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ONTOGENY AND BIOCHEMICAL PROPERTIES OF GLUCOCORTICOID RECEPTORS IN MID-GESTATION MOUSE EMBRYOS

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SUMMARY

Adult hepatic and mid-gestation embryonic cytosol fractions prepared from two inbred strains of mice, A/J and C57BL/6J, contain macromolecules which specifically bind [^{3}H]-dexamethasone with high affinity as determined by Scatchard analysis. The dexamethasone-binding macromolecules appear to be proteins since binding activity is destroyed by proteolytic enzymes but not by incubation with nucleases, lipase or collagenase. Binding is dependent upon the integrity of −SH groups. Both natural and synthetic glucocorticoids compete with labeled dexamethasone for the binding sites in adult hepatic and embryonic cytosols. [^{3}H]-Triamcinolone acetonide–receptor complexes in adult hepatic and embryonic cytosols sediment as single broad peaks between 7 to 10*S* after velocity sedimentation on 10–30% glycerol gradients and elute as two distinct binding components following DEAE–cellulose chromatography. [^{3}H]-Corticosterone-binding components have also been detected in adult hepatic and embryonic cytosols. Based upon physical and chemical properties, these macromolecules resemble corticosteroid-binding globulin (transcortin) in mouse serum. Ontogenically, receptor proteins could first be detected in day 12 embryonic cytosols when the concentration of receptor sites was significantly higher in A/J embryos (approximately two-fold) than in C57BL/6J embryos. By days 13 and 14 the receptor concentration was equivalent in both.

Glucocorticoids have been shown to be involved in the pre- and postnatal development of a variety of tissues such as intestine and lung [1–3] and in the development of specific proteins in the liver and neural retina [4, 5]. In fact, the appearance of specific cytoplasmic glucocorticoid receptors in these fetal tissues at defined periods has been implicated as one factor which might determine the onset of hormonal responsiveness in these tissues to glucocorticoids [3, 6]. Such studies have demonstrated that fetal glucocorticoid receptors physically resemble their counterparts in adult glucocorticoid target cells [3, 7, 8]. The ontogeny of glucocorticoid receptors has been studied in several species where they were identified in tissues obtained from fetuses at three to five days prior to birth [1–5].

In order to determine the time in development when this specific gene product first appears, we chose to examine mid-gestation mouse embryos rather than discrete fetal organs which arise at later stages in development from embryonic cells. Furthermore, although such receptors have been characterized in fetal tissues from prenatal rat, rabbit and sheep, they have not yet been identified in the mouse, nor in any embryonic tissues in those species previously mentioned. Moreover, since the level of corticosoterone, the major naturally occurring adrenal corticoid in rodents [9], is substantially elevated in the maternal serum during pregnancy, it is of interest to determine the amount of embryonic receptors present during mid-pregnancy as the embryo is exposed at this time to unusually high amounts of corticosterone originating from the maternal and possibly placental and embryonic compartments [10, 11].

Two inbred strains of mice, A/J and C57BL/6J, were chosen for analysis because they represent strains which exhibit marked differences in glucocorticoid-induced teratogenesis [12–14]. For example, after the exogenous administration of glucocorticoids over days 11 through 14 of gestation, 100% of the offspring of treated A/J mice develop cleft palate, whereas only 20–25% of offspring of similarly treated C57BL/6J mice have cleft palate [12, 13]. The incidence of cleft palate is highest after a single maternal injection *in vivo* of triamcinolone acetonide on days 11 or 12 of gestation, while the frequency drops if the steroid is administered on either days 13 or 14 [15, 16]. Although we recognize that analysis of steroid receptors in whole embryos during the steroid-sensitive period, days 11 through 14 of gestation, may not be a sensitive or specific test for receptors in the developing secondary palate, whole embryos

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were used due to the inadequate size of palatal tissues for biochemical analysis at the critical period of steroid sensitivity.

The results of this report demonstrate: (1) that glucocorticoid receptors appear in the embryo as early as day 12, a time prior to the development of most fetal organs, (2) that such embryonic receptors physiochemically resemble cytoplasmic receptors found in an adult glucocorticoid target organ, the liver and (3) even with the limitations imposed by the tissue source, that there is a quantitative difference in the amount of embryonic receptor protein in these two strains on one critical day during the steroid-sensitive period.

EXPERIMENTAL

Materials. Nonradioactive steroids were purchased from Sigma Chemical Co. and prepared as 1 mM stock solutions in absolute ethanol. Chromatographically pure [1,2-^{3}H]-dexamethasone (28 Ci/mmol)*, [1,2,4-^{3}H]-triamcinolone acetonide (36 Ci/mmol), and [1,2,6,7-^{3}H]-corticosterone (82 Ci/mmol) were obtained from Amersham/Searle. Timed pregnant A/J and C57BL/6J mice which were obtained on the 10th day of gestation were purchased from Jackson Laboratories, Bar Harbor, Maine.

Preparation of cytosol. Whole mouse embryos or maternal livers were obtained from pregnant animals at days 11 through 14 of gestation (day of detection of vaginal plug is designated day 0 of gestation). Most experiments used pooled embryos from several litters or a single liver lobe obtained from comparably staged pregnant animals. Placental and extraembryonic membranes were dissected from the embryos as previously described [17]. Embryonic or hepatic tissues were washed several times in cold phosphate-buffered saline (0.85% NaCl, pH 7.4) and homogenized in 2–3 vol. of Buffer I (10 mM Tris–HCl, 1.5 mM EDTA and 0.5 mM dithiothreitol, pH 7.4). Following homogenization, 2.0 M sucrose prepared in Buffer I was added to the homogenates to a final concentration of 0.5 M. Homogenates were centrifuged at 105,000 *g* for 60 min at 0–4°C to obtain crude cytoplasmic supernatant fractions (cytosol). Aliquots of the cytosol fractions were used for the binding assays.

Preparation of serum. Maternal blood (0.2 ml) was collected from day 12 pregnant mice by cardiac puncture. Clotted blood was centrifuged at 1500 *g* for 15 min to obtain serum.

* The trivial names used are: dexamethasone, 9α-fluoro-16α-methyl-11β, 17, 21-trihydroxy 1,4-pregnadiene-3, 20-dione; triamcinolone acetonide, 9α-fluoro-11β, 16α, 17, 21-tetrahydroxy-1, 4 pregnadiene-3, 20-dione-16, 17-acetonide; corticosterone, 11β, 21-dihydroxy 4-pregnene-, 3, 20-dione; tetrahydrocortisone, 3α, 17, 21-trihydroxy-5β-pregnane-11, 20-dione; cortisol, 11β 17, 21-trihydroxy 4-pregnene-3, 20-dione; cortisone, 17, 21 dihydroxy-4-pregnene-3, 11, 20-trione; 17β-estradiol, 1, 3, 5 (10)-estratrien-3, 17β-diol; progesterone, 4-pregnene-3, 20-dione; testosterone, 17β-hydroxy-4-androsten-3-one.

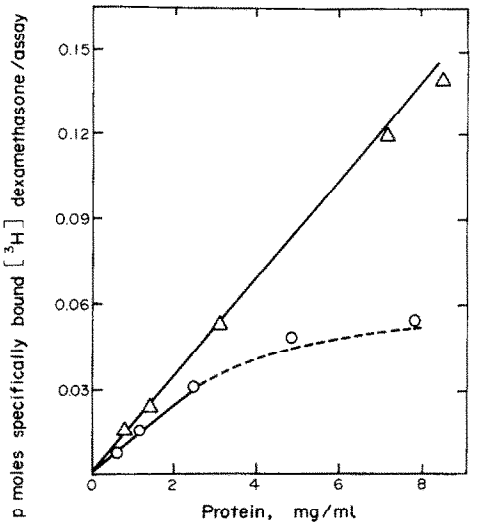

Fig. 1. Specific binding of [^{3}H]-dexamethasone to day 14 A/J hepatic (△) and embryonic (○) cytosols as a function of protein concentration. [^{3}H]-dexamethasone (3.8×10^{-8} M) was incubated for 120 min at 0°C in the absence or presence of nonradioactive dexamethasone (1×10^{-5} M) with various dilutions of cytosol. Bound [^{3}H]-dexamethasone was assayed by charcoal absorption technique. Specific binding represents the binding observed in the presence of radioactive dexamethasone minus that observed in the presence of unlabeled dexamethasone. Each point represents the mean value of duplicate determinations.

