J. steroid Biochem. Vol. 23, No. 4, pp. 437–443, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

GLUCOCORTICOID RECEPTORS IN MURINE VISCERAL YOLK SAC AND LIVER DURING DEVELOPMENT

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(*Received* 25 *February* 1985)

Summary—Specific binding of triamcinolone acetonide was analyzed in cytosols from developing mouse visceral yolk sac and fetal, neonatal and adult liver. In the visceral yolk sac, binding capacity increased from 1×10^3 sites/cell on day 10 to maximal levels (9×10^3 sites/cell) on day 14 of gestation. In fetal liver, binding sites were low (2×10^3 sites/cell) from day 14 to 18, increased rapidly after birth to approx. 1.7×10^4 sites/cell by day 9 postpartum and were present at approx. 3×10^4 sites/cell in adult liver. Scatchard analysis of the data indicated the presence of a single class of cytosolic binding sites of limited capacity and high affinity ($K_d = 2$–4 nM). The level of specific nuclear binding 2 h after injection of [^{3}H]triamcinolone acetonide was proportional to the number of cytosolic binding sites/cell for each tissue tested. The physicochemical characteristics of cytosolic glucocorticoid–receptor complexes were examined by DEAE-Sephadex A-50 column chromatography. "Unactivated" complexes from visceral yolk sac, fetal and adult liver eluted at approx. 0.4 M KCl. Heat "activated" complexes from fetal and adult liver eluted at approx. 0.25 M KCl, whereas those from visceral yolk sac eluted in the prewash fractions (0.02 M potassium phosphate buffer). These results provide evidence that quantitative but not qualitative changes in glucocorticoid receptors occur during liver development and also establish the presence of glucocorticoid receptors in the midgestation mouse visceral yolk sac.

INTRODUCTION

Glucocorticoid hormone responsive target tissues can be distinguished from non-target tissues by virtue of the presence of specific cytosolic receptors which bind glucocorticoids with high-affinity and limited capacity [for review, see 1]. Cytosolic glucocorticoid–receptor complexes are thought to go through a temperature dependent "activation" process which results in their nuclear binding due to increased affinity for specific sequences in the DNA. Nuclear binding of the hormone–receptor complexes in turn results in altered gene transcription [for review, see 2 and 3].

Recent studies indicate the apparent existence of at least two forms of the "activated" glucocorticoid receptor which can be distinguished by physicochemical properties, tissue specificity [2, 18], and biological function [23]. The major glucocorticoid binder in liver and other tissue may thus be different from that in kidney and colon [23, 24].

Glucocorticoid receptors are present in a number of embryonic and fetal tissues including lung [4], neural retina [5], and secondary palate [6]; and glucocorticoids have been shown to effect differentiation of these tissues. We are interested in possible glucocorticoid effects on development of mouse visceral yolk sac and fetal liver. The visceral yolk sac is an extra-embryonic membrane which surrounds the embryo and fetus during the latter half of gestation. It consists of an outer layer of endoderm cells and an inner layer of mesoderm cells [7]. The visceral yolk sac has several biological functions. It is the site of hematopoiesis during early development [8] and is involved in selective transmission of maternal antibodies and serum proteins [9, 10]. The endoderm cells of the visceral yolk sac synthesize several proteins which are later produced by fetal and neonatal liver, notably alphafetoprotein, metallothionein [11], albumin [12], and transferrin [13]. Expression of the alphafetoprotein and metallothionein genes can be regulated by glucocorticoid hormones. In neonatal liver, alphafetoprotein synthesis can be repressed [14], whereas in adult liver, metallothionein synthesis can be induced [15] by glucocorticoids. The visceral yolk sac and fetal liver thus offer potentially attractive model systems for examining glucocorticoid effects on gene expression during development. In this study, we present evidence which indicates that visceral yolk sac and fetal liver do contain glucocorticoid receptors and the ontogeny and some of the biochemical properties of these receptors are delineated.

