Dexamethasone inhibits α -fetoprotein gene transcription in neonatal rat liver and isolated nuclei

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ABSTRACT

The effect of dexamethasone on rat α -fetoprotein (AFP) expression has been further examined. Quantitation of serum AFP levels from newborns treated with dexamethasone showed a dose-response relationship between the quantity of dexamethasone administered and the reduction in AFP serum level. RNA blots, utilizing cloned AFP cDNA as probe, showed a marked reduction in AFP mRNA in dexamethasone treated livers. The extent of AFP-mRNA depletion was correlated with dexamethasone dosage. The effect of dexamethasone on AFP mRNA concentration was relatively rapid; a substantial reduction occurred 12 hours after a single injection. The effect of dexamethasone appeared to be irreversible as hormone withdrawal did not cause AFP mRNA levels to rise. One putative AFP nuclear RNA precursor was identified which rapidly disappeared following dexamethasone treatment. AFP mRNA synthesis was also diminished in nuclei transcribed *in vitro*. The direct inhibitory effect of glucocorticoid hormone on AFP gene transcription was demonstrated in a reconstituted cell-free nuclear system.

INTRODUCTION

α -Fetoprotein (AFP) is an oncodevelopmental protein which is being studied as a model of the gene expression involved in development and oncogenesis. In mammals, AFP is produced by the developing liver and yolk sac. Shortly after birth, AFP serum levels begin to decline until AFP is barely detectable in the adult (1). An interesting feature of AFP expression is that AFP is produced by the adult regenerating liver and the neoplastic liver (2,3). In a number of systems it has been found that the ability to produce AFP is dependent on the amount of AFP mRNA available for translation (4-9). Thus, it is generally believed that transcriptional regulation may be a key control step in AFP gene expression.

Glucocorticoids have been shown to affect the levels of various proteins by increasing mRNA levels (10,11). Examples of the glucocorticoid suppression of gene transcription are more scarce. However, dexamethasone has been shown to inhibit the production of β -globin mRNA in Friend erythroleukemia cells

(12) and procollagen mRNA in neonatal chickens and chicken fibroblasts (13). Glucocorticoid treatment of mouse pituitary tumor cells causes a reduction in the amount of translatable mRNA coding for ACTH (14). In previous reports, we have shown that the administration of dexamethasone to newborn rats and mice can significantly depress the serum levels of AFP following a short course of treatment (15-17). Nucleic acid hybridization experiments further indicate that dexamethasone reduces the quantity of AFP mRNA available for translation (16-18).

We now report some additional observations on the effect of dexamethasone on AFP gene expression. Utilizing the RNA blotting procedure, we show that dexamethasone acts in a dose dependent manner to lower levels of rat liver AFP mRNA. The hormone effect appears to be irreversible since withdrawal does not cause an increase in AFP mRNA. In addition to mature AFP message, dexamethasone acts rapidly to decrease the amount of a putative AFP mRNA precursor. In vitro reconstituted cell-free transcription experiments in isolated nuclei indicate dexamethasone inhibits AFP mRNA transcription in vitro. The results presented in this paper strongly suggest that dexamethasone inhibits the transcription of AFP mRNA.

MATERIALS AND METHODS

Animals and Treatments.

Three day old Sprague-Dawley rats were received from Charles River Breeding Laboratories (Wilmington, MA). Except where indicated, 2 µg/g body weight of dexamethasone (Elkins-Sinn, Inc.) were given on the next day. The rats received a single intraperitoneal injection of dexamethasone in isotonic saline once daily. A typical injection volume was 50 microliters which was well tolerated. Control animals were given isotonic saline. For RNA isolations and serum AFP determinations, an average of six or more rats in each group were sacrificed by decapitation. Blood and liver samples were pooled.

Buffalo rats weighing 150-200 gm were adrenalectomized under ether anesthetic. After 2 weeks recovery from surgery, 2×10^7 Morris hepatoma 7777 cells were transplanted into their thigh muscle. Animals were then divided into two groups. One group received dexamethasone intraperitoneally at 2 µg/gm body weight daily for 10 days and the other received only saline.

Isolation of RNA.