Measurement of specific binding. Aliquots of the cytosol fractions containing protein concentrations within the linear portion for specific binding (see Fig. 1) were incubated with 1–100 nM [^{3}H]-dexamethasone at 0°C for 2 h or as indicated. Samples containing the same concentration of [^{3}H]-dexamethasone plus an excess of nonlabeled dexamethasone (10^{-5} M) as a competitor were run in parallel to correct for nonspecific binding [18, 19]. Following the incubation, free and bound dexamethasone were separated by the addition of 0.05 ml of a dextran-charcoal suspension (5% activated charcoal, Sigma Chemical Co., and 0.5% dextran T_{70} suspended in Buffer I) to each sample. The samples were mixed and centrifuged at 1500 *g* for 15 min at 4°C. Duplicate aliquots (0.1 ml) of the supernatant from each sample were mixed with 10 ml of scintillation fluid (Hydromix, Yorktown Research) and counted in a Packard Tri-Carb model 3375 liquid scintillation spectrometer with an efficiency of 33% for [^{3}H] and corrected for quenching by the method of channels ratio of the external standard. Another aliquot was analyzed for protein by the method of Lowry *et al.*[20] using bovine serum albumin as a standard. To calculate the concentration of receptor–steroid complexes, the data was analyzed by the method of Scatchard[21]. The apparent dissociation constant (K_D) of the receptors for dexamethasone was calculated from the negative value of the slopes of the Scatchard plots. Slopes and abscissa intercepts from nonlinear Scatchard plots were deter-

mined by linear regression analysis on a Honeywell model 316 computer.

Measurement of nuclear uptake of [^{3}H]*-dexamethasone by whole mouse embryos.* Day 12 mouse embryos were washed in 5 ml of cold NCTC-109 medium (Microbiological Associates, Bethesda, MD). Pooled embryos (approximately 10–15) were incubated for 2 h at 37°C on a shaking water bath in 3 ml of NCTC-109 medium containing [^{3}H]-dexamethasone (1×10^{-8} M). Parallel incubations with the same concentration of [^{3}H]-dexamethasone plus an excess of nonlabeled dexamethasone (10^{-5} M) were carried out to determine nonspecific binding of the labeled hormone. Following incubation, the embryos were washed several times at 4°C with Buffer II (10 mM Tris–HCl, 1 mM $MgCl_2$ and 0.25 M sucrose, pH 7.6) and then homogenized in 2 ml of the same buffer. A nuclear fraction (pellet) and a cytosol fraction (supernatant) were prepared by centrifuging the homogenates at 1000 *g* for 10 min at 4°C. The pellets were washed three times with 5 ml of cold Buffer II, each wash followed by centrifugation at 1000 *g* for 10 min at 4°C. The washed nuclear pellets were purified by homogenization in 2 ml of Buffer III (10 mM Tris–HCl, 1 mM $MgCl_2$ and 2.2 M sucrose, pH 7.6). The nuclear and crude cytosol fractions were then centrifuged at 50,000 *g* for 60 min at 4°C. The DNA content in the nuclear fractions was determined by the diphenylamine procedure [22] using calf thymus DNA as a standard. The amount of [^{3}H]-dexamethasone which was specifically bound to the nuclear fraction was expressed on a per mg DNA basis. Purified cytosol fractions were treated with 0.05 ml of a dextran-charcoal suspension to separate free from macromolecular-bound steroids. Aliquots of the supernatants were taken for protein determination and for calculation of the amount of [^{3}H]-dexamethasone which was specifically bound to cytosol proteins.

Analytical procedures. Glycerol density gradient centrifugation was performed in 4 ml linear 10–30% glycerol gradients in Buffer I in a Beckman Model L3-50 ultracentrifuge using a SW56 rotor at 45,000 rev./min for 15 h at 4°C. Samples for analysis, either serum or tissue cytosol fractions in Buffer I, were incubated for 2–5 h at 0°C with either [^{3}H]-dexamethasone (3.5×10^{-8}), [^{3}H]-corticosterone (1.2×10^{-8}) or [^{3}H]-triamcinolone acetonide (2.8×10^{-8} M) in the absence or presence of 10^{-5} M nonlabeled homologous steroids. The cytosols were then treated with charcoal to remove unbound steroid. Aliquots of these cytosols (1–4 mg protein in 0.25 ml) were mixed with 0.1 ml crystalline bovine serum albumin (10 mg per ml) and layered onto the gradients. Following centrifugation, the tubes were pierced through the bottom and the contents removed by forcing a solution of 50% glycerol up through the bottom of each tube. The absorption of the effluent was monitored continually at 280 nm and 0.1 ml fractions were collected. From each fraction a 0.05 ml aliquot was added to scintillation fluid and assayed for radioactivity. The sedimentation coefficients of the steroid-binding components were estimated by comparison with that of bovine serum albumin (BSA, 4.6*S*) and calculated by the method of Martin and Ames[23].

For column chromatography, DEAE-52-cellulose (Whatman DE-52 cellulose) glass columns, 1.3 × 34 cm with bed volumes of 11.8 ml were equilibrated and packed in Buffer IV which contained 0.002 M Na_2HPO_4, pH 7.3. Day 12 embryonic or adult hepatic cytosols prepared in Buffer I were prelabeled with [^{3}H]-triamcinolone acetonide (2.8×10^{-8} M) in the absence or presence of 10^{-5} M nonlabeled triamcinolone acetonide for 2.5 h at 0°C. Aliquots of the labeled cytosols (2–4 mg of protein) which had previously been treated with charcoal to remove unbound steroid were applied to the columns and washed with Buffer IV at 4°C at a flow rate of 40 ml/h. After washing with 20 ml of Buffer IV, proteins were eluted with 100 ml of a linear gradient of 0–0.20 M Na_2HPO_4. Fractions of 2.5 ml were collected and aliquots (1.0 ml) were used to assay for radioactivity and protein. The conductivity of each fraction was measured and used to estimate the molarity of the gradient.

RESULTS

[^{3}H]-*Dexamethasone binding to mouse embryonic and adult hepatic macromolecules*

Cytosols from A/J or C57BL/6J adult livers or embryos were found to show specific binding of dexamethasone which was proportional to the protein concentration over the range of 0.5–8.5 mg of protein per ml of cytosol for hepatic cytosols and from 0.5 2.5 mg of protein for embryonic cytosols (Fig. 1). Saturation experiments (see below) were carried out with aliquots of either hepatic or embyronic cytosols containing protein concentrations within the linear portion for specific binding. Figure 2 depicts the kinetics of the association and dissociation of specific [^{3}H]-dexamethasone binding to day 14 A/J or C57BL/6J adult hepatic or embryonic cytosol components. The binding of the steroid in hepatic cytosols approached maximum equilibrium after 120 min, whereas only 60 min was required for the maximum binding of the steroid to components in the embryonic cytosols. Furthermore, although the amount of [^{3}H]-dexamethasone bound to the hepatic components relative to that bound to embryonic components is high in relationship to those illustrated in Fig. 1, the relative differences are within the range of binding (see Fig. 1 and Table 5) for tissues obtained at this particular stage in gestation.

The reversibility of the binding reaction for the cytosol preparations is also indicated in Fig. 2. The addition of an excess of nonlabeled dexamethasone (10^{-4} M) to assays of previously formed [^{3}H]-dexamethasone cytosol-complexes resulted in a rapid displacement of bound hormone (inset, Fig. 2) [24].

