Pergamon

0960-0760(93)E0059-G

J. Steroid Biochem. Molec. Biol. Vol. 48, No. 5/6, pp. 467–474, 1994
Copyright © 1994 Elsevier Science Ltd
Printed in Great Britain. All rights reserved
0960-0760/94 $7.00 + 0.00

Antagonistic Effects of Retinoic Acid and Triiodothyronine in the Expression of Corticoid-binding Globulin (CBG) by Cultured Fetal Hepatocytes

E. L. Elfahime, J. M. Félix and B. Koch*

Institut de Physiologie, CNRS URA 1446, Université Louis Pasteur, 21 Rue René Descartes, 67084 Strasbourg Cédex, France

Cultures of rat fetal hepatocytes were used to investigate the effects and interplay of triiodothyronine (T_3) and retinoic acid (RA) in the regulation of gene expression of CBG, compared to that of α-fetoprotein (AFP). The cultured cells showed cytological features typical to hepatocytes and actually synthesized CBG and AFP, as evidenced from *in situ* hybridization with specific radioactive probes. Time course studies indicated that CBG (but not AFP) binding capacity in culture medium and cell mRNA levels disappeared with a half-life of about 2 days, thereby reflecting the decrease previously seen in hepatic CBG mRNA contents during embryonic life. The K_d values for CBG binding were unchanged under these conditions. Culturing of hepatocytes in the presence of T_3 resulted in dose-dependent stimulations of both medium CBG and cell mRNA levels, with an EC_{50} concentration of about 10^{-9} M. In sharp contrast, RA caused a reduction in CBG biosynthesis ($IC_{50} = 1.7 \times 10^{-7}$ M) and, in addition, antagonized the stimulatory influence of T_3. Under the same experimental conditions, AFP synthesis failed to be affected in a similar fashion. We conclude that thyroid hormones and RA directly act on hepatocytes to specifically regulate the expression of CBG in an antagonistic way.

J. Steroid Biochem. Molec. Biol., Vol. 48, No. 5/6, pp. 467–474, 1994

INTRODUCTION

Corticoid-binding globulin (CBG) or transcortin is a plasma glycoprotein that binds glucocorticoids, as well as progesterone, with high affinity [1, 2]. The macromolecule serves important biological functions in that it controls the levels of the unbound moiety of steroids which is readily diffusible into target-cells. In addition, being internalized by cells, the binder is able to modulate in a more subtle way the translocation of the steroid–receptor complexes to the nucleus of pituitary cells [3] and thus their interaction with specific response elements. Finally, CBG also shows the ability to interact with cell membrane receptors [4] and, eventually, to regulate adenylate cyclase activity [5]. In this context, it has recently been shown that CBG may be produced and/or accumulated in a variety of tissues, including not only the liver, but also the pancreas, the placenta and the kidney of fetal and neonatal mice, at which level the protein may possibly play important autocrine/paracrine functions [6].

*Correspondence to B. Koch.
Received 16 Sep. 1993; accepted 14 Dec. 1993.

In adult rats the plasma concentration of CBG is mainly under the control of thyroid hormones and glucocorticoids, which exert stimulatory and inhibitory effects, respectively [1, 7]. Sex steroids also play a role, although their effect is thought to be partly mediated through the hypothalamic–pituitary–thyroid axis [7] and/or GH [8]. In fetal rats, however, indirect evidence suggests that the CBG gene may be regulated in a different and more complicated way, because pharmacological blockade of thyroid function failed to markedly affect fetal plasma CBG levels [9]. In fetal livers, the CBG gene is actually expressed at very high levels, while its expression declines sharply at the time of birth [10, 11]. That fall in hepatic CBG mRNA contents is reflected by a similar decay in serum CBG concentrations in fetuses and neonates, as reported previously [9, 12–14].

Studies on regulation by hormones or other putative regulatory factors of CBG production in cultured hepatocytes have, to our knowledge, not yet been reported; though cultures of fetal hepatocytes have successfully been used to show the ability of the tissue to synthesize CBG and α-fetoprotein (AFP [15, 16]).

The aims of the present investigation were, first, to determine whether triiodotyronine (T_3) acts directly on gene expression and secretion of CBG in primary cultures of fetal hepatocytes. Second, because recent evidence suggests some analogy between T_3- and retinoic acid (RA)-response elements and indicates that receptors for these two compounds can actually form heterodimers [17–19], we examined whether RA may synergize with or antagonize the effect of thyroid hormones. In an initial step, however, we meant to validate our culture model system by assessing the functional activity of hepatocytes and localizing CBG mRNA in cells by means of *in situ* hybridization (ISH). Our results provide evidence for selective antagonistic effects between RA and T_3 in the expression of the CBG gene in fetal hepatocytes, while in contrast these factors failed to affect in a similar way the regulation of the AFP gene, examined in parallel control studies.

MATERIALS AND METHODS

Animals and reagents

Wistar female rats (230–250 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. To induce hypothyroidism, pregnant dams were given methimazole (Calvo *et al.*, 1991) in drinking water (0.02%) along with daily s.c. injections of the drug (40 μg/100 g), from gestational day 10 to day 16. Methimazole, T_3 and all-*trans* RA, as well as collagenase (type IV), DNase-I, collagen, Williams' medium E (WE) and steroids were purchased from Sigma Chimie (La Verpillière, France). [α-^{33}P]deoxyadenosine triphosphate ([α-^{33}P]dATP; 3000 Ci/mmol) from Isotopchim-France, [α-^{32}P]dCTP (3000 Ci/mmol), [1,2-^{3}H(*N*)]corticosterone (51 Ci/mmol) and [6,7-^{3}H(*N*)]estradiol (48 Ci/mmol) from New England Nuclear (Du Pont de Nemours, France). Terminal deoxynucleotidyl-transferase, yeast tRNA (RNase-free) and proteinase K were from Boehringer (Mannheim, Germany). Spin columns were obtained from 5Prime-3Prime, Inc. (Boulder, U.S.A.).