Baby rat livers were excised and immediately immersed in liquid nitrogen. The pooled tissue was rapidly homogenized by a Polytron in 10 volumes of 7.5 M guanidine hydrochloride, pH 7.0/0.1 M mercaptoethanol/0.5% (w/v) sarkosyl, and

precipitated overnight by the addition of 2 volumes of ice cold 95% (v/v) ethanol as described by Chirgwin *et al.* (19). The RNA was collected in a SS 34 rotor (9,000 x g) at -20°C, resuspended in guanidine hydrochloride, then precipitated for three to four hours with 95% (v/v) ethanol. The pellet was again resuspended in guanidine hydrochloride and small volumes were layered over 5.7 M cesium chloride/0.1 M ethylene diaminetetraacetic acid (EDTA), pH 7.0. In some instances homogenized samples were placed directly over 5.7 M cesium chloride when volume permitted. The samples were centrifuged at 36,000 rpm in a Beckman SW 50.1 rotor for 16 hours at 20°C. Pellets were dissolved in 0.01 M Tris, pH 8.0/0.001 M EDTA then extracted twice with phenol/chloroform/isoamyl alcohol (49:49:2) followed by two additional extractions with chloroform/isoamyl alcohol (24:1). The total RNA in the final supernatant was recovered by precipitation with 95% (v/v) ethanol.

Isolation of Nuclear RNA.

Nuclei were isolated by the citric acid procedure described by Busch (20). The nuclei were then lysed in 7.5 M guanidine-HCl/0.2% (w/v) sodium dodecyl sulfate (SDS)/2X SSC (0.30 M NaCl, 0.03 M sodium citrate) and layered over a cushion of 5.7 M cesium chloride. The samples were spun at 36,000 rpm in a SW 50.1 rotor for 16 hours and the RNA pellets were collected and further purified as described above.

Determination of AFP Concentration.

The concentration of serum AFP was determined by a modification of the rocket immunoelectrophoresis technique of Laurell (21) as described in our previous paper (22). Rabbit antisera anti-rat AFP was diluted 100 fold in 1% (w/v) agarose/10 mM sodium barbital, pH 8.6. AFP serum concentrations were derived by comparison to purified AFP standards.

RNA Blot Analysis.

The AFP cDNA clone (pRAF87) and albumin cDNA clone (pRSA57) were originally described by Sargent *et al.* (23). Plasmids were labeled to high specific activity ($1-2 \times 10^8$ cpm/ μ g) with [32 P]dCTP (Amersham, ~3000 Ci/mmol) according to the procedure of Weinstock *et al.* (24). RNA was dissolved in 50% (v/v) formamide/10 mM sodium acetate/10 mM morpholinisulfonic acid, pH 7.0/6% (v/v) formaldehyde and heated to 60°C for 15 minutes. The samples were electrophoresed through 1.5% (w/v) horizontal agarose gels containing 6% (v/v) formaldehyde at 40 mAmps for 12 hours, then blotted to nitrocellulose paper according to Thomas (25). Prehybridization, hybridization and washing procedures were identical to those of Thomas (25). Generally $1-2 \times 10^6$ cpm/ml of labeled cDNA probe was used.

Dot Hybridization.

Total RNA samples isolated by the guanidine-HCl procedure were dotted onto nitrocellulose sheets in 10X SSC using a Schleicher and Schuell Filtration Manifold (SRC-96). The nitrocellulose filters were treated identically to the RNA blots during prehybridization and hybridization. Following hybridization, the filters were autoradiographed and the dots were cut out and counted in Filtron-X (National Diagnostics).

Isolation of Nuclei and In Vitro Transcription.

Individual livers or spleens were homogenized gently in a loose Teflon homogenizer in 5 volumes of 2.2 M sucrose/5 mM MgCl₂/20 mM Tris-HCl, pH 8.0/10 mM dithiothreitol (DTT)/0.2% (v/v) Triton X-100. The homogenate was then spun for 5 minutes in a Beckman microfuge. The nuclear pellet was washed two times in 25% (v/v) glycerol/5 mM MgCl₂/10 mM dithiothreitol/20 mM Tris-HCl, pH 8.0. The nuclei thus obtained were free of any microscopically visible cytoplasmic contamination and could be stored in the washing buffer for up to one week at -70°C without any loss of transcriptional activity.

