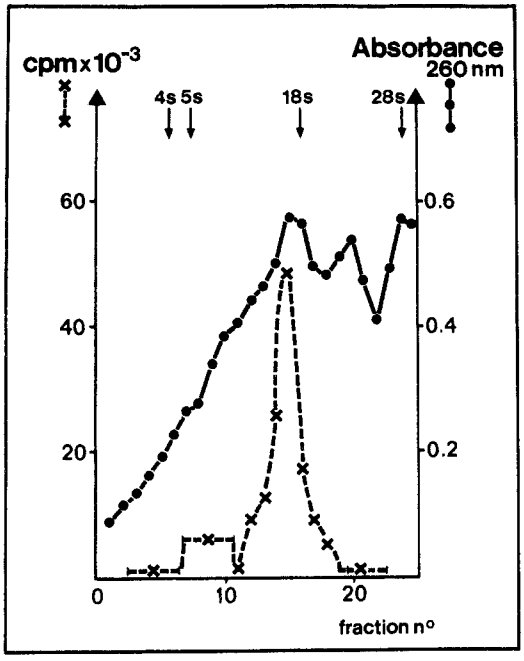

FIGURE 3: Sucrose density gradient ultracentrifugation of poly(A+) RNA from livers of pregnant guinea pigs. Liver poly(A+) RNA (300 μg) was centrifuged through a 15–30% linear sucrose gradient. The optical density at 260 nm was recorded. Arrows indicate the position of 5S, 18S, and 28S RNA. After ethanol precipitation and washing, aliquots (see Materials and Methods) from each fraction were assayed in the wheat germ system for CBG mRNA activity (correction for nonspecific precipitation was performed as described under Materials and Methods). Fractions 3–6, 7–10, and 19–22 were pooled; fractions 11–18 were assayed individually.

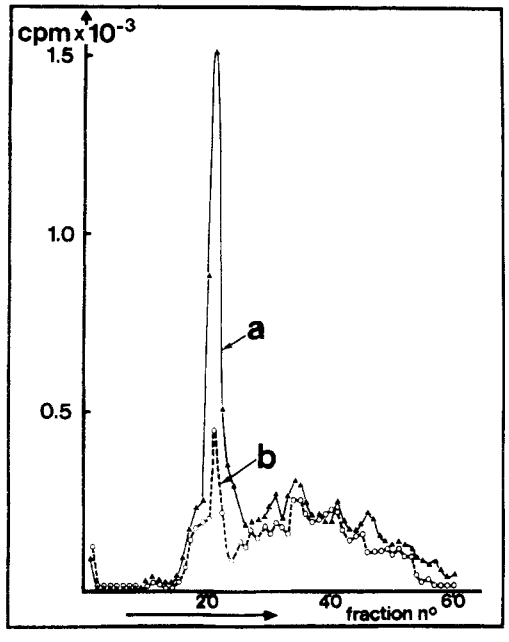

FIGURE 4: Concentration of translatable messenger RNA for CBG in livers of pregnant and nonpregnant guinea pigs. Poly(A+) mRNA was isolated from livers of pregnant (a) and nonpregnant (b) female guinea pigs. After translation in the wheat germ system (5.4 μg of RNA per 90 μL of incubation mixture), the peptides were analyzed by immunoprecipitation with anti-CBG serum, followed by Na-DodSO$_4$–polyacrylamide gel electrophoresis. Total protein synthesis ([^{3}H]leucine precipitated by trichloroacetic acid) was 92 500 and 90 900 cpm/90 μL of incubation mixture for pregnant and nonpregnant guinea pig RNAs, respectively.

steroid binding protein (PBP, progesterone binding protein) (Milgrom et al., 1970; Burton et al., 1971; Perrot & Milgrom, 1978). Thus, it may be interesting to compare the sites of synthesis, the role, and the regulation of these two proteins.

Schneider & Slaunwhite (1971) and Rozen & Volchek (1970) have previously purified cavian CBG by classical methods. During the completion of this work, Mickelson & Westphal (1978) have published a method based on affinity chromatography, followed by gel filtration.