Primary cultures of rat fetal hepatocytes

Fetuses were obtained from 15- to 16-day-old pregnant rats and fetal livers were dissected out under sterile conditions and placed in ice-cold Hepes buffer, composed of (in mM): 25 Hepes, 137 NaCl, 5 KCl, 0.7 Na_2HPO_4, 0.36 $CaCl_2$, 10 glucose and 0.1% bovine serum albumin (BSA), at pH 7.3. Tissues were incubated in the same medium containing collagenase (50 mg/100 ml) and DNase-I (10 μg/ml) for 20 min at 37°C, followed by gentle dissociation with the aid of a plastic Pasteur pipette. Tissue pieces were allowed to settle and isolated cells in the supernatant were saved and the whole process was repeated thrice. The resulting final cell suspension was filtered through a 100-μm nylon screen and the cells pelleted down at 50 *g* for 2 min. The supernatant, which mainly contained non-parenchymal cells, was discarded and the cell pellet was resuspended in HB and recentrifuged. This step was repeated 3 more times. The cells were finally resuspended in WE medium, enriched with 5% fetal calf serum, 1 μM insulin and antibiotics. To prevent possible inactivation of thyroid hormone receptors, hepatocytes were cultured in the presence of 0.3 mM 2-mercaptoethanol [20]. Cells were plated in 6-well cluster plates, precoated with collagen, and incubated at 37°C in 5% CO_2–95% air. To remove unattached cells, the medium was replaced with fresh medium after 3 h of culture. WE culture fluid, containing T_3, RA or corresponding vehicles, were then changed daily.

One day prior to harvesting hepatocytes and incubation fluids, cells were washed twice with serum-free WE medium (containing 0.1% BSA) and incubated in the same medium supplemented, or not, with test substances. T_3 and RA were added as a 1000-fold concentrated stock-solution in 0.05 M NaOH or dimethyl sulfoxide, respectively.

Cells, cultured for 1, 3 and 5 days, were counted after being dislodged from culture wells and dissociated with the aid of trypsin in phosphate-buffered saline (PBS; 0.15 mg/ml), containing 1 mM EDTA.

In situ hybridization (ISH)

The cells were seeded over glass coverslips (precoated with collagen) lodged in Costar dishes (35 × 10 mm) and cultured for 1 day. They were fixed with 4% paraformaldehyde for 1 h at room temperature and rinsed thrice with phosphate-buffered saline for 5 min. The samples were then dehydrated with graded alcohol, air dried and stored at −75°C until used for ISH.

The CBG and AFP probes were synthetic single stranded oligodeoxynucleotides, complementary to the rat CBG and AFP sequences and corresponding to the amino acids 29–42 [21] and 126–139 [22], respectively. The oligoprobes (2–4 pmol) were labeled with 50 μCi [α-^{33}P]dATP and 100 U terminal deoxynucleotidyl-transferase, in 50 μl buffer composed of (in mM): 100 potassium cacodylate, 1.5 $CoCl_2$, 0.2 dithiothreitol, at pH 7.2. After being filtered through a G25 Spin column to get rid of unincorporated tracer, the specific activity of the probe was calculated to be about 1×10^4 Ci/mmol.

Prior to ISH, the glass coverslips were incubated at 37°C in 50 mM Tris buffer (pH 7.5), containing 5 mM EDTA and 1 μg/ml proteinase K for 15 min. They were rinsed in PBS and fixed as described above. The glass coverslips were then quartered so that the same population of cells on each piece could be hybridized with either CBG or AFP radioactive oligoprobes and further tested for specificity in the presence of 100-fold concentrations of respective unlabeled probes.

Hybridization was carried out for 15–18 h at 35°C in a medium composed of: 0.6 M NaCl, 1 mM EDTA, 50% formamide, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA),

250 μg/ml yeast tRNA, 200 μg/ml denaturated herring sperm DNA and 10% dextran sulfate. Probe concentrations were 2–4 $\times 10^{-9}$ M.

The sections were then washed for 30 min at room temperature, first in 0.5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate at pH 7.4) and then in 0.25 × SSC, followed by a final wash in 0.25 × SSC at 50°C. They were dehydrated and dried, before being dipped in K5 emulsion (Ilford) and exposed for 3 weeks at 4°C prior to development.

For microscopic examinations, the cells were fixed as described above and stained by means of the May–Grünewald–Giemsa procedure.

Northern blots

Total RNA contents were extracted from cultured hepatocytes and the resulting samples (15 μg) were used for Northern blot analysis, as reported previously [11].

For Northern blot analysis of CBG mRNA, the oligoprobe was labeled with 50 μCi [α-^{32}P]dCTP to a specific activity of approx. 1 $\times 10^4$ Ci/mmol [11]. For that of AFP mRNA, we used a 0.7 kb rat AFP cDNA [22], which we further amplified and purified. A 50 ng aliquot of AFP cDNA probe was labeled with [α-^{32}P]dCTP with aid of the multiprime DNA-labeling system from Boehringer, as indicated by the manufacturer and further purified by gel filtration through a Sephadex G50 column. The specific activity of the probe was 0.5–1.0 $\times 10^9$ cpm/μg.