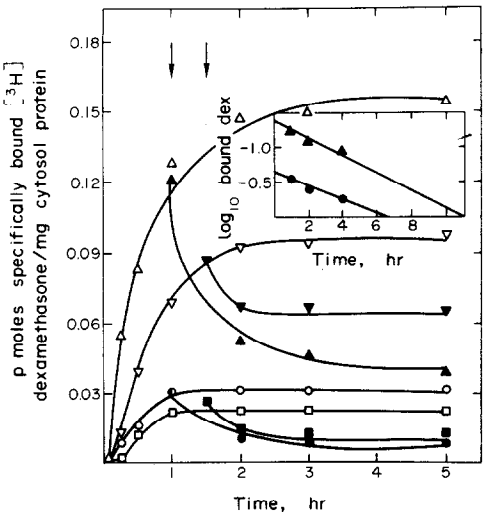

Fig. 2. Kinetics of [^{3}H]-dexamethasone association and dissociation with the specific cytoplasmic binding components. Aliquots of either day 14 hepatic cytosol obtained from A/J (△) or C57BL/6J (▽) mice; or embryonic cytosol obtained from A/J (○) or C57BL/6J (□) mice were incubated with [^{3}H]- dexamethasone (5×10^{-8} M) in the absence or presence of unlabeled dexamethasone (1×10^{-5} M) at 0°C. At various time intervals, samples of each cytosol were withdrawn and assayed for specific binding. As indicated (arrows), an excess of nonradioactive dexamethasone (1×10^{-4} M) was added to a parallel set of the cytosol preparations and the specific binding was assayed in each set at various time intervals (closed symbols) following the addition of unlabeled steroid. The *inset* represents the dissociation data for A/J hepatic (▲) and embryonic (●) cytosols in which the $\log_{10}$ (specifically bound steroid) was plotted as a function of time after adding nonradioactive dexamethasone. Each point is the mean value of duplicate determinations.

Specific binding of [^{3}H]*-dexamethasone as a function of steroid concentration*

To estimate the number and relative affinity of the [^{3}H]-dexamethasone-binding sites, cytosols prepared from day 11 through 14 A/J or C57BL/6J adult livers or embryos were incubated with various concentrations of [^{3}H]-dexamethasone for 2 h at 0°C. Figure 3(a) illustrates one of several such binding curves obtained from a day 12 A/J adult hepatic cytosol preparation. A Scatchard plot (inset, Fig. 3(a)) of the specific binding yielded a straight line, consistent with only a single class of specific binding sites. The apparent equilibrium dissociation constant (K_D) for the [^{3}H]-dexamethasone-macromolecular complex was 2.4×10^{-8} M at 0°C. Assuming that each receptor site binds one molecule of steroid [25], the concentration of binding sites determined from the abscissa of this plot is 0.12 pmol per mg cytosol protein. Qualitatively similar saturation binding curves and Scatchard plots to that of Fig. 3(a) were obtained from adult hepatic cytosol preparations obtained from all stages of gestation of A/J and C57BL/6J mice, as well as from embryonic cytosol preparations obtained from day 12 through day 14 C57BL/6J mice and day 14 A/J mice.

In contrast, the binding curves for day 12 and 13 A/J embryonic cytosols were biphasic. Figure 3(b) illustrates one such example suggesting that at least two separate binding sites exist in A/J embryos at this age. Extrapolation of the two components of this curve to the abscissa allowed estimation of an apparent K_D of 2.2×10^{-8} M for the high affinity site and of 1.3×10^{-7} M for the low affinity binding site. The concentration of receptor sites was 0.02 and 0.06 pmol per mg cytosol protein for the high and low affinity sites, respectively. It is noteworthy, that the propor-

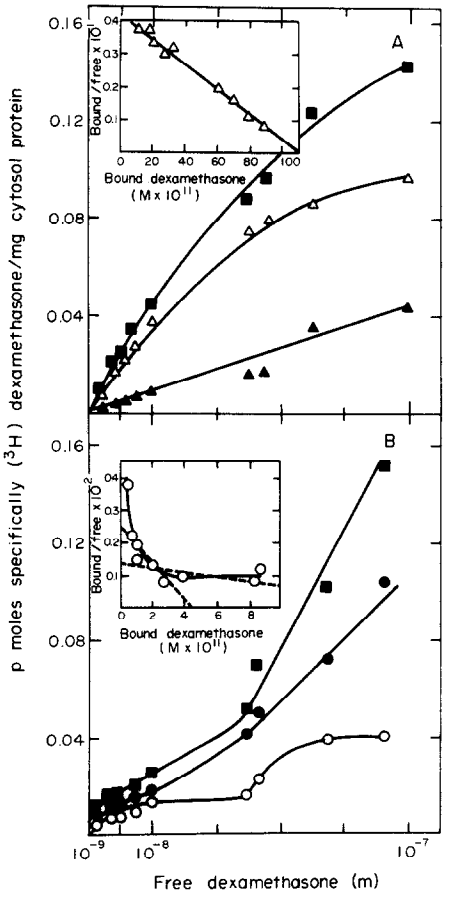

Fig. 3. Specific binding of [^{3}H]-dexamethasone to cytosols as a function of the steroid concentration. Various concentrations of [^{3}H]-dexamethasone were incubated for 120 min at 0°C with aliquots of A, day 12 A/J hepatic (△) or B, day 12 A/J embryonic (○) cytosol in the absence (total, ■—■) or presence (closed figures) of nonlabeled dexamethasone (1×10^{-5} M) to correct for nonspecific binding. Specifically bound [^{3}H]-dexamethasone (open figures) was determined by the charcoal assay. *Insets*, Scatchard plots of the binding data, showing the ratio of bound to free [^{3}H]-dexamethasone as a function of the amount of specifically bound hormone. Each point represents the mean value of duplicate determinations.

tion of nonspecific binding in day 12 embryonic cytosols accounts for approximately 60–70% of the total binding of [^{3}H]-dexamethasone, whereas in hepatic cytosol preparations the nonspecific binding accounts for only 20–30% of the total binding capacity. Furthermore, in contrast to hepatic cytosols, a substantial portion of this "background" binding is non-linear especially at the higher steroid concentrations. The physiological significance of this nonspecific component is not known. Although high and low affinity receptor sites for cortisol also have been identified in cytosols prepared from embryonic chick neural retina and from adult rat liver tissues [5, 26], the physiological role of these two classes of receptors in relation to the mode of action of glucocorticoids is presently unknown.

Effect of sulfhydryl reagents, hydrolytic enzymes and temperature on the [^{3}H]*-dexamethasone and* [^{3}H]*-corticosterone-binding proteins in hepatic and embryonic cytosols and serum*

Pretreatment of cytosols with nucleases, lipase or collagenase did not reduce the subsequent binding of dexamethasone, whereas protease pretreatment caused a marked reduction of the specific binding of dexamethasone to both hepatic and embryonic cytosols (date not included). Digestion by protease indicated that both the hepatic and embryonic binding components were proteins.

Cytosols or serum obtained from either A/J or C57BL/6J mice were then pretreated with various sulfhydryl reagents at 1.0 mM concentrations (Table 1). The specific binding of dexamethasone to hepatic or embryonic cytosols from either strain of mice was markedly inhibited under these conditions. There was no detectable binding of dexamethasone in the serum samples. In contrast, there was substantial specific [^{3}H]-corticosterone binding in the serum samples and in the cytosol preparations. This binding activity, however, was not impaired by sulfhydryl reagent pretreatment. In fact, pretreatment with mercuribenzoate caused a significant increase in corticosterone binding above the control values. A similar phenomenon has been observed in the ability of iodoacetamide and *p*-chloromercuriphenyl sulfonic acid to enhance cortisol binding in rat muscle and liver cytosol preparations, respectively [27, 28].

Preincubation of A/J hepatic or embryonic cytosols at 45°C for 15 min, reduced the amount of specific [^{3}H]-dexamethasone binding (Table 2). However, specific [^{3}H]-corticosterone binding in either hepatic or embryonic cytosols and serum was unaffected by this treatment.

Based upon these criteria it was concluded that dexamethasone is bound to receptor components whereas corticosterone was preferentially bound to serum corticosteroid-binding globulin (transcortin) in the cytosol preparations.