EXPERIMENTAL

Chemicals

Molybdate and triamcinolone acetonide (TA)†

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†The abbreviations used are: TA, triamcinolone acetonide, α-fluoro-11β,21-dihydroxy-16α,17-[1-methylethulidene *bis*(oxy)]pregna-1,4-diene-3,20-dione; DMSO, dimethylsulfoxide; CBG, corticosteroid binding globulin; VYS, visceral yolk sac.

were purchased from Sigma Chemical Company. Dextran T-70 and DEAE-Sephadex A-50 were obtained from Pharmacia and Norit A charcoal from Pfanstiehl Laboratories, Inc. [^{3}H]TA (32.8 Ci/mM) was from New England Nuclear.

Animals

Tissue samples were removed from CD-1 mice (Charles River Laboratories) and used immediately as described below. Gestational age was calculated from the day on which a vaginal plug was found, designated day 0 of gestation. Adult tissues were removed from 60- to 90-day old female mice.

Determination of TA binding capacity in cytosols

Samples were homogenized in 5 vol of 10% glycerol, 10 mM Tris pH 7.4, 1.5 mM EDTA, 5 mM DTT (freshly prepared), and 10 mM molybdate. Aliquots (100 μl) of this total homogenate were analyzed for DNA content using the diphenylamine reaction [16] and cytosols were prepared by centrifugation at 30,000 rpm (100,000 g) for 30 min at 4°C in the SW55 TI rotor (Beckman). The lipid layer was discarded and the supernatant carefully removed from the pellet. TA binding capacity was determined on triplicate aliquots of cytosol (250 μl) by incubating them in 1.5 ml microfuge tubes at 4°C for 4 h (unless otherwise indicated) with 30 nM [^{3}H]TA (1 μCi/ml) plus or minus at 200-fold excess of unlabeled TA. For Scatchard [17] analysis, duplicate aliquots of cytosol (250 μl) were incubated with [^{3}H]TA ranging in final concentration from 0.2 to 15 nM plus or minus a 200-fold excess of unlabeled TA. Free hormone was removed by the additon of an equal volume (250 μl) of 0.05% Dextran T-70; 1% Norit A (charcoal) in 10% glycerol; 10 mM Tris, pH 7.4; and 1.5 mM EDTA. Samples were centrifuged for 2 min in a microfuge (Eppendorf) following a 15 min incubation at 4°C. An aliquot (300 μl) of the charcoal-extracted cytosol was mixed with 10 ml aquasol and radioactivity was determined at 30–50% counting efficiency for ^{3}H using a Beckman 9800 scintillation counter; all data were expressed in disintegrations per minute (dpm). Non-specific binding (i.e. that hormone bound in the presence of a 200-fold excess of unlabeled TA) ranged from 10 to 70% of total binding depending on the tissue and stage of development. Specifically bound hormone was calculated by subtracting non-specific binding from total binding. Calculation of the average number of specifically bound receptor sites per cell assumed one molecule of hormone bound per receptor molecule and 6.4 pg DNA/cell.

Injection of TA and determination of specific nuclear binding

Mice were injected subcutaneously with 4 mg/kg of [^{3}H]TA plus or minus a 100-fold excess of unlabeled TA. TA was injected in 100 μl DMSO (100 μg; 100 μCi; sp. act. 260 mCi/mM) in adult mice and in 10 μl DMSO (10 μg; 10 μCi) for neonates. Tissues were removed 2 h after injection, homogenized at 4°C in 50 vol of nuclear isolation buffer (0.25 M sucrose; 10 mM Tris, pH 7.4; 5 mM $MgCl_2$; 0.1% nonidet P40) and nuclei were pelleted by centrifugation at 1000 g for 8 min. After 2 washes in 5 ml of nuclear isolation buffer, the nuclei were resuspended in 600 μl of this buffer. DNA content was determined on aliquots (50 μl) of the final nuclear suspension using the diphenylamine reaction [16] and radioactivity was determined on aliquots (200 μl) of the suspension by liquid scintillation counting. Non-specific binding represented about 15% of the total binding in these experiments, and receptor sites per cell were calculated as described above.