Measurement of CBG and AFP mRNAs

Hybridization conditions for the CBG oligoprobe were those described under ISH, while the AFP cDNA probe was hybridized at 42°C for 15–18 h in the medium used for ISH. The final stringency of medium was 0.1 SSC, 0.1% sodium dodecyl sulfate at 50°C for 30 min.

Hybridization was carried out, in a first step, to allow detection of CBG mRNA. The nitrocellulose filters were then processed to remove the CBG probe [23], before being hybridized with the AFP cDNA probe. Quantitation of CBG and AFP mRNAs was performed as reported previously [11].

Measurement of CBG and AFP binding parameters in culture medium

Aliquots of 0.2 ml of incubation media were labeled with 1.4 and 1.8 pmol of either [^{3}H]corticosterone or [^{3}H]estradiol, in the absence or presence of various concentrations of unlabeled steroids. Parallel incubations contained a 500-fold excess of cold steroids to permit correction for non-specific binding. In the case of [^{3}H]estradiol binding, we showed that cold testosterone, unlike cold 17β-estradiol, failed to displace the tracer, indicating that the binding was not due to sex hormone-binding globulin (which combines both sex steroids). Assay tubes were first held at 30°C for 20 min and then maintained in an ice-cold water bath for 2 h. Bound and unbound moieties were separated by adding 50 μl of ice-cold dextran-coated charcoal for 10 min (0.5% dextran T70 and 5% charcoal in 20 mM Tris–HCl buffer, pH 7.2). Tubes were centrifuged at 1500 *g* for 10 min and 150 μl aliquots of the supernatants were counted for bound radioactivity, with a counting efficiency of about 35%.

Statistics and steroid binding analysis

Statistical evaluation of results was peformed with analysis of variance, followed by the non-parametric Student–Newman–Keuls post test for multiple comparisons, using GRAPHPAD's software "Instat2". Binding data were analyzed with aid of the program "Ebda" (ELSEVIER-BIOSOFT) and EC_{50}/IC_{50} values were calculated using GRAPHPAD's "InPlot". Data reported in figures are from representative experiments, which were replicated 3–4 times with similar results.

RESULTS

Cytological characterization of cultured hepatocytes and ISH

After 1 day in culture, the majority of cells appeared as typical hepatocytes [Fig. 1(a)],, with basophilic cytoplasm, a well-developed Golgi apparatus and a nucleus with low-condensed chromatin and 1–3 nucleolus. A few cells, showing the features of hematopoietic cells in the way of differentiation, were also present. After 3 and 5 days [Fig. 1(b and c)], hepatocytes appeared to overgrow the culture dish, while small aggregates of erythropoietic cells, at different stages of terminal differentiaton [inset to Fig. 1(b)], were scarce. Cell counts (after trypsinization of cultures) showed a doubling of the number of cells between days 1 and 3, which plateaued thereafter.

ISH with CBG and AFP probes [Fig. 1(d and f), respectively] revealed positive reactions in hepatocytes. Silver grains were mainly localized over the basophilic (RNA-rich) part of the cytoplasm, with a few grains over the Golgi apparatus and almost none over the nucleus. Incubation of cells in the presence of an excess of respective cold probes [Fig. 1(e and g)] nearly abolished the labeling.

Time course of cell production of CBG and AFP

Both the binding capacity (B_{max}) of CBG in culture medium and cell content of CBG mRNA fell dramatically between culture days 1 and 5, with a final drop of about 90% (Fig. 2). The calculated half-life of CBG disappearance in culture medium was found to be $t_{1/2} = -\ln 2/\text{slope} = 2.5$ days. In contrast, the time course of AFP production showed a significantly different pattern, as it was unchanged in cells cultured for 1–3 days and decreased by only about 30% by day 5. As revealed by Scatchard plot analyses, the binding affinities for CBG were not significantly affected in the course of cell culture: apparent dissociation constants (K_d) were calculated to be, respectively,