Competition of various steroids for the specific binding of [^{3}H]*-dexamethasone and* [^{3}H]*-corticosterone in hepatic and embryonic cytosols and serum*

Various unlabeled steroids in 1000 fold excess were screened for their ability to compete with [^{3}H]-dexamethasone or [^{3}H]-corticosterone binding in hepatic or embryonic cytosols obtained from day 12 mice (Table 3A). The specific binding of [^{3}H]-dexamethasone to receptor sites in both hepatic and embryonic cytosols from either A/J or C57BL/6J mice was effectively inhibited by unlabeled dexamethasone, triam-

Table 1. Effect of sulfhydryl-blocking reagents on the specific binding of [^{3}H]-steroids to cytosol and serum macromolecules

	A/J			C57BL/6J		
Reagent	Embryonic	Hepatic	Serum	Embryonic	Hepatic	Serum
A						
None, [^{3}H]-dexamethasone	100(0.243)	100(0.126)	0	100(0.051)	100(0.062)	0
p-chloromercuribenzoate	15	0.65	—	12	4.3	—
p-chloromercuriphenyl sulfonic acid	6.6	1.9	—	19.6	2.6	—
N-Ethylmaleimide	15.4	5.4	—	16.5	26	—
B						
None, [^{3}H]-corticosterone	100(0.685)	100(0.678)	100(2.45)			100(2.98)
p-chloromercuribenzoate	172	210	181	—	—	153
p-chloromercuriphenyl sulfonic acid	100	95	118	—	—	120
N-Ethylmaleimide	87	128	126	—	—	117

Aliquots of day 13 A/J or C57BL/6J embryonic or hepatic cytosols (0.3 ml) or day 12 serum (0.005 ml) were incubated at 4°C for two h with or without 1 mM of the indicated reagents. Following treatment, aliquots of either treated or untreated cytosols or serum were incubated for 2 h at 4°C with either: (a) 10 nM [^{3}H]-dexamethasone in the absence or presence of an excess (10^{-5} M) of nonlabeled dexamethasone; or (b) 10 nM [^{3}H]-corticosterone in the absence or presence of an excess (10^{-5} M) of nonlabeled corticosterone. The specific binding of either [^{3}H]-steroid was determined by the charcoal technique. Values represent the mean of two separate experiments and are expressed as the percent of the value observed for specifically bound [^{3}H]-steroid in control samples. Values in parentheses represent the pmoles of specifically bound steroid per mg cytosol or serum proteins in control samples.

Table 2. Effect of temperature on the specific binding of [^{3}H]-steroids to cytosol and serum macromolecules

Pretreatment	[^{3}H]-Dexamethasone			[^{3}H]-Corticosterone		
	Embryonic	Hepatic	Serum	Embryonic	Hepatic	Serum
4°, Control	100(0.01)	100(0.063)	0	100(0.346)	100(0.183)	100(4.18)
45°, 15 min	1.6	0	—	113	111	134

Aliquots of day 12 A/J embryonic or hepatic cytosols (0.3 ml) or serum (0.005 ml) were preincubated at 45°C for 15 min. Aliquots of heated or untreated cytosols or serum were incubated for 2 h at 4°C with either: (a) 10 nM [^{3}H]-dexamethasone in the absence or presence of an excess (10^{-5} M) of nonlabeled dexamethasone; or (b) 10 nM [^{3}H]-corticosterone in the absence or presence of an excess (10^{-5} M) of nonlabeled corticosterone. The specific binding of either [^{3}H]-steroid was determined by the charcoal technique. Values represent the mean of two separate experiments and are expressed as a percent of the specifically bound [^{3}H]-steroid in cytosols or serum not heated, but preincubated at 4°C. Values in parentheses represent the pmoles of specifically bound steroid per mg cytosol or serum protein in control samples.

cinolone acetonide, corticosterone and cortisol. In contrast, the inactive analog tetrahydrocortisone (THF) was not effective as a competitor. Cortisone competed with [^{3}H]-dexamethasone binding in the embryonic cytosol preparations, but was substantially less effective as a competitor in the hepatic cytosol preparations. Progesterone was moderately effective competing with dexamethasone for binding sites in embryonic cytosols and completely effective in this respect in hepatic cytosols. 17β-estradiol competed for dexamethasone binding in both hepatic and embryonic cytosols obtained from A/J mice, but was much less effective than dexamethasone in hepatic or embryonic cytosols obtained from C57BL/6J mice. Testosterone failed to compete for dexamethasone binding sites in the embryonic cytosols, but partially competed for such sites in the hepatic cytosol preparations.

Several of these steroids were tested for their ability to compete for the specific binding of [^{3}H]-corticosterone to macromolecular components in cytosol or serum (Table 3B) since the experiments conducted with sulfhydryl-blocking reagents and thermal inactivation suggested that such corticosterone binding components in the cytosol samples resembled transcortin. Likewise, the large amount of [^{3}H]-corticosterone binding to both hepatic and embryonic cytosol components (3–5 fold over [^{3}H]-dexamethasone

Table 3. Effect of various unlabeled steroids on [^{3}H]-dexamethasone and [^{3}H]-corticosterone binding to cytosol and serum macromolecules

Unlabeled steroid	A/J			C57BL/6J		
	Embryonic	Hepatic	Serum	Embryonic	Hepatic	Serum
A						
None, [^{3}H]-Dexamethasone	100(0.029)	100(0.169)	0	100(0.043)	100(0.130)	0
Dexamethasone	0	0	—	0	0	—
Triamcinolone Acetonide	0	0.2	—	14	0	—
Corticosterone	14	0.7	—	32	5	—
Cortisone	11	46	—	25	50	—
Cortisol	0	0.42	—	3	4	—
Tetrahydrocortisone	103	105	—	109	94	—
Progesterone	38	3.6	—	25	13	—
17β-Estradiol	22	29	—	54	67	—
Testosterone	123	35	—	99	42	—
B						
None, [^{3}H]-Corticosterone	100(0.090)	100(0.416)	100(1.07)	100(0.102)	100(0.111)	100(1.97)
Corticosterone	0	0	0	0	0	0
Dexamethasone	86	103	137	103	128	97
Cortisone	54	111	110	81	99	80
Tetrahydrocortisone	125	130	138	142	129	104
Progesterone	10.4	32	79	20	59	56
17β-Estradiol	140	154	168	165	169	123
Testosterone	31	110	159	61	226	117

Aliquots of either day 12/A/J or C57BL/6J embryonic or hepatic cytosols (0.3 ml) or serum (0.005 ml) were incubated for 2 h at 4°C in the presence of either 10 nM [^{3}H]-dexamethasone or 10 nM [^{3}H]-corticosterone. The ability of an excess (10^{-5} M) of various nonlabeled steroids to compete for the specific binding of either [^{3}H]-steroid to macromolecular components was determined by the charcoal technique and calculated according to Baxter and Tomkins[19]. "Background" radioactivity (radioactivity in the homologous competitor-containing incubation that resisted charcoal treatment) was subtracted from each value and the difference is expressed as the percent of the value (minus "background") obtained on incubation with [3H]-dexamethasone or [^{3}H]-corticosterone in the absence of competitor (control, 100% value). All values represent the mean of two to three separate experiments. Values in parentheses represent the pmoles of specifically bound steroid per mg cytosol or serum protein in control samples.

binding, see Tables 1B and 3B) suggests that serum contamination accounts for the majority of corticosterone binding in these preparations. Dexamethasone failed to compete for the binding of [^{3}H]-corticosterone in hepatic or embryonic cytosols and serum from either strain of mice. In fact, the steroid competition profiles in both hepatic and embryonic cytosols exhibited considerable similarity to the profiles obtained from the serum preparations. However, there were some significant exceptions. Progesterone and testosterone were partial competitors of corticosterone binding to the embryonic cytosol components, while less effective in hepatic or serum samples. In fact, THF, 17β-estradiol and testosterone enhance the total binding of [^{3}H]-corticosterone to embryonic, hepatic and serum components. The reason(s) for these effects is not entirely apparent. However, it should be mentioned that attempts to saturate both tissue and serum binding components, presumably transcortin, with varying concentrations of [^{3}H]-corticosterone have been unsuccessful. It is possible that high concentrations of these competing steroids are preferentially saturating nonspecific binding sites and thereby facilitating the subsequent binding of [^{3}H]-corticosterone to transcortin.