In vitro nuclear transcription was performed with addition of HeLa whole cell extract (HWCE) as described elsewhere (26). The HWCE was prepared according to the method of Manley *et al.*, (27). In vitro transcription reaction mixture contained: 20 mM Hepes, pH 7.9, 5 mM MgCl₂, 150 mM KCl, 0.1 mM EDTA, 4 mM DTT, 25% (v/v) glycerol/1 mM ATP/0.5 mM GTP/0.5 mM CTP/100 μ Ci [α -³²P]UTP (600 Ci/mM), 10⁸ nuclei/ml, and 100 μ g HWCE in 100 μ l reaction mixture. In some experiments, 100 μ g/ml of partially purified glucocorticoid receptor complexes (hepatoma cytosol) was added. The reaction mixture was incubated at 25°C for 30 minutes. The mixture was then adjusted to 0.5% (w/v) SDS, 5 mM EDTA, and 20 μ g/ml of proteinase K was added at 37°C and allowed to incubate for 15 minutes. The nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (49:49:2), adjusted to 0.2 M NaCl, and precipitated overnight in two volumes of 95% (v/v) ethanol at -20°C. The pellet was collected, lyophilized, and dissolved in DNase buffer (0.1 M NaCl/5 mM MgCl₂/10 mM Tris-HCl, pH 7.5) and 20 μ g/ml of affinity column purified DNase was added for 1 hour at 37°C. Another Proteinase K digestion was followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. The [³²P]RNA obtained was hybridized to nitrocellulose filters to which 5 μ g of EcoRI restricted plasmid pBR322 or cloned AFP cDNA (pRAF87) and cloned albumin cDNA (pRSA57) was bound.

The immobilization of DNA onto nitrocellulose was the method of Landes *et al.*, (28). The filters were prehybridized in 5x Denhardt's solution (0.1%

bovine serum albumin, 0.1% Ficoll and 0.1% polyvinyl pyrrolidone)/5x SSC/50% (v/v) formamide/50 mM sodium phosphate, pH 6.5/250 µg/ml sonicated and denatured salmon sperm testis DNA in a sealed plastic bag overnight at 42°C. The filter was then hybridized with 1x Denhardt's solution/5x SSC/200 µg/ml *E. coli* tRNA/50% (v/v) formamide and radiolabeled RNA transcript. The hybridization was carried out at 42°C for 20 hours. Following hybridization the filters were washed extensively in 0.1 x SSC for 30 minutes at room temperature and then treated with 20 µg/ml of heat treated RNase A. The filters were then washed at 50°C in 0.1 X SSC, 0.2% SDS until the radioactivity in the washing buffer approached background (usually 1-2 hours). The filters were dissolved in Filtron-X (National Diagnostics) and counted in a Beckman LS-3133-P scintillation counter with an open window setting.

Hepatoma Cytosol Preparation.

Morris hepatoma 7777 was homogenized in 3 volumes of 1 mM EDTA/20 mM sodium phosphate/10% (v/v) glycerol/50 mM KCl, pH 7.0. The homogenate was centrifuged for 10 minutes at 12,100 x g. The supernatant was collected and centrifuged again at 10,500 x g for 90 minutes. After centrifugation, the clear cytosol was carefully removed. Four and three tenths milliliters saturated $(\text{NH}_4)_2\text{SO}_4$ was added to 10 ml of cytosol and stirred on ice for 2 hrs. After spinning at 17,300 x g for 15 min, the pellets were dissolved in small volume and then dialyzed against 1000 volume of 20 mM Hepes, pH 7.9/150 mM KCl/5 mM MgCl_2 /0.1 mM EDTA/4 mM DTT/20% (v/v) glycerol for 20 hrs with 3 changes at 4°C. The 30% $(\text{NH}_4)_2\text{SO}_4$ precipitated and dialyzed hepatoma cytosol proteins were used as a source of glucocorticoid hormone-receptor complexes.

Glucocorticoid Receptor Assay.