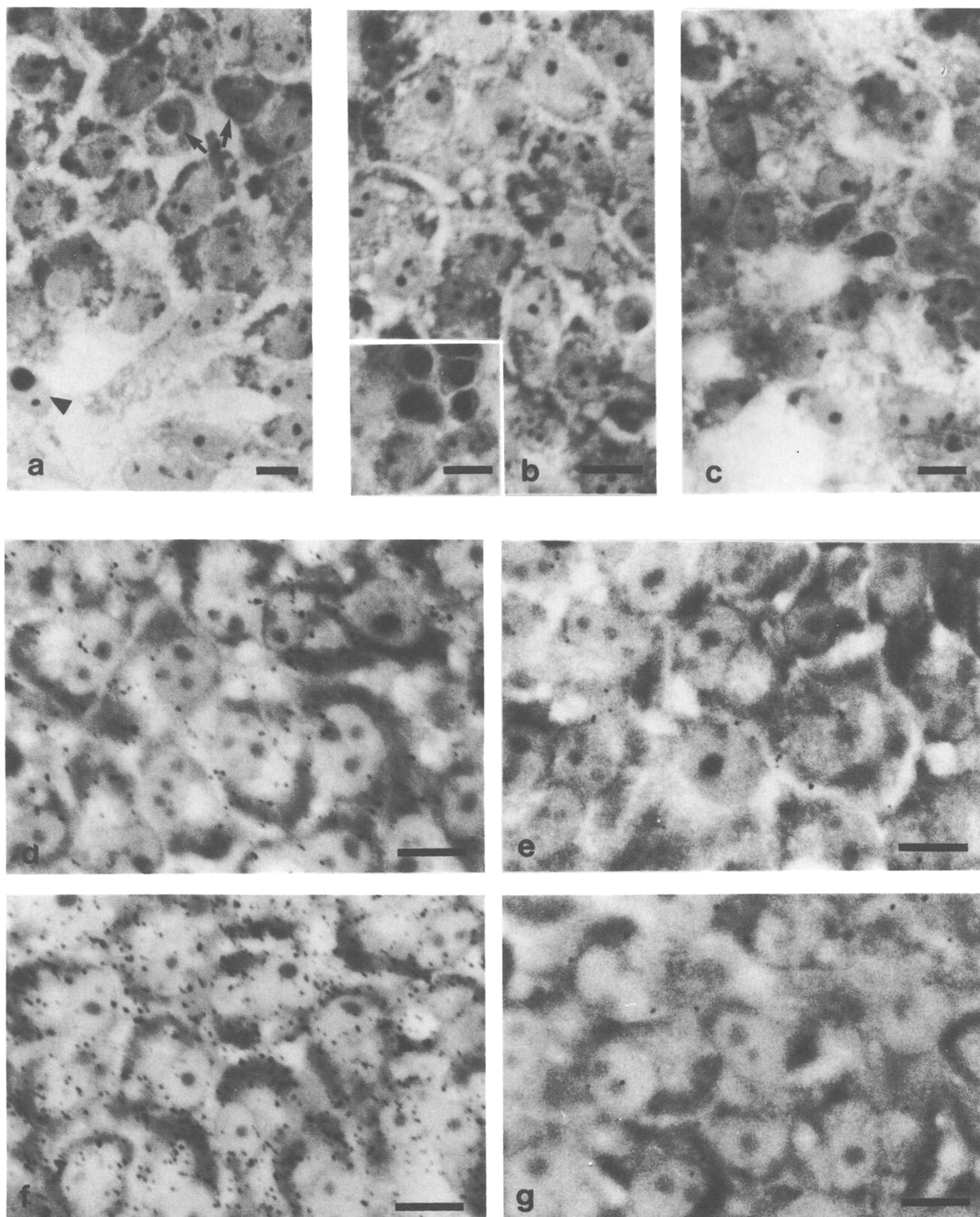

Fig. 1. Cytological aspects and ISH labeling of cultured fetal hepatocytes. Shown are cells cultured for 1, 3 and 5 days (a, b and c, respectively); the inset to b corresponds to a small aggregate of erythropoietic cells. ISH with radiactive probes for CBG (d and e) and AFP (f and g). Non-specific labeling, after incubation of cells with an excess of cold probes, is shown in e (CBG) and g (AFP). Arrows point to hematopoietic cells at early stages of terminal differentiation. Arrow head singles out acidophilic erythroblast.

$2.1 \pm 0.6 \times 10^{-8}$ M by day 1 and $3.2 \pm 1 \times 10^{-8}$ M by day 3. These values were close to that of plasma CBG of adult rats ($K_d = 3.3 \pm 0.9 \times 10^{-8}$ M).

CBG production appeared to be restricted to hepatocytes, because measurements carried out in incubation medium of non-parenchymal cells (cultured for 1

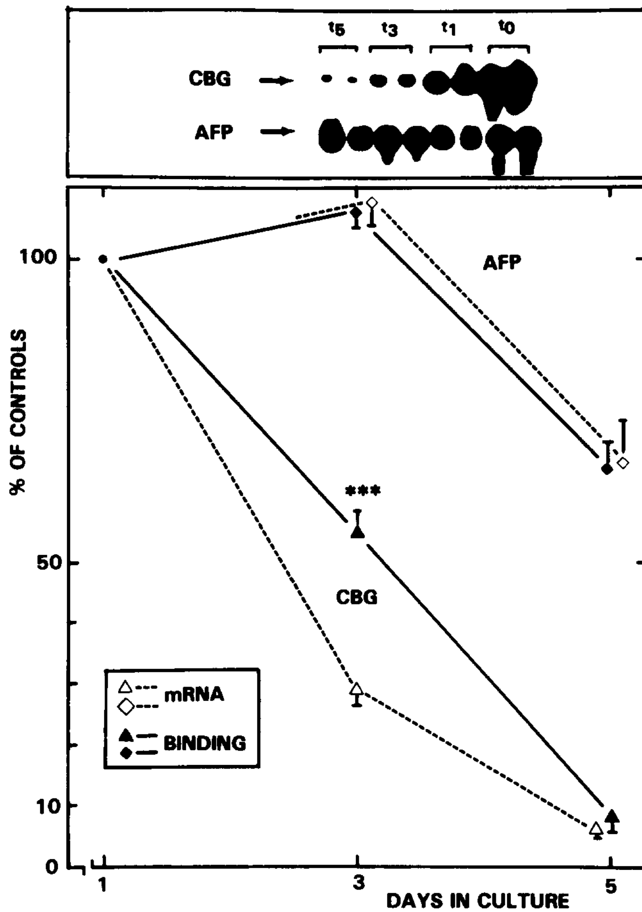

Fig. 2. Time course of CBG and AFP biosynthesis in cultured hepatocytes. Binding capacities in culture medium and mRNA levels for CBG and AFP are represented and expressed as percents of control values recorded after 1 day in culture (corrected for a constant number of 10^6 cells or 15 μg RNA). Values are means ± SEM of 4 culture dishes. The concentrations of binding sites for CBG and AFP in control media were 8.2 ± 0.8 pmol and 0.7 ± 0.5 nmol, respectively. On top is shown a representative autoradiograph of a Northern blot for RNA extracts of hepatocytes cultured for 1 day (t1), 3 days (t3) and 5 days (t5), compared to freshly dispersed cells (t0).