To exclude the possibility that the competing steroids were being metabolized in the hepatic and embryonic cytosols, and that a metabolite(s) was antagonizing the binding of [^{3}H]-dexamethasone to the receptors, embryonic and hepatic cytosols were incubated with either radioactive dexamethasone, corticosterone, progesterone or 17β-estradiol (testosterone was not tested). After a 3 h incubation, the radioactive compounds in the samples were extracted with anhydrous ethyl ether, and subjected to thin-layer chromatography in the solvent system, chloroform–ethanol (95:5, v/v). The labeled compounds from each sample were found to migrate with authentic steroid markers indicating there was no metabolism.

Velocity sedimentation and DEAE chromatographic profiles of [^{3}H]*-dexamethasone and* [^{3}H]*-triamcinolone acetonide-cytosol complexes*

To further characterize the glucocorticoid receptors, hepatic or embryonic cytosols were incubated with [^{3}H]-triamcinolone acetonide. The protein-bound complexes were subjected to 10–30% glycerol density gradient centrifugation. Triamcinolone acetonide was chosen because it has been reported to form more stable steroid–receptor complexes than dexamethasone in rat muscle, rat hepatoma and mouse fibroblast cytosol preparations [29–31]. Day 12 A/J or C57BL/6J hepatic cytosols labeled with [^{3}H]-triamcinolone acetonide (2.8×10^{-8} M) display components sedimenting between 8 and 10*S* which represent specifically bound hormone since they were substantially reduced when the cytosols were incubated in the presence of an excess of unlabeled triamcinolone acetonide (Fig. 4(a)). Cytosols from day 12 A/J or C57BL/6J embryos exhibited peaks of radioactivity in the 7 to 8*S* regions of the gradient (Fig. 4(b)). Fig. 4(b) also shows the results for A/J embryonic cytosols incubated with an excess of unlabeled triamimolone acetonide in which complete competition was observed; essentially identical results were obtained with C57BL/6J embryonic cytosols.

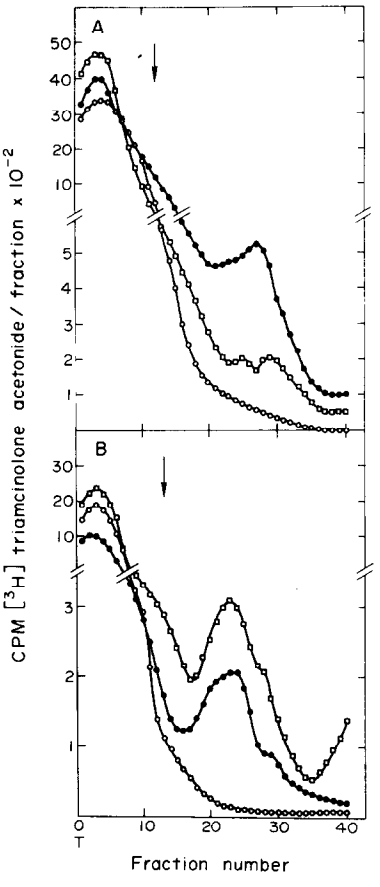

Fig. 4. Glycerol density gradient sedimentation patterns of cytoplasmic [^{3}H]-triamcinolone acetonide–macromolecular complexes from hepatic and embryonic tissues. Cytosols were incubated for 5 h at 0°C with 2.8×10^{-8} M [^{3}H]-triamcinolone acetonide. Following incubation, the samples were treated with charcoal to remove the unbound labeled steroid. Aliquots of supernate (0.25 ml) were layered onto 10–30% glycerol gradients prepared in Buffer I and centrifuged for 15 h at 44,000 rev./min at 4°C in a SW 56 rotor. Fractions were collected from the top (T) of the tubes and assayed for radioactivity and protein. *Solid arrows*, the sedimentation position of bovine serum albumin (BSA, 4.6*S*). A, day 12 A/J (●, 3 mg cytosol protein) of C57BL/6J (□, 1.7 mg cytosol protein) hepatic cytosols incubated with [^{3}H]-triamcinolone acetonide; (○) day 12 A/J hepatic cytosol incubated with [^{3}H]-triamcinolone acetonide + 10^{-5} M unlabeled triamcinolone acetonide. B, day 12 A/J (●, 1.4 mg cytosol protein) or C57BL/6J (□, 1.6 mg cytosol protein) embryonic cytosols incubated with [^{3}H]-triamcinolone acetonide; (○) day 12 A/J embryonic cytosol incubated with [^{3}H]-triamcinolone acetonide + 10^{-5} M unlabeled triamcinolone acetonide.

Serum labeled with [^{3}H]-corticosterone (1.2×10^{-8} M) and subjected to density gradient centrifuga-

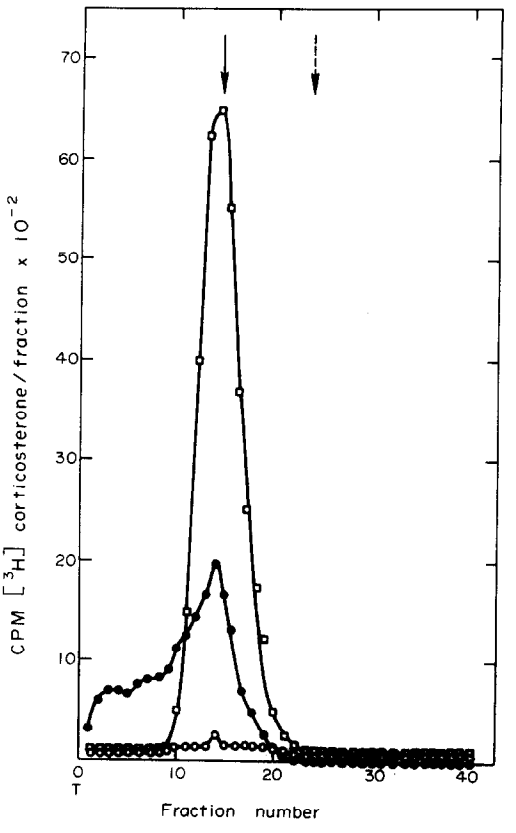

Fig. 5. Glycerol density gradient profiles of [^{3}H]-corticosterone binding components. Aliquots of day 12 A/J serum (□, 0.429 mg serum protein) or embryonic cytosol (●, 1.9 mg cytosol protein) were incubated with 1.2×10^{-8} M [^{3}H]-corticosterone for 5 h at 0°C. Samples were charcoal treated and processed as described in Fig. 4. (○) day 12 A/J embryonic cytosol incubated with [^{3}H]-corticosterone + 10^{-5} M unlabeled corticosterone. *Solid arrow*, BSA sedimentation position; *broken arrow*, the 7*S* region of the gradient.

tion exhibited a single peak of radioactivity with a sedimentation constant of 4.6*S* (Fig. 5). Hepatic (not shown) or embryonic cytosols processed under identical conditions showed peaks of radioactivity in the same region of the gradient. The inability to detect the larger glucocorticoid receptor protein in hepatic or embryonic cytosols which had been incubated with [^{3}H]-corticosterone may indicate either that the binding of [^{3}H]-corticosterone to this form of the receptor like dexamethasone might be unstable, or that binding to the receptor is being masked by the presence of a large excess of serum transcortin in the cytosol preparations. The latter alternative appears to be more likely since the physiochemical properties of the [^{3}H]-corticosterone-binding components in hepatic and embryonic cytosols closely resemble those reported for serum transcortin.