DEAE-Sephadex A-50 chromatography of "unactivated" and "activated" cytosol

Cytosols were prepared from pooled fetal livers and visceral yolk sacs as described above, except that molybdate was omitted from all buffers. Following incubation at 4°C for 2 h with 30 nM [^{3}H]TA, cytosols were extracted with Dextran-coated charcoal which resulted in a 2-fold dilution and maintained at 4°C for 30 min ("unactivated") or heated to 25°C for 30 min ("activated") before chromatography.

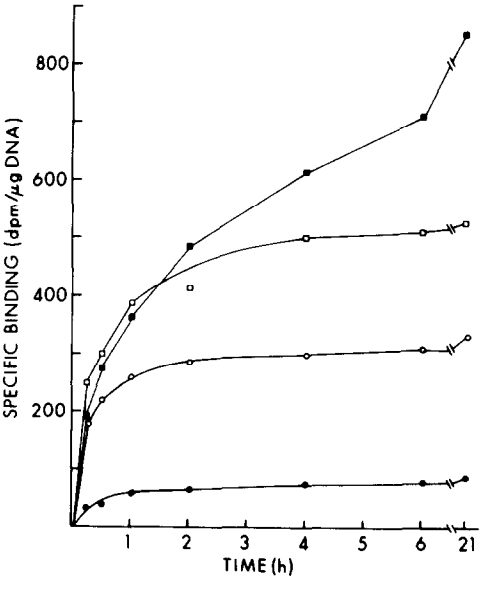

Fig. 1. Time course for specific binding of triamcinolone acetonide to cytosol. Cytosols prepared in the presence of 10 mM molybdate and 5 mM DTT, from fetal liver (●), neonatal liver (○), adult liver (□), and lactating female liver (■) were incubated at 4°C with 30 nM [^{3}H]TA plus or minus a 200-fold excess of unlabeled TA. Samples were extracted with Dextran-coated charcoal at indicated times and specifically bound hormone was determined by subtracting non-specific binding (that bound in the presence of a 200-fold excess of unlabeled hormone) from total binding. DNA content was determined from aliquots of the total homogenate taken before cytosol preparation (centrifuged at 100,000 g for 30 min).

DEAE-Sephadex A-50 columns (7 ml bed volume) were prepared in 12 ml disposable plastic syringes. The bottom 2 ml of Sephadex contained 0.5 ml of Dextran-coated charcoal to absorb free hormone during chromatography [18]. Columns were equilibrated at 4°C with 0.02 M potassium phosphate buffer, pH 6.8. Cytosol (0.5 to 0.7 ml) was applied, and a prewash of 16 ml of 0.02 M potassium phosphate buffer was passed through the column at a flow rate of 1 ml/min (8 fractions of 2 ml each were collected). Bound radioactivity was eluted with a linear 0–1 M KCl gradient, and 100 fractions of 0.5 ml each were collected. Aliquots (300 μl) were analyzed for radioactivity and conductivity of aliquots (100 μl) was determined using a conductivity meter (Radiometer) and compared with solutions of known KCl molarity.

RESULTS

The specific TA binding capacity of cytosols was determined in buffers containing 10 mM molybdate plus 5 mM DTT which promote stabilization of glucocorticoid receptors and allow for exchange of exogenous TA for endogenous receptor-bound glucocorticoids [19–21]. Cytosolic TA binding sites in fetal, neonatal, and adult liver were essentially completely occupied by exogenous TA (30 nM) during a 4 h incubation at 4°C (Fig. 1). Further incubation up to 21 h resulted in a slight increase (< 10%) in specific binding indicating that the large majority of cytosolic binding sites in these tissues were unoccupied by endogenous glucocorticoids. Similar results were obtained with cytosol from visceral yolk sac (VYS) [not shown]. In the lactating female liver, a large increase (> 40%) in specific TA binding occurred between 4 and 21 h of incubation which indicated the presence of a significant population of endogenous cytosolic hormone–receptor complexes.