day) failed to reveal the presence of any CBG binding and mRNA content, nor did co-incubation of non-parenchymal cells with hepatocytes alter CBG production in the latter (not shown). Taken together with ISH studies, our data clearly localize CBG biosynthesis in hepatocytes and thus support previous results based on immunological approaches [15, 16]. The latter results, however, did not provide conclusive evidence for CBG biosynthesis because it is well documented that the protein can merely be taken up by and concentrated in some tissues [6, 24, 25].

Effect of T_3 and RA on CBG and AFP binding

Culturing hepatocytes in the presence of T_3 and RA resulted in opposite and dose-dependent effects on CBG binding capacities in incubation media (Fig. 3). There was about a 1.8-fold increase in B_{max} over basal level in response to 10 nM T_3, with a calculated half-maximum value of $EC_{50} = 0.6 \pm 0.2 \times 10^{-9}$ M. Conversely, in response to 10 μM RA, the concentration of binding sites fell by about 73% compared to control values; half-maximum inhibition being achieved with $IC_{50} = 1.7 \pm 0.6 \times 10^{-7}$ M. Scatchard plot analysis of binding data revealed that T_3 and RA actually affected CBG B_{max}, but left K_d unchanged (not shown).

Interestingly, in sharp contrast with the effects on CBG, we found that AFP binding capacity in media of hepatocytes maintained in the presence of either T_3 or RA failed to be changed in a similar way (Fig. 3).

Effect of blockade of maternal and fetal thyroid functions on CBG biosynthesis

We used methimazole to block thyroid function in pregnant rats to determine if these hormones may be key factors involved in the regulation of the elevated CBG gene expression in fetal liver. Long-term treatment of dams with this drug caused a small, but significant ($P < 0.02$), decrease in CBG binding capacity in incubation medium of 1-day-old cultured hepatocytes, compared to controls ($B_{max} = 15.9 \pm 0.8$

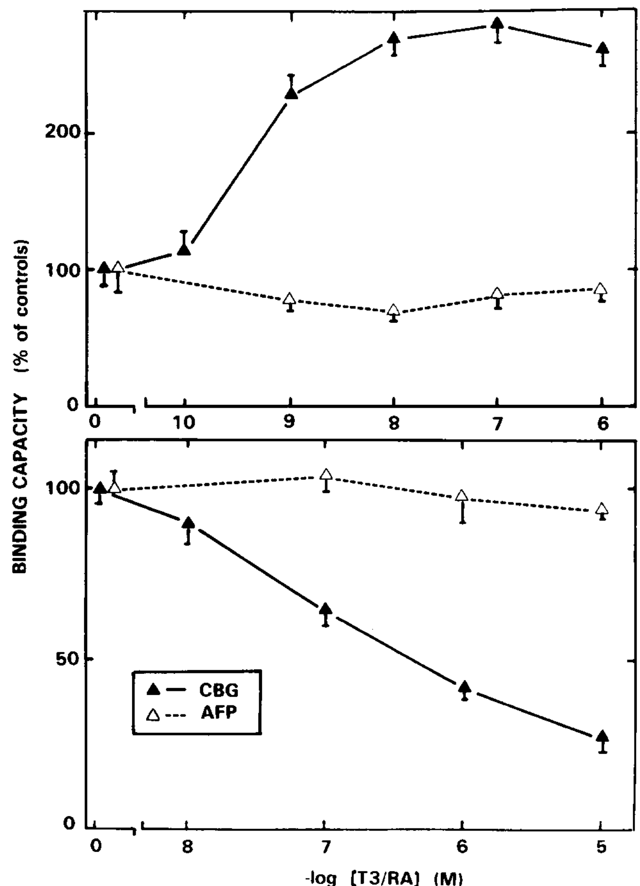

Fig. 3. Dose-dependent effects of T_3 and RA (upper and lower panels, respectively) on concentrations of binding sites for CBG and AFP in culture medium. Data are means ± SEM of 4 observations in a representative experiment. They are corrected for 10^6 cells/dish and expressed as percents of control (vehicle-treated) cells. Duration of treatments was 3 days.

vs 20.0 ± 0.5 pmol/10^6 cells, respectively). That small effect, however, was apparently not associated with a significant change in cellular accumulation of CBG mRNA (37.2 ± 5.1 vs 30.9 ± 4.3 A.U., $n = 4$, in livers of control and treated rats, respectively).

Effects of RA and T_3, alone and in combination, on CBG and AFP biosynthesis

Our data clearly show that both the stimulatory and the inhibitory effects of T_3 and RA on CBG binding capacities [Fig. 4(c)] were associated with parallel and significant ($P < 0.01$) changes in cellular mRNA productions [Fig. 4(a)]. Moreover, co-incubation of cells in the presence of RA and T_3 reversed the stimulatory influence of the latter on both CBG B_{max} and mRNA formation ($P < 0.001$).