DEAE-cellulose chromatography has been utilized previously to resolve glucocorticoid binding components in chick neural retina and adult rat liver [5, 32, 33], progesterone and androgen binding components in chick oviduct tissue and hamster ductus deferens tumor cells, respectively [34, 35]. For comparison therefore, labelled cytosols from either day 12 A/J or C57BL/6J mice adult livers were applied to DEAE 52-cellulose columns. Proteins were eluted with a linear gradient of Na_2HPO_4 (Fig. 6(a)). Free steroid eluted at 0.01 M Na_2HPO_4 (peak A). Peak B (0.04–0.045 M Na_2HPO_4) which followed the free hormone peak may represent nonspecifically bound hormone since the radioactivity in this peak was not displaced by unlabeled triamcinolone acetonide. Two remaining peaks (C and D) were consistently found in the hepatic cytosols and eluted at 0.11 and 0.18 M Na_2HPO_4, respectively. When hepatic cytosols were labeled in the presence of an excess of nonlabeled triamcinolone acetonide (10^{-5} M), the binding was reduced in peaks C and D by 85–90% suggesting that peaks C and D contained specific binding components for [^{3}H]-triamcinolone acetonide*. There was 2 to 4-fold more hormone bound in peaks C plus D in A/J hepatic cytosols than in the C57BL/6J cytosols.

Embryonic cytosols treated in a similar manner exhibit elution profiles on DEAE-cellulose identical to that of the adult hepatic cytosols (Fig. 6(b)). Peaks C and D were again found to represent specifically bound hormone. There was more specific binding activity in peak D in day 12 A/J embryonic cytosols (approximately 81% more) than in peak D obtained from day 12 C57BL/6J cytosols.

Peak C and D in either adult hepatic or embryonic cytosols represent specific intracellular receptor components and not serum contaminants since serum which had been labeled with [^{3}H]-triamcinolone acetonide and subjected to DEAE-cellulose chromatography failed to exhibit any binding components in the regions of the salt gradient where peaks C and D elute (Fig. 7). However, serum which had been labeled with [^{3}H]-corticosterone exhibited a defined peak of radioactivity eluting at 0.08 M Na_2HPO_4 (Fig. 7).

Nuclear uptake of [^{3}H]*-dexamethasone in whole mouse embryos*

Physiological effects produced by glucocorticoids result from the interaction of cytoplasmic glucocorticoid–receptor complexes with specific acceptor sites in the nucleus of the target cell(s) [3, 7, 8]. To determine whether there were specific nuclear receptors for dexamethasone in the embryo, whole day 12 mouse embryos were labeled at 37°C for 2 h with [^{3}H]-dexa-

* Dr. Yoshihiro Sakaue and Dr. Y. Zubairi have recently observed that the cytoplasmic glucocorticoid receptors obtained from rat liver, rat hepatoma cells (HTC) and mouse L cells display similar elution profiles on DEAE-cellulose columns as that obtained in mouse embryonic and hepatic cytosols (personal communications).

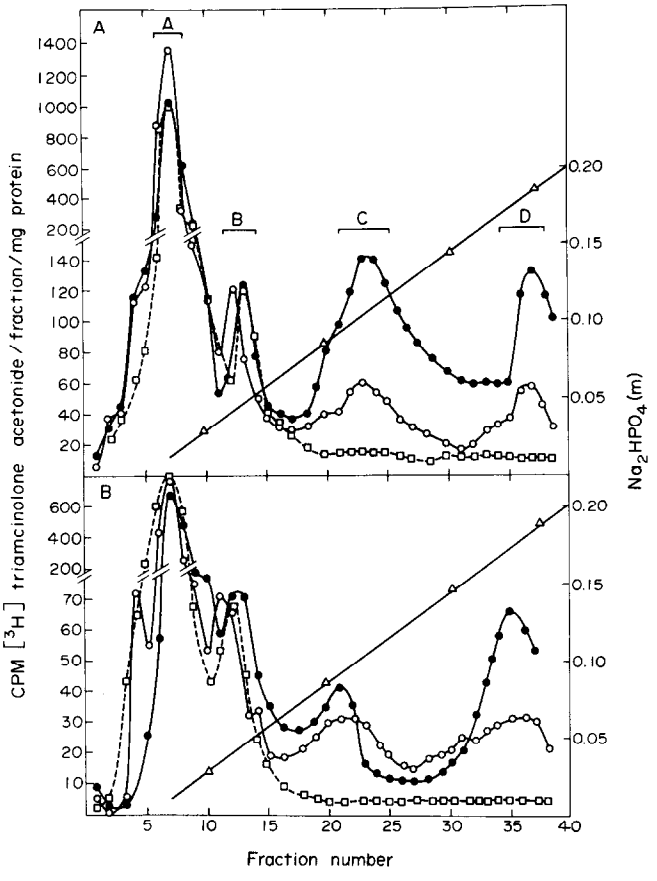

Fig. 6. DEAE-cellulose chromatographic profiles of [^{3}H]-triamcinolone acetonide cytoplasmic binding proteins. Aliquots of cytosol were incubated with 2.8×10^{-8} M [^{3}H]-triamcinolone acetonide in the absence or presence of 10^{-5} M unlabeled triamcinolone acetonide for 3 h at 0°C. Samples were then charcoal treated, and aliquots (0.4 ml) were applied to the columns, washed with 0.002 M Na_2HPO_4 buffer, and eluted with a linear gradient of Na_2HPO_4 (△—△). Aliquots (1.0 ml) of 2.5 ml fractions were either counted for radioactivity or used for protein determination. A, day 12 A/J (●, 4.25 mg cytosol protein) or C57BL/6J (○, 4.2 mg cytosol protein) hepatic cytosols incubated with [^{3}H]-triamcinolone acetonide. (□), day 12 A/J hepatic cytosol incubated with [^{3}H]-triamcinolone acetonide + 10^{-5} M unlabeled triamcinolone acetonide. B, day 12 A/J (●, 3.7 mg cytosol protein) or C57BL/6J (○, 3 mg cytosol protein) embryonic cytosols incubated with [^{3}H]-triamcinolone acetonide. (□), day 12 A/J embryonic cytosol incubated with [^{3}H]-triamcinolone acetonide + 10^{-5} M unlabeled triamcinolone acetonide. Letters are used to designate the observed peaks of radioactivity.

methasone (1×10^{-8} M). The distribution of specifically bound steroid between the cytosol and nuclear fractions was determined. There was demonstrable nuclear binding of dexamethasone in both strains (Table 4). Although the amount of steroid bound per mg DNA differed somewhat at this stage in gestation, the differences were more pronounced in the cytosol sites between the two strains, 0.045 and 0.006 pmol per mg cytosol protein for A/J and C57BL/6J, respectively.

Ontogeny of dexamethasone receptors in embryonic cytosols

The dexamethasone-binding components in the embryonic cytosols represent glucocorticoid receptor proteins as they share similar properties with receptors characterized from other glucocorticoid target tissues [3, 7, 8]. Equilibrium binding to receptor sites was determined on day 11 through 14 embryonic cytosols, using 10^{-9}–10^{-7} M [^{3}H]-dexamethasone. Table 5 represents the data obtained from Scatchard analyses (see for example Fig. 3) from several experiments. No specific binding was detected in cytosols from day 11 embryos but was first detected in cytosols obtained from day 12 embryos. The concentration of cytoplasmic receptors in A/J embryonic cytosols expressed on a per mg cytosol protein basis was highest on day 12, and declined significantly on days 13 and 14. However, expressed on a per embryo basis to normalize the increase in cell protein in the developing embryo, the receptor concentration increased from days 12 through 14 of gestation. On day 12 only, A/J embryos were found to contain about 2 fold more receptor sites expressed on either a per mg protein or per embryo basis than C57BL/6J embryos. This difference in receptor concentration on