Specific TA binding sites in developing VYS and liver cytosol were determined after a 4 h incubation at 4°C with 30 nM TA (Fig. 2). Two-fold dilution

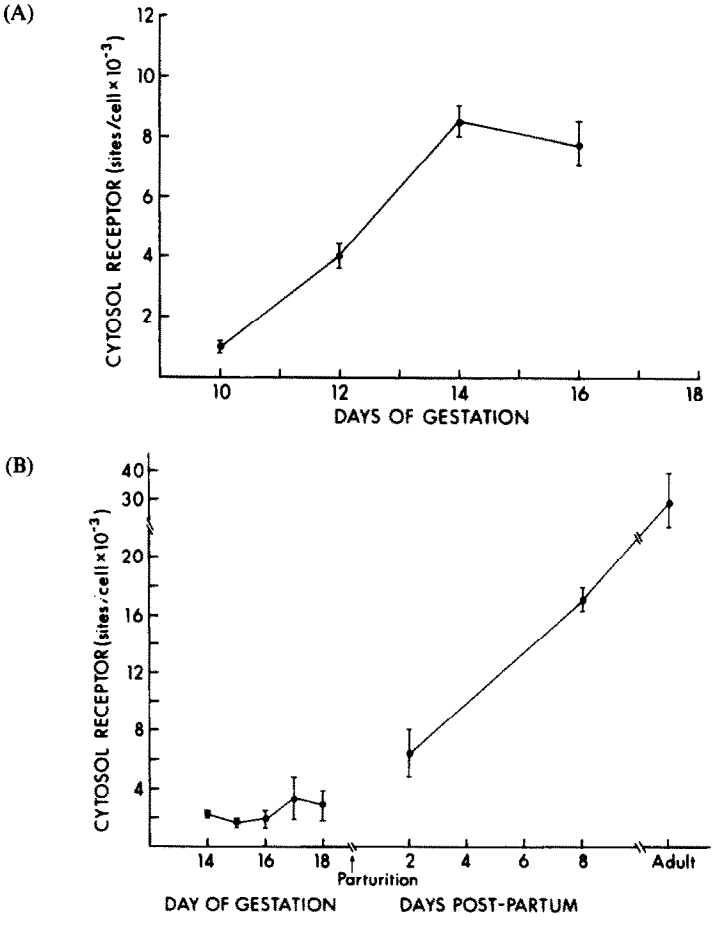

Fig. 2. Changes in cytosolic triamcinolone acetonide binding capacity during visceral yolk sac and liver development. Visceral yolk sac (A) and liver (B) cytosols prepared in the presence of 10 mM molybdate and 5 mM DTT were incubated for 4 h at 4°C with 30 nM [^{3}H]TA plus or minus a 200-fold excess of unlabeled TA. Samples were extracted with Dextran-coated charcoal, and specifically bound hormone was determined as described in the legend to Fig. 1. Values are ± SD of at least 6 determinations. DNA content was determined from aliquots of the total homogenate taken before cytosol preparation. Receptor sites per cell were calculated as described in Experimental.

of cytosol before incubation had no effect on the number of receptor sites/cell assayed under these conditions, whereas a 5-fold dilution of cytosol resulted in a significant reduction in binding capacity (not shown). The number of TA receptor sites/cell in VYS increased dramatically between day 10 and 14 of gestation, after which it leveled off (Fig. 2A). The level of receptor in day 10 VYS was near the limit of detection of the assay employed (1×10^3 sites/cell). Changes in cytosolic TA binding sites in liver (Fig. 2B) showed a different developmental pattern than those in VYS. The number of receptor sites/cell in fetal liver were low (10- to 20-fold less than in adult liver) throughout gestation. The number of cytosolic receptor sites/cell in liver increased rapidly after birth, reaching a level of about 1.7×10^4 sites/cell by day 9 post-partum. The specific binding capacity of various mixtures of fetal and adult liver cytosol, made before incubation with TA, was predictably additive (not shown) indicating that fetal liver cytosol did not contain an "inhibitor" of TA binding (i.e. free corticosterone or protease).