Interestingly enough, we found that treatment of hepatocytes with RA and T_3 failed to alter the density of AFP binding sites and mRNA accumulation in a way similar to that of CBG [Fig. 4(b and d)]. However, in the presence of T_3 there was a slight, although consistent, decrease in AFP binding in culture medium [Fig. 4(d)].

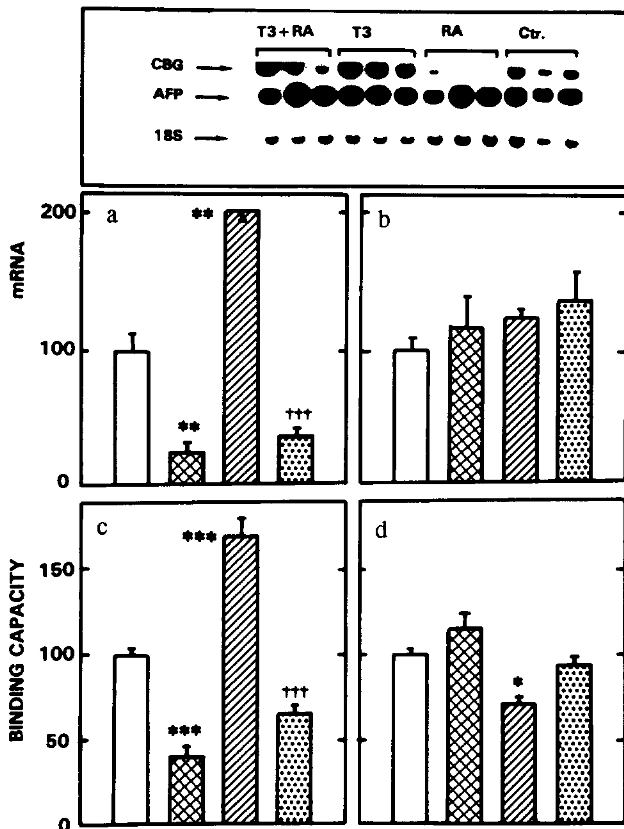

Fig. 4. Effects of RA and T_3, alone and in combination, on CBG (a and c) and AFP (b and d) biosynthesis. Hepatocytes were exposed for 3 days to either vehicle (□) or 10^{-5} M RA (■), 10^{-7} M T_3 (▨) and both compounds (▦). Data, which correspond to mRNA levels (a, b) and concentrations of binding sites (c, d), are means ± SEM of 4 observations and are expressed as percents of untreated cells. On top is shown a representative autoradiograph for RNA extracts of control cells (Ctr.) and cells treated with RA, T_3 and T_3 + RA. The 18S ribosomal RNA shows the actual amount of applied material, as shown previously [11]. The concentrations of binding sites for CBG and AFP in control dishes were 6.3 ± 0.4 pmol and 0.8 ± 0.3 nmol, respectively. (***) (**) (*): $P < 0.001$; 0.01; and 0.05, respectively, compared to controls. (†††): $P < 0.001$ compared to T_3-treated cells.

DISCUSSION

The present study provides the first evidence that thyroid hormones and RA act directly and in an opposite fashion on hepatocytes to regulate the biosynthesis of CBG. We indeed found T_3 to stimulate and RA to inhibit CBG production on its own and to reverse the stimulatory influence of the thyroid hormone as well. Interestingly, these regulatory factors seem to be aimed specifically at the CBG gene, because the AFP gene failed to be affected in a similar way.

The observation that adult rats administered with T_4 show elevated levels of plasma CBG concentration was first established by Labrie *et al.* [7] and later confirmed in both adult and neonatal rats [1, 9, 12–14]. A more recent study [26] reports in addition that *in vivo* administration of T_4 caused an increase in stability and accumulation of hepatic CBG mRNA, without affecting the rate of transcription. This stimulatory effect of thyroid hormones on CBG formation could be either exerted directly or indirectly on the liver and mediated by factors induced outside the liver, as seems to be the case for estrogens [7, 8]. However, we clearly show here that hepatocytes, at least those of fetal origin, actually appear to be direct targets for thyroid hormones in their regulatory effect on CBG expression. Treatment of cultured fetal hepatocytes with T_3 indeed resulted in enhanced CBG biosynthesis, as apparent from elevations of both corticoid binding capacity in culture medium and tissue accumulation of CBG mRNA. The EC_{50} value for this effect was fairly close to the K_d of thyroid hormone receptors (TRs [27]). The question of whether the hormonal action is due to an increase in CBG mRNA stability or enhanced transcriptional rate remains to be established. It is not known either whether hormonal treatment of hepatocytes modifies for instance carbohydrate composition of the molecule, as seems to be the case in the effect of glucocorticoids on CBG in embryonic liver of the sheep [28].

Although our demonstration that T_3 regulates CBG biosynthesis in rat fetal liver is in accord with the presence of specific TRs in that tissue [29] and the presence of both T_4 and T_3 at early embryonic stages [30, 31], it seems unlikely that thyroid hormones could play a major role during embryonic life. Indeed, the highly-induced expression of the CBG gene in fetal hepatocytes [10, 11] was shown here to only be slightly dampened as a result of pharmacological blockade of maternal and fetal thyroid functions, as already suggested by us previously [9]. This thus leaves open the intriguing question of what type of factor(s) may actually be responsible for the dramatic stimulation of CBG expression in fetal liver.