The protein synthesized in vitro in the wheat germ system had a molecular weight of 37 000 whereas circulating CBG has a molecular weight of 52 000 (NaDodSO$_4$–polyacrylamide gel electrophoresis (this study) or 45 000 [analytical ultracentrifugation, see Mickelson & Westphal (1978)]. This difference was compatible with the presence in the plasma CBG of 25% (Schneider & Slaunwhite, 1971) or 28% (Mickelson & Westphal, 1978) carbohydrates.

Previous studies on the site of synthesis of CBG have given mainly indirect evidence on its hepatic origin. The presence of a messenger RNA for CBG in relatively high concentration in the liver indicates that this organ is probably at the origin of circulating CBG. It is unknown, however, if CBG may also be synthesized in other cells.

The increased concentration of messenger RNA for CBG during pregnancy introduces the question of the hormonal control of this protein [Rosenthal et al. (1969); general reviews in Westphal (1971) and Wagner (1978)]. The ontogeny of CBG and the origin of fetal CBG are also unsolved problems which now become amenable to analysis.

By poly(U)–Sepharose chromatography, followed by sucrose gradient ultracentrifugation, it is possible to obtain RNA preparations enriched in CBG messenger and directing the synthesis of polypeptides of which about 20% are CBG. Specific probes for CBG messenger RNA could thus be obtained either by using specific immunoprecipitation of polysomes to further purify the mRNA or by cloning double-stranded cDNA prepared from partially purified messenger.

The various plasma proteins binding steroid hormones and vitamin D metabolites have been thoroughly studied in respect to their ligand specificity and physicochemical properties. However, their biological role and the mechanisms which regulate their concentration are still poorly understood. Moreover, to approach the problems of the evolution of these plasma proteins and of their relationship with intracellular binding proteins (receptors), it would be necessary to evaluate the similarities and differences of the nucleic acids which code for them. The possibility of detecting, measuring, and probably transcribing into complementary DNAs the messenger RNAs for these proteins could give new insights into some of these problems.

Acknowledgments

We thank J. F. Savouret for advice and N. Malpoint for typing the manuscript.

References

Atger, M., & Milgrom, E. (1977) *J. Biol. Chem. 252*, 5412–5418.

Burton, R. M., Harding, G. B., Rust, N., & Westphal, U. (1971) *Steroids 17*, 1–16.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

De Kloet, E. R., & McEwen, B. S. (1976) *Biochim. Biophys. Acta 421*, 115–123.

Feldman, D., Funder, J. W., & Edelman, I. S. (1973) *Endocrinology 92*, 1429–1441.

Giannopoulos, G. (1976) *J. Steroid Biochem. 7*, 553–559.

Grabar, P., & Williams, C. A. (1953) *Biochim. Biophys. Acta 10*, 193–215.