The measurement of steroid occupied and unoccupied receptors was performed using the exchange assay described by Banerji and Kalimi (29). Briefly, liver or 7777 tumor cytosols were prepared by homogenization in 3 volumes(w/v) of 10 mM Tris pH 7.4, 10 mM DTT, and 0.25 M sucrose. Following a low speed spin (3000 x g) to pellet nuclei, the supernatant was centrifuged at 140,000 xg for 1 hr at 0°C to obtain cytosol. Glycerol was added to 10% (v/v) and NaCl to 0.1 M to allowed prolonged storage(30). Aliquots of cytosol (200 µl) were incubated with 30 nM of [^3H] dexamethasone (New England Nuclear, Boston, MA), at 0°C in the presence or absence of nonlabeled dexamethasone (10^{-5} M). Specific binding was determined by the addition of dextran-charcoal to remove excess free steroid. The removal of endogenous steroid was accomplished by a 90 minute preincubation of the cytosol in the presence of 0.5 mM p-hydroxymercuribenzoate (pHMB) at 0°C. Following this incubation,

free steroid was removed by dextran-charcoal absorption, and receptor binding regenerated by the addition of DTT to 50 mM. Specific [^3H] dexamethasone binding was then measured as above. Protein determination was performed as described by Lowry (31).

RESULTS

Our initial observations on the suppressive action of dexamethasone on AFP expression were made using dosages of dexamethasone of 2 micrograms per gram of body weight (17). We were interested in the effect smaller doses of dexamethasone would have on the suppression of AFP synthesis and if there was a relationship between the dosage of dexamethasone administered and the reduction in AFP level. This experiment has not been done before. We treated 4-day old rats with increasing concentrations of dexamethasone (up to 2 $\mu\text{g}/\text{gm}$) for a period of 4 days. These animals were then sacrificed and their sera analyzed for AFP concentration by rocket immunoelectrophoresis. As shown in Figure 1A, as little as 0.05 $\mu\text{g}/\text{g}$ of dexamethasone was effective in reducing

Figure 1: Relationship of the decline in AFP serum level and liver AFP mRNA content with dexamethasone dosage. Three day old rats received 4 injections of dexamethasone in saline once daily and were killed on day 8. (A) Serum AFP was assayed by rocket immunoelectrophoresis and each sample represents 5 to 8 rats. (B) Total RNA was isolated from pooled livers. Northern blot hybridization was performed as described in Methods. These results were then quantitated by densitometric scanning. Each RNA sample contains 10 μg of total RNA.

the AFP serum level from approximately 2.75 mg/ml to 1.7 mg/ml. The reduction of serum AFP appeared to reach maximal effect at concentrations as low as 0.5 $\mu\text{g/gm}$, showing little increase in inhibition of AFP serum concentrations at dexamethasone concentrations up to 2 $\mu\text{g/gm}$.

The autoradiograph of a typical RNA blot in Figure 1B shows the relative AFP mRNA abundance among various amounts of hormone treated samples. The 2100 nucleotide AFP mRNA band probed with cloned AFP cDNA is clearly reduced in the dexamethasone treated samples. In a fashion similar to the reduction of AFP serum levels, the AFP mRNA concentration decreased with increasing hormone dosage. An appreciable reduction in AFP mRNA was observed in the sample treated with 0.05 $\mu\text{g/g/day}$.

A time course study was undertaken to determine the rate at which dexamethasone causes a reduction in AFP mRNA sequences. The reduction of AFP mRNA by dexamethasone can be detected by 12 hours after hormone treatment (Figure 2). There is little change of AFP mRNA content in liver during the first 6 hours of treatment. However, the concentration of tyrosine aminotransferase (TAT) mRNA increased nearly three-fold during this period of time. This reduction of AFP mRNA sequences within 24 hrs of dexamethasone administration agrees with previously reported data (17,18). The estimated time for a 50%

Figure 2: Time course of dexamethasone effect on AFP mRNA content in 3 day old rat livers. Total liver RNAs were isolated from rats killed at various times after a single (2 $\mu\text{g/g}$) injection of dexamethasone. The level of AFP mRNA (●), and TAT mRNA (○) were determined by dot hybridization analysis. Dots were cut from the nitrocellulose filter and counted as described in Methods. Values are expressed as a percentage of control points $\pm$ standard error. Albumin mRNA hybridization (not shown) was performed in a separate experiment to insure equal amounts of RNA were applied at each time point.

Figure 3: Effect of dexamethasone withdrawal on AFP serum level and liver AFP mRNA. Four day old rats were divided into 3 groups and treated with saline (●), dexamethasone (1 μ g/g/day) (▲), or with 2 injections of dexamethasone (1 μ g/g/day) then withdrawn (△). Groups of 