Plasma CBG concentrations undergo a striking decrease from late embryonic to early postnatal stages [9],

which is paralleled by a sharp fall in fetal liver CBG mRNA levels as well. [10, 11] A striking observation in the present study was that CBG production in cultured fetal hepatocytes showed a quite similar decline, with a calculated $t_{1/2}$ of about 2 days, close to the value found in the plasma of neonatal rats [9]. It seems unlikely that this phenomenon could be due to unfavorable cell culture conditions, because (i) cytological examination, coupled with ISH, showed the cells to replicate and be functionally active; and (ii) biosynthesis of AFP was almost unaffected under the same experimental conditions. It may rather be due to a lack in the culture medium of appropriate and specific stimulating factors or actually may reflect a genetically preimprinted mechanism in relation with some potent inhibitory factors. Among the latter, glucocorticoids are well known as repressors of CBG biosynthesis in the rat [1, 7, 21]. Here, we show in addition that RA may play a similar role, as this compound not only was shown here to depress CBG production on its own, but also to antagonize the stimulatory efect of T_3. A similar antagonism between T_3 and RA in the down-regulation of an isoform of TR mRNA in GH_3 pituitary cells has been described recently [17].

RA and its metabolites are part of a complex group of so-called retinoids, which play important roles in cell differentiation and embryogenesis [19]. RA receptors (RARs) show homology with the superfamily of receptors for steroids, thyroid hormones and vitamin D [32, 33]. The mechanism of action of RA in the regulation of CBG mRNA may involve interaction with RARs, which are actually present in fetal livers [34], or with RXRs after RA has been metabolized to more potent forms, such as 9-*cis* RA [18, 35]. These receptors also have the potential to interact with TRs [18, 36, 37] to form heterodimers, thereby interfering with thyroid hormone response elements and modulating the cell's sensitivity to these ligands. The possibility that RA has to be metabolized to more active forms (the cell's concentration of which is unknown) and/or the binding of RA to various intracellular retinoid-binding proteins [19], most probably accounts for the fact that fairly high amounts of RA are needed to be effective in the present study, as well as in others [17, 18].

The results indicate that thyroid hormones and RA directly act on cultured fetal hepatocytes to regulate CBG gene expression in an antagonistic fashion. The effects seem to be specifically targeted at CBG, because the biosynthesis of AFP was unaffected under the same experimental conditions. Whether these factors change CBG mRNA stability or the rate of transcription has yet to be established.

Acknowledgements—We are grateful to J. L. Danan (Paris) for the generous supply of the AFP probe. We also thank Mrs L. LePersonnic and F. Herzog for their excellent technical assistance. Supported in part by the "Fondation pour la Recherche Médicale" and "Fondation de la Recherche en Hormonologie", contract No. 67.93.05.

REFERENCES

1. Westphal U.: *Steroid–Protein Interactions II. Monogr. Endocr.*, Vol. 27. Springer-Verlag, Berlin (1986).
2. Hammond G. L.: Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins. *Endocrine Rev.* **11** (1990) 65–91.
3. Koch B., Sakly M., Lutz-Bucher B. and Briaud B.: Glucocorticoid binding and control of ACTH secretion. *J. Physiol. (Paris)* 77 (1981) 923–933.
4. Koch B., Lutz-Bucher B., Briaud B. and Milahe C.: Specific interaction of corticosteroids with binding sites in the plasma membranes of the rat anterior pituitary gland. *J. Endocr.* **79** (1978) 215–222.
5. Rosner W.: The function of corticoid-binding globulin and sex hormone-binding globulin: recent advances. *Endocrine Rev.* **11** (1990) 80–91.
6. Scrocchi L. A., Orava M., Smith C. L., Han V. K. M. and Hammond G. L.: Spatial and temporal distribution of corticosteroid-binding globulin and its messenger in embryonic and fetal mice. *Endocrinology* **132** (1993) 903–909.
7. Labrie F., Pelletier G., Labrie R., Ho-Km M. A., MacIntosh B. and Fortier C.: Liaison transcortine–corticostérone et contrôle de l'activité hypophysosurrénalienne chez le Rat: Interactions hypophyse–thyroïde–surrénales–gonades. *Ann. Endocr. (Paris)* **29** (1968) 29–43.
8. Jansson J. O., Oscarsson J., Mode A. and Ritzen E. M.: Plasma growth hormone pattern and androgens influence the levels of corticosteroid-binding globulin in rat serum. *J. Endocr.* **122** (1989) 725–732.
9. Koch B.: Fractions libre de la corticostérone plasmatique et réponse hypophysosurrénalienne au stress durant la période post-natale chez le Rat. *Horm. Metab. Res.* **1** (1969) 301–308.
10. Smith C. L. and Hammond G. L.: Ontogen of corticosteroid-binding globulin biosynthesis in the rat. *Endocrinology* **128** (1991) 983–988.
11. Elfahime E. L., Plante M., René F., Félix J. M. and Koch B.: Biosynthesis of hepatic corticosteroid-binding globulin: ontogeny and effect of thyroid hormone. *J. Steroid Biochem. Molec. Biol.* **41** (1992) 135–140.
12. Nunez E. A., Behassayag C., Savu L., Valette M. F. and Jayle M. F.: Serum binding of some steroid hormones during development in different animal species. *Ann. Biol. Anim. Biochem. Biophys.* **16** (1976) 491–501.
13. Henning S. J.: Plasma concentration of postnatal development of corticosteroid-binding globulin. *Am. J. Physiol.* **240** (1981) E402–406.
14. D'Agostino J. and Henning S. J.: Postnatal development of corticosteroid-binding globulin: effect of thyroxine. *Endocrinology* **111** (1982) 1476–1482.
15. Vranck R., Plas C., Ali M., Martin M. E. and Nunez E. A.: Rat corticosteroid-binding globulin biosynthesis by fetal hepatocytes in culture. *J. Steroid Biochem.* **23** (1985) 195–199.
16. Ali M., Vranck R. and Nunez E. A.: Origin of corticosteroid-binding globulin in fetal rat. *J. Biol. Chem.* **261** (1986) 9915–9921.
17. Davis K. D. and Lazar M. A.: Selective antagonism of thyroid hormone action by retinoic acid. *J. Biol. Chem.* **267** (1992) 3185–3189.
18. Kliever S. A., Umesono K., Mangelsdorf D. J. and Evans R. M.: Retinoid X receptors interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D_3 signalling. *Nature* **355** (1992) 446–449.
19. Ross A. C.: Cellular metabolism and activation of retinoids: roles of cellular retinoid-binding proteins. *FASEB J.* **7** (1993) 317–327.
20. Yamamoto N., Inoue A., Takahashi K. P., Li Q., Nakamura H., Tagami T., Sasaki S., Imura H. and Morisawa S.: Loss of thyroid hormone receptor activity in primary cultured rat hepatocytes is reversed by 2-mercaptoethanol. *Biochem. J.* **281** (1992) 669–673.
21. Smith C. L. and Hammond G. L.: Rat corticosteroid binding globulin: primary structure and messenger ribonucleic acid levels in the liver under different physiologcal conditions. *Molec. Endocr.* **3** (1989) 420–426.
22. Jagodzinsky L., Sargent T., Yang M., Glackin C. and Bonner J.: Sequence homology between RNAs encoding rat α-fetoprotein and rat serum albumin. *Proc. Natn. Acad. Sci. U.S.A.* **78** (1981) 3521–3525.
23. Meinroth J. and Wahl G.: Hybridization of nucleic acids