Guidollet, J., & Louisot, P. (1969a) *Acta Endocrinol. (Copenhagen) 62*, 468–476.
Guidollet, J., & Louisot, P. (1969b) *Clin. Chim. Acta 23*, 121–132.
Hunter, A. R., Farrell, P. J., Jackson, R. J., & Hunt, T. (1977) *Eur. J. Biochem. 75*, 149–157.
Koblinsky, M., Beato, M., Kalimi, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7897–7904.
Koch, B., Lutz, B., Briaud, B., & Mialhe, C. (1976) *Biochim. Biophys. Acta 444*, 497–507.
Kroll, J. (1968) *Scand. J. Clin. Lab. Invest. 22*, 79–81.
Laurell, C. B. (1965) *Anal. Biochem. 10*, 358–361.
Le Gaillard, F., Racadot, A., Racadot-Leroy, N., & Dautrevaux, M. (1974) *Biochimie 56*, 99–108.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Marcu, K., & Dudock, B. (1974) *Nucleic Acids Res. 1*, 1385–1397.
Mayer, N., Kaiser, N., Milholland, N., & Rosen, F. (1975) *J. Biol. Chem. 250*, 1207–1211.
Mickelson, K. E., & Westphal, U. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 992.
Milgrom, E., & Baulieu, E. (1970) *Endocrinology 87*, 276–287.
Milgrom, E., Atger, M., & Baulieu, E. (1970) *Nature (London) 228*, 1205–1206.
Orenstein, L. (1964) *Ann. N.Y. Acad. Sci. 121*, 321–349.
Ouchterlony, O. (1958) in *Progress in Allergy* (Kallos, P., Ed.) Vol. 5, pp 1–78, Karger, Basel and New York.
Perrot, M., & Milgrom, E. (1978) *Endocrinology 103*, 1678–1685.
Roberts, B. E., & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330–2334.
Rosen, J. M., Woo, S. L. C., Holder, J. W., Means, A. R., & O'Malley, B. W. (1975) *Biochemistry 14*, 69–78.
Rosenthal, H. E., Slaunwhite, W. R., Jr., & Sandberg, A. A. (1969) *Endocrinology 85*, 825–830.
Rozen, V. B., & Volchek, A. G. (1970) *Probl. Endokrinol. 16*, 81–85.
Sandberg, A. A., & Slaunwhite, W. R., Jr. (1959) *J. Clin. Invest. 38*, 1290–1298.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Scheidegger, J. J. (1955) *Int. Arch. Allergy Appl. Immunol. 7*, 103–110.
Schneider, S. L., & Slaunwhite, W. R., Jr. (1971) *Biochemistry 10*, 2086–2093.
Slaunwhite, W. R., Jr., & Sandberg, A. A. (1959) *J. Clin. Invest. 38*, 384–391.
Tse, T. P. H., & Taylor, J. M. (1977) *J. Biol. Chem. 252*, 1272–1278.
Uriel, J. (1960) in *Analyse Immunoelectrophoretique*, p 33, Masson et Cie, Paris.
Vaitukaitis, J., Robbins, J. B., Nieschlag, E., & Ross, G. T. (1971) *J. Clin. Endocrinol. Metab. 33*, 988–991.
Wagner, R. (1978) *Acta Endocrinol. (Copenhagen), Suppl. 218*, 1–73.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Weiser, J. N., Do, Y. S., & Feldman, D. (1979) *J. Clin. Invest. 63*, 461–467.
Westphal, U. (1971) *Steroid Protein Interactions*, Springer-Verlag, West Berlin and New York.

Variable Proton Conductance of Submitochondrial Particles†

M. Catia Sorgato* and Stuart J. Ferguson‡

ABSTRACT: The relationship between the rate of substrate oxidation and the protonmotive force (electrochemical proton gradient) generated by bovine heart submitochondrial particles has been examined. Unexpectedly, oxidation of succinate generated a higher protonmotive force than the oxidation of NADH, although the rate of proton translocation across the membrane was inferred to be considerably lower with succinate as substrate. The data suggest that the flow of electrons through site 1 of the respiratory chain may increase the conductance of the mitochondrial membrane for protons. Upon reduction of the rate of succinate oxidation by titration with malonate, the protonmotive force remained essentially constant until the extent of inhibition was greater than 75%. The general conclusion from this work is that a constant passive membrane conductance for protons cannot be assumed.

The conservation of energy, released via respiratory chain electron flow, as an electrochemical proton gradient (protonmotive force = Δp) is the major feature of the chemiosmotic mechanism in mitochondria as well as bacteria and chloroplasts (Mitchell, 1966). Many important contributions seem to have confirmed the link between respiration and protonmotive force (Boyer et al., 1977), but still there is little evidence on the factors that can modulate respiration, particularly if and how the protonmotive force itself can influence the rate of respiration and consequently the rate of proton translocation.

An important role between respiration rate and magnitude of the electrochemical proton gradient is played by the membrane; Mitchell's concept is that of a "passive" membrane (Mitchell, 1978) whose low effective proton conductance [0.11 nequiv of H^+ min^{-1} (mg of protein)$^{-1}$ mV^{-1}] (Mitchell & Moyle, 1967a) should be protonmotive force independent and therefore limit the state 4 respiration in mitochondria (Mitchell, 1968). More recently, however, Nicholls (1974a, 1977) found that the dielectric characteristics of the membrane, thus the proton conductance, can change under different

† From the Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom. *Received March 7, 1979; revised manuscript received May 29, 1979.* M.C.S. was supported by a long-term European Molecular Biololgy Organization Fellowship.

* Address correspondence to author at the C.N.R. Unit of the Physiology of Mitochondria, Via Marzolo 3, 35100 Padova, Italy.
‡ Present address: Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

0006-2960/79/0418-5737$01.00/0