Scatchard analysis [17] of binding data indicated the presence of a single class of high affinity binding sites in VYS, fetal and neonatal liver (Fig. 3). Similar results were obtained with adult liver cytosol ($K_d = 3.1$ nM; not shown). In each case, the apparent dissociation constant (K_d) for the hormone receptor interaction at 4°C ranged between 2 and 4 nM.

In another series of experiments, mice were injected with [^{3}H]TA (plus or minus a 100-fold excess of unlabeled TA) at a dose which resulted in 10^{-6} M serum concentrations of TA within 0.5 h of injection [22]. Nuclei were isolated from VYS, fetal, neonatal, and adult liver 2 h post-injection, and specifically bound TA was determined as described in the Experimental section (Table 1). In each tissue examined, the amount of specifically bound nuclear TA was proportional to the number of cytosolic binding sites assayed *in vitro* (compare Table 1 and Fig. 2A, B). However, the absolute number of nuclear bound receptor sites/cell after *in vivo* injection of TA was roughly 2-fold greater than the number of cytosolic receptor sites/cell assayed *in vitro*. Although the reason for this discrepancy is unclear, the results indicated that the TA receptor complexes in VYS and liver could be "activated" *in vivo* to the nuclear binding form described by others [2, 3].

Table 1. Nuclear bound triamcinolone

Tissue	Age	Sites/Cell
Adult liver		63,000
Neonatal liver	3 days	23,000
Fetal liver	17 days	4,600
	16 days	4,000
	14 days	2,900
Visceral yolk sac	14 days	16,000

Mice (2 adults or 6 neonates) were injected subcutaneously with 4 mg/kg of TA plus or minus a 100-fold excess of unlabeled TA to control for non-specific binding. Nuclei were prepared 2 h after injection and specifically bound hormone was determined as described in Experimental. DNA was measured by the diphenylamine reaction [16] and nuclear bound sites/cell were calculated using a sp. act. of 260 mCi/mM and a value of 6.4 pg DNA/nucleus.

Bound [^{3}H]TA in cytosol from fetal liver and VYS was further characterized by DEAE-Sephadex A-50 column chromatography (Fig. 4). Bound [^{3}H]TA in cytosol maintained at 4°C ("unactivated") eluted as two peaks during chromatography (Fig. 4A, C). The initial peak in the prewash fractions (0.02 M potassium phosphate buffer) represented non-specifically bound TA as this binding was not displaced by a 100-fold excess of unlabeled TA (Fig. 4C). The second peak which eluted at approx. 0.4 M KCl in cytosol from both VYS and fetal liver represented TA receptor complexes. A similar elution profile was obtained with cytosol from adult liver (not shown)

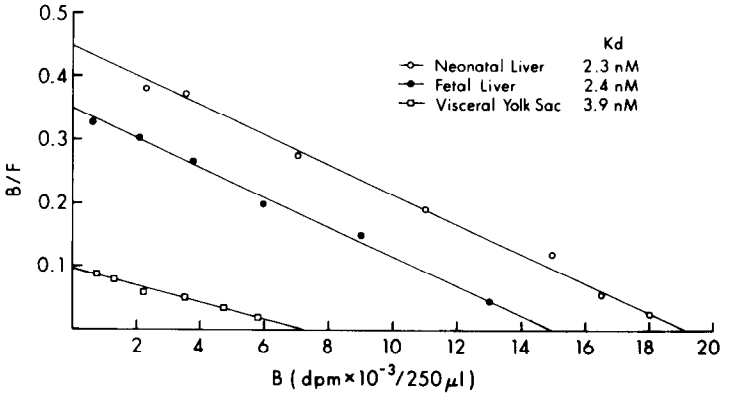

Fig. 3. Scatchard analysis of triamcinolone acetonide binding to cytosol. Visceral yolk sac (□), fetal liver (●), and neonatal liver (○) cytosols were prepared in the presence of 10 mM molybdate and 5 mM DTT and incubated for 4 h at 4°C with [^{3}H]TA, ranging in concentration from 0.2 to 15 nM plus or minus a 200-fold excess of unlabeled TA. Samples were extracted with Dextran-coated charcoal, and specifically bound hormone was calculated as described in the legend to Fig. 1. Data were plotted according to the method of Scatchard [17]. B and F refer to specifically bound and free hormone concentrations, respectively.