immobilized on solid supports. *Ann. Biochem.* **38** (1984) 267–284.
24. Koch B., Lutz B., Briaud B. and Mialhe C.: Heterogeneity of pituitary glucocorticoid binding: evidence for a transcortin-like compound. *Biochim. Biophys. Acta* **444** (1976) 497–507.
25. Siiteri P. K., Murai J. T., Hammond G. L., Nisker J. A., Raymoure W. J. and Kuhn R. W.: The serum transport of steroid hormones. *Recent Prog. Horm. Res.* **38** (1982) 457–510.
26. Smith C. L. and Hammond G. L.: Hormonal regulation of corticosteroid binding globulin biosynthesis in the male rat. *Endocrinology* **130** (1992) 2245–2251.
27. Brent G. A., Moore D. D. and Larsen P. R.: Thyroid hormone regulation of gene expression. *A. Rev. Physiol.* **53** (1991) 17–35.
28. Berdusco E. T. M., Hammond G. L., Jacobs R. A., Grolla A., Akagi K., Langlois D. and Challis J .R. G.: Glucocorticoid-induced increase in plasma corticosteroid binding globulin levels in fetal sheep is associated with increased biosynthesis and alterations in glycosylation. *Endocrinology* **132** (1993) 2001–2008.
29. Rodd C., Schwartz H. L., Strait K. A. and Oppenheimer J. H.: Ontogeny of hepatic nuclear triiodothyronine receptor isoforms in the rat. *Endocrinology* **131** (1992) 2559–2564.
30. Obregon M. J., Mallol J., Pastor R., Morreale de Escobar G. and Escobar Del Rey F.: L-thyroxine and 3,5,3′-triiodothyronine in rat embryos before onset of fetal thyroid function. *Endocrinology* **114** (1984) 305–307.
31. Obregon M. J., Ruiz De Ona C., Calvo R., Escobar Del Rey F. and Morreale de Escobar G.: Outer ring iodothyronine deiodinases and thyroid hormone economy: responses to iodine deficiency in the rat fetus and neonate. *Endocrinology* **129** (1991) 1663–1673.
32. Petkowitch M., Brand N. J., Krust A. and Chambon P.: A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature* **330** (1987) 444–450.
33. Giguère V., Ong E. S., Segui P. and Evans R. M.: Identification of a receptor for the morphogen retinoic acid. *Nature* **240** (1987) 889–895.
34. Wan Y. J. Y., Wang L. and Wu T. C.: Expression of retinoic acid receptor genes in developing rat livers and hepatoma cells. *Lab. Invest.* **66** (1992) 646–651.
35. Heyman R. A., Mangelsdorf D. J., Dyck J. A., Stein R. B., Eichele G., Evans R. M. and Thaller C.: 9-*Cis* retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68** (1992) 397–406.
36. Rosen E. D., O'Donnel A. L. and Koenig R. J.: Ligand-dependent synergy of thyroid hormone and retinoid X receptors. *J. Biol. Chem.* **267** (1992) 22010–22013.
37. Yen P. M., Sugawara A. and Chin W. W.: Triiodothyronine (T_3) differentially affects T_3-receptor/retinoic acid receptor and T_3-receptor/retinoid-X receptor heterodimer. *J. Biol. Chem.* **267** (1993) 23248–23252.