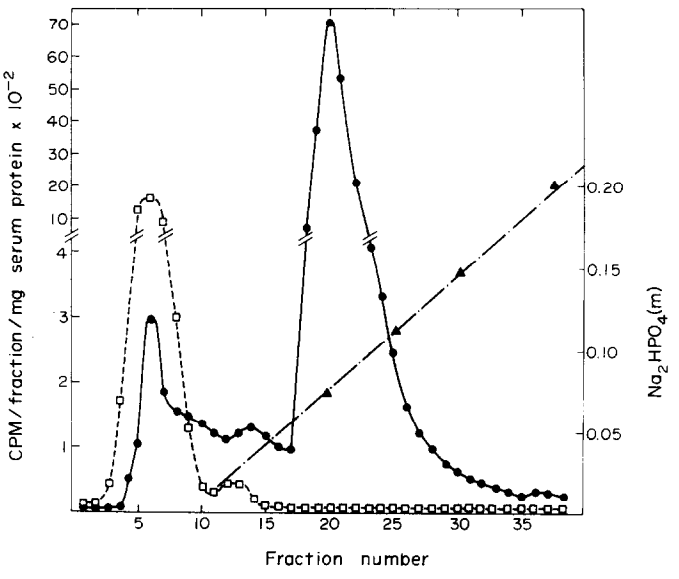

Fig. 7. DEAE-cellulose chromatographic profile of either [^{3}H]-triamcinolone acetonide or [^{3}H]-corticosterone binding components in serum. Aliquots of day 12 C57BL/6J serum were incubated with 2.8×10^{-8} M [^{3}H]-triamcinolone acetonide (□) or 1.2×10^{-8} M [^{3}H]-corticosterone (●) for 3 h at 0°C. Samples (1.78 mg serum protein) were charcoal treated and processed as described in Fig. 6. Columns were eluted with a linear gradient of Na_2HPO_4 (▲—▲).

day 12 is apparently due to the presence of the low affinity-high capacity dexamethasone sites in the day 12 A/J embryonic cytosols. Excluding the low affinity dexamethasone sites observed in day 12 and 13 A/J embryonic cytosols (K_D, $1.2–1.5 \times 10^{-7}$ M), the apparent K_D of the high affinity sites for dexamethasone does not change significantly from days 12 through 14 of gestation (range: $1.2–1.7 \times 10^{-8}$ M).

In contrast to the marked fluctuation with age in dexamethasone receptors in A/J embryonic cytosols, the concentration of cytoplasmic receptor sites expressed on a per mg cytosol protein basis in C57BL/6J embryos remains fairly constant. However, when expressed on a per embryo basis, the concentration of receptor sites increased in a manner similar to that observed in A/J embryos. Likewise, the apparent K_D of the receptor sites for dexamethasone in C57BL/6J embryonic cytosols does not significantly fluctuate or differ from the high affinity sites in the A/J embryonic cytosols.

Several types of controls were carried out to exclude trivial explanations which might account for these differences in receptor levels between the two strains in the embryonic cytosols over this developmental period. Mixing experiments between day 12 A/J and C57BL/6J embryonic cytosols failed to demonstrate any soluble activators or inhibitors in the A/J or C57BL/6J cytosols (Table 6). To determine if the low receptor site concentration observed in day 14 A/J or C57BL/6J embryonic cytosols might be due to a high concentration of endogenous steroids in the isolated cytosol preparations competing with the binding of [^{3}H]-dexamethasone for receptor sites, cytosols were preabsorbed with an activated dextran-charcoal suspension. This treatment did not alter the concentration of receptor sites (data not included).

The presence of a protease activity could not account for the decrease in receptor concentration observed in A/J embryos, because the protease inhibitor, trasylol [36] when included in the homogenization buffer and the cytosols analyzed did not alter the concentration of receptor sites in or between day 14 embryonic cytosols prepared from either A/J or C57BL/6J mice.

The average number of receptor sites in the embryonic cytosols was compared with the concentration of receptors in maternal livers over the same developmental period (Table 5). Interestingly, the receptor concentration in day 12 A/J hepatic cytosols was approximately 77% higher than that in day 12

Table 4. Subcellular distribution of specifically bound [^{3}H]-dexamethasone in whole mouse embryos *in vitro*

	Specific binding of [^{3}H]-dexamethasone	
Embryonic tissue	Cytosol pmol/mg protein	Nuclei pmol/mg DNA
A/J	0.045	0.255
C57BL/6J	0.006	0.373

Day 12 embryos were incubated for 2 h at 37°C in NCTC-109 medium with 10 nM [^{3}H]-dexamethasone in the absence or presence of an excess (10^{-5} M) nonlabeled dexamethasone. Purified nuclei were prepared and assayed for specifically bound radioactivity and DNA as described under "Experimental Procedure." Specifically bound [^{3}H]-dexamethasone in the cytosol was estimated by the charcoal assay. Values represent the mean of two experiments and are expressed as pmoles of specifically bound hormone per mg of cytosol protein or DNA, respectively.

Table 5. Ontogeny of dexamethasone binding affinity and capacity in mouse embryonic and hepatic cytosols

Gestation age (days)	Geneotype	No. of experiments	Tissue	Average K_D (nM)	Average r (pmol)	Average (pmol/embryo)
11	A/J	2	Embryonic	—	0	0
	C57BL/6J	2	Embryonic	—	0	0
	A/J	2	Hepatic	8.7	0.074	—
12	A/J	7	Embryonic	17 ± 4.3 (150 ± 33)	0.02 ± 0.001 (0.06 ± 0.008)	0.038 ± 0.02 (0.114 ± 0.015)
	C57BL/6J	5	Embryonic	25 ± 7.4	0.03 ± 0.01	0.075 ± 0.025
	A/J	3	Hepatic	16 ± 6.1	0.11 ± 0.01	—
	C57BL/6J	2	Hepatic	22	0.062	—
13	A/J	3	Embryonic	12 ± 3.4 (120 ± 47)	0.02 ± 0.001 (0.03 ± 0.008)	0.07 ± 0.004 (0.11 ± 0.028)
	C57BL/6J	3	Embryonic	20 ± 4.9	0.042 ± 0.01	0.231 ± 0.055
	A/J	2	Hepatic	26	0.10	—
	C57BL/6J	2	Hepatic	25	0.08	—
14	A/J	4	Embryonic	15 ± 3.3	0.033 ± 0.01	0.267 ± 0.081
	C57BL/6J	5	Embryonic	12 ± 2.6	0.031 ± 0.001	0.220 ± 0.007
	A/J	3	Hepatic	28 ± 6.8	0.069 ± 0.02	—
	C57BL/6J	3	Hepatic	22 ± 0.7	0.078 ± 0.02	—

Aliquots of embryonic or hepatic cytosols from A/J or C57BL/6J tissues obtained from different stages in gestation were incubated with 1–100 nM [^{3}H]-dexamethasone in the absence or presence of an excess (10^{-5} M) of unlabeled dexamethasone for 2 h at 4°C. Specific binding of [^{3}H]-dexamethasone was determined as described under "Experimental Procedure." The mean ± S.E.M. and the number of separate experiments are shown for each stage. Data was analyzed by the method of Scatchard[21] to calculate the equilibrium dissociation constant (K_D) and the concentration of receptor sites (r) expressed on a per mg of protein basis or on a per embryo basis. Values in parentheses represent the average K_D or receptor concentration for the low affinity, high capacity binding sites.

C57BL/6J hepatic cytosols. In contrast, there was no significant difference between the hepatic cytosols on days 13 and 14. The apparent K_D for dexamethasone in both A/J and C57BL/6J hepatic cytosols were approximately equivalent and comparable to the K_D of the high affinity–low capacity sites in the embryonic cytosol preparations.

DISCUSSION

These studies demonstrate the presence of glucocorticoid-binding macromolecules in the cytoplasmic fractions of mid-gestation mouse embryos. The macromolecular binding activity in both hepatic and embryonic cytosols represent specific receptors for dexamethasone. They bind the steroid with high affinity and exhibit saturability, and their physiochemical properties resemble those described for the cytoplasmic binding proteins from other adult glucocorticoid target tissues [3, 7, 8, 18, 19, 28, 29]. Although similar cytoplasmic receptors have been detected in various fetal rat and rabbit tissues just prior to parturition [1–4, 6], this is the first report to demonstrate cytoplasmic glucocorticoid receptors which can undergo nuclear translocation in early mammalian embryos at a time when most embryonic tissues are just starting organogenesis. The presence of glucocorticoid receptors in the embryo during mid-gestation may provide a role for glucocorticoids in the initial stages of differentiation of a variety of tissues.