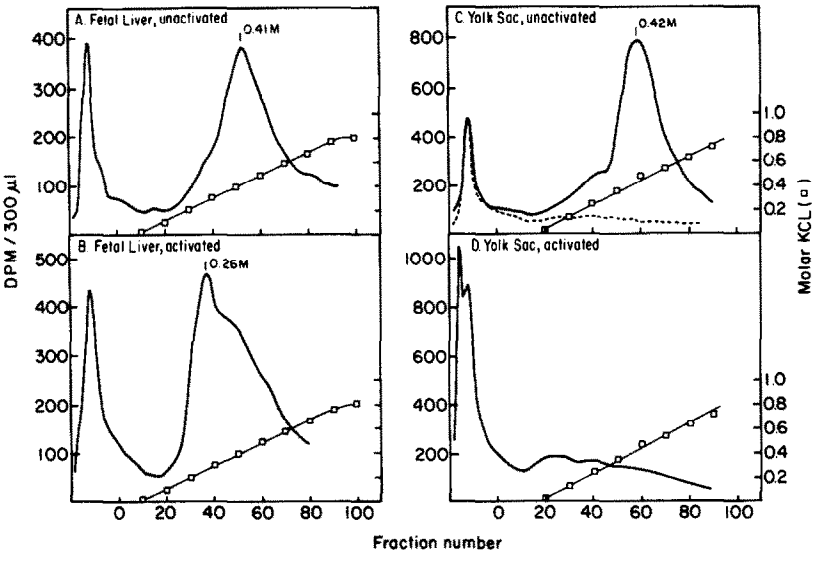

Fig. 4. DEAE-Sephadex A-50 chromatograms of bound [^{3}H]triamcinolone acetonide in fetal liver and visceral yolk sac cytosol. Cytosols from fetal liver (A and B) and visceral yolk sac (C and D) were incubated at 4°C for 2 h with 30 nM [^{3}H]TA. After extraction with Dextran-coated charcoal, cytosol was maintained at 4°C for 30 min ("unactivated"; A and C) or heated to 25°C ("activated"; B and D) before chromatography. Eluted [^{3}H]TA represents bound hormone because there was a plug of Dextran charcoal at the bottom of the column which adsorbed free hormone during chromatography. ---- (C), elution of [^{3}H]TA bound in the presence of a 200-fold excess of unlabeled TA (non-specific binding). Initial unnumbered fractions represent the prewash (8 fractions of 2 ml each); subsequent numbered fractions (100 fractions of 0.5 ml each) represent a linear 0–1 M KCl gradient.

and is characteristic of "unactivated" glucocorticoid receptor complexes from several adult tissues [2, 18, 23, 24].

"Activation" of glucocorticoid receptors has been shown to be a temperature-dependent process which results in an increase in the positive charge of the hormone–receptor complex [2, 3]. "Activated" and "unactivated" hormone–receptor complexes were resolved by DEAE-Sephadex A-50 chromatography [18, 23]. Bound [^{3}H]TA in fetal liver cytosol heated to 25°C for 30 min ("activated") before chromatography eluted as two major peaks. Elution of non-specifically bound TA was essentially unchanged, whereas specifically bound TA eluted at 0.22–0.26 M KCl (Fig. 4B). A similar chromatographic profile was obtained with "activated" adult liver cytosol (not shown). In contrast to results obtained with liver cytosols, the bound [^{3}H]TA in heat "activated" cytosol from VYS consistently eluted in the prewash fractions (Fig. 4D).

DISCUSSION

Mouse VYS and fetal liver provide useful model systems for studying the regulation of gene expression during development. Both tissues actively express the metallothionein and alphafetoprotein genes [11], which are glucocorticoid responsive [14, 15] and have been characterized in some detail at the molecular level [25, 26]. Since glucocorticoids are thought to act via receptor-mediated effects on gene transcription, it was of interest to investigate the status of glucocorticoid receptors in developing VYS and liver. Analysis of cytosolic receptors was carried out under conditions which allow for exchange of exogenous glucocorticoid for endogenous receptor-bound glucocorticoids [21]. In light of the fact that maternal serum corticosterone levels are extremely high ($\sim 10^{-6}$ M) during the latter half of pregnancy [27, 28], exchange assay conditions are of critical importance if reliable measurements of receptor concentration are to be made. Most (99%) of this maternal corticosterone, however, is bound to CBG, and fetal serum corticosterone levels are much lower ($\sim 10^{-9}$ M) than maternal serum levels [29–31]; fetal serum and amniotic fluid also contain high levels of CBG [30]. No evidence was obtained in this study for the existence of a significant population of cytosolic glucocorticoid receptor complexes in the fetal tissues examined, which supports the contention that accurate measurements of cytosolic glucocorticoid receptor levels were reported herein. Furthermore, the levels of nuclear bound [^{3}H]TA after injection of labeled TA indicated that each tissue examined contained glucocorticoid receptors which could undergo the "activation" process *in vivo*, which is indicative of functional glucocorticoid receptors [2, 3].

The midgestation mouse VYS was shown herein to contain glucocorticoid receptors which indicates that this extraembryonic membrane is a potential target tissue for glucocorticoid hormones. Human fetal membranes (amnion) have also been reported to

contain glucocorticoid receptors [32]. At present, nothing is known about glucocorticoid effects on the mouse VYS. It is interesting to note that maximal VYS glucocorticoid receptor levels (9×10^3 sites/cell) were found on day 14 of gestation, which is coincident with maximal relative rates of secretory protein synthesis by this membrane [33]. We observed that heat "activated" hormone–receptor complexes from VYS eluted in the prewash fractions from DEAE-Sephadex A-50 columns. A similar elution profile for "activated" glucocorticoid receptors from rat colon and kidney (binder IB) has been reported by others [18, 23, 24].

Glucocorticoid receptor levels in fetal liver were low throughout gestation but increased rapidly after birth. A similar developmental pattern has been reported for rat liver glucocorticoid receptors [34, 35]. Between days 12 and 15 of gestation, the fetal liver is the major erythropoietic organ, and the majority of cells in the liver are of the erythropoietic lineage [36, 37]. It seems likely that the glucocorticoid receptors in the fetal liver at this stage were also present in the erythropoietic cells. Evidence for low levels of glucocorticoid receptors in the erythropoietic cells of fetal rat liver has been reported [38]. The glucocorticoid receptors in day 17 fetal liver, where the majority of fetal liver cells are hepatocytes, had the same apparent dissociation constant (K_d) as did receptors from neonatal and adult liver. Furthermore, no evidence was found for physicochemical differences between fetal and adult liver glucocorticoid receptor complexes ("unactivated" or "activated"), which is in contrast to results reported for the rat liver receptor during development [39]. However, the possible existence of physicochemical differences between fetal and adult liver receptors which were not detected by the techniques employed in this study cannot be ruled out. The presence of glucocorticoid receptors in fetal liver (albeit at low levels) indicates the potential responsiveness of this organ to glucocorticoids. Deposition of glycogen in fetal mouse liver can be influenced by glucocorticoids after day 16 of gestation [40], whereas, in fetal rat, injected glucocorticoids are unable to induce hepatic tyrosine aminotransferase [41].

In summary, the results presented herein establish that the visceral yolk sac and fetal liver contain glucocorticoid receptors which bind TA with high affinity and limited capacity. Maximal levels of glucocorticoid receptors were found in the day 14 visceral yolk sac, whereas liver glucocorticoid receptors increased dramatically after birth. The results, although preliminary in nature, further indicated that the "activated" visceral yolk glucocorticoid receptors from VYS may differ from those in mouse liver.

Acknowledgements—I wish to thank Wayne Balkan and Edith Morrow for technical assistance and Susan DeBrunner-Bass for typing the manuscript.

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