Table 6. Specific binding of [^{3}H]-dexamethasone in mixed embryonic cytosols

	pmol/mg cytosol protein			
	Observed		Expected	
Embryonic Cytosol	Exp. I	Exp. II	Exp. I	Exp. II
A/J	0.015	0.0197	—	—
C57BL/6J	0.010	0.0118	—	—
A/J + C57BL/6J	0.023	0.032	0.025	0.0315

Aliquots (0.4 ml) of A/J or C57BL/6J day 12 embryonic cytosols were incubated either separately or together with 10 nM [^{3}H]-dexamethasone in the absence or presence of an excess (10^{-5} M) of nonlabeled dexamethasone for 2 h at 4°C. Dexamethasone which was specifically bound to cytosol macromolecules was determined by the charcoal technique.

The dexamethasone and triamcinolone-binding proteins in both hepatic and embryonic cytosols have been distinguished from the corticosterone-binding components in these cytosols and in mouse serum by several criteria. In contrast to the dexamethasone receptors in these tissues, corticosterone binding components resembled serum transcortin [27, 28] since both are not sensitive to sulfhydryl-blocking reagents or to temperature inactivation and do not bind dexamethasone or cortisone. Furthermore, [^{3}H]-corticosterone binding found in serum resembled both physically and on the basis of competition with other steroids the corticosterone binding components in the tissue cytosols. However, the dexamethasone receptors are sensitive to these treatments sharing similar

properties with other glucocorticoid receptor proteins, and exhibit specificity for both natural and synthetic glucocorticoids.

The triamcinolone acetonide–protein complex in hepatic cytosols sediments as a broad peak between 8 to 10*S* on glycerol gradients in low salt buffer. The dexamethasone-labelled complex seems unstable under these conditions as has been demonstrated for these receptors in rat muscle and mouse L cells [29, 31]. The elution profile of these triamcinolone acetonide receptor complexes from DEAE-cellulose exhibits a remarkable similarity to the profiles obtained on similar columns for the progesterone and androgen receptors [34, 35]. Whether these two peaks functionally correspond to the A and B monomer subunits of the progesterone receptor [34] remains to be determined. However, in rat liver and rat hepatoma (HTC) cells, two such peaks are found which do not seem to behave as do the progesterone receptor components [37]. In rat liver and HTC cells, the receptor component eluting at low salt concentrations (peak C) can bind to isolated nuclei, DNA and chromatin while the high-salt eluting component (peak D) lacks binding activity. However, if this second component is preincubated at 37°C then binding can be demonstrated. Presumably these components might represent activated and inactivated forms of the cytosol receptor [37]. The apparent absence of a second receptor peak (peak D) in rat liver as observed by Beato and Feigelson[32] might be due to the application of different elution buffers (NaCl vs Na_2HPO_4), the use of different synthetic glucocorticoids (dexamethasone vs triamcinolone acetonide) as probes, or the stability of the complexes once formed with either steroid to withstand preparative separation. In fact, pH and ionic strength appear to be critical factors in maintaining the intact dexamethasone–receptor complex [33]. Although no significant qualitative differences were noted between the hepatic cytosols obtained from day 12 A/J or C57BL/6J mice, the amount of specifically bound hormone associated with peaks C and D was approximately 2-fold higher in A/J hepatic cytosols than in C57BL/6J cytosols.

Comparing embryonic cytosol receptors with hepatic cytosol receptors, it was found that the similarities in elution profiles following DEAE-cellulose chromatography as well as the sedimentation patterns of the embryonic and hepatic receptors from both A/J or C57BL/6J mice were more striking than their differences. Interestingly, the amount of specifically bound [^{3}H]-triamcinolone acetonide which was associated with peak D in day 12 A/J and C57BL/6J embryonic cytosols was strain dependent with approximately 81% more binding activity in this peak in A/J cytosols than in C57BL/6J cytosols, which agrees with the data obtained from Scatchard plots of steroid binding in crude cytosol preparations. This difference appeared to be due to the presence of a low affinity-high capacity binding site for dexamethasone. Whether peak D in the A/J cytosols from the DEAE-cellulose column contains this second class of binding sites or represents an inactive form of the receptor remains to be established.

Although the physical properties of the receptors in the embryo and liver were similar, the competition experiments suggest that subtle differences may exist in the specificity of these receptors for various steroids. Cortisone and the sex steroids, progesterone, 17β-estradiol and testosterone compete differently with dexamethasone for receptor site binding in hepatic and embryonic cytosols. These steroids have been classified as anti-inducers in rat HTC cells [18, 19]. It has been suggested that such antagonists bind to the glucocorticoid receptor at a site distinct from the glucocorticord site so as to reduce the affinity of the receptor for active glucocorticoids [38].

The data on receptor site concentration should be cautiously interpreted since it has been suggested that the charcoal assay detects only free binding sites and not sites occupied by endogenous steroids [3]. For example, the failure to detect specific binding of dexamethasone in the cytosols of day 11 embryos might not be due to an absence or reduction in receptors, but rather to the occupation of binding sites by endogenous steroids. During pregnancy the maternal plasma corticosterone level in the mouse increases some 60 fold between days 11 and 15 of gestation [9, 10] while the plasma level of progesterone increases approximately 12-fold over the same period [39]. Differences in receptor concentration in embryos from two strains of mice or between embryos at different stages in gestation could merely reflect changes in free receptor sites due to fluctuations in the circulating plasma levels of endogenous steroids. This may account for the apparent absence of differences in receptor levels in day 13 or 14 A/J and C57BL/6J embryos. However, the data obtained in comparing charcoal-absorbed cytosols with untreated cytosols argues against this possibility. Moreover, receptor activity was measured in cytosols with dexamethasone and triamcinolone acetonide both of which should readily exchange with endogenous glucocorticoids for receptor binding because of the higher affinity and slower release rate of synthetic glucocorticoids [31]. Considering the limitations imposed upon these results by the sensitivity of the assay techniques; the small amount of embryonic tissue available for analysis and the physiological status of the mice, we nevertheless feel that the number of cytoplasmic receptor sites estimated under these conditions provides a reliable criterion of comparison between these two strains of mice.

This study has demonstrated that mid-gestation mouse embryos possess specific high affinity glucocorticoid receptors which physically resemble the receptors in the adult liver, but which exhibit differences in the binding of various steroids to these receptors. Ontogenically the difference in receptor levels observed on day 12 between A/J and C57BL/6J

embryos *may be* related to the enhanced sensitivity of A/J embryos to steroid-induced cleft palate since the initial stages in the formation of the secondary palate occur betwen days 11 and 12 of gestation and since maximal sensitivity to steroids coincides with this event [15]. However, due to the small amount of palatal tissue in 12 or 13 day embryos, direct analysis of this tissue was technically unfeasable. Although a quantitative difference in receptor concentrations between the two strains on a single day has been observed in whole embryos which may be related to the lethal effect of glucocorticoids on early embryogenesis, the relationship between this finding and a response induced in a specific tissue, the palate (i.e. cleft palate) may be coincidental. Certainly identification and quantification of such receptors in isolated palatal tissue as a function of strain would lend support to the hypothesis that glucocorticoids may directly influence the growth of the palatal shelves, and function in both normal and/or abnormal development of this organ depending upon the concentrations of endogenous or exogenous steroid and time during development during which the palate is exposed to the hormone. It is noteworthy in this respect that maternal plasma corticosterone levels in the mouse increase between days 11 and 14 of gestation and peak on day 15, the stage at which palatal shelf closure normally occurs [9].

Preliminary evidence indicates that on day 14 maxillary or palatal mesenchyme cells obtained from A/J or C57BL/6J embryos possess high affinity glucocorticoid receptor proteins, and that A/J mesenchyme cells have two to three fold more receptor proteins per cell than C57BL/6J mesenchyme cells [40]. This quantitative difference has been found in both cells maintained *in vitro* as primary cultures or in freshly dissociated cell suspensions. Experiments are in progress to characterize these palatal glucocorticoid receptors and to determine the biological effect of glucocorticoids on these cells *in vitro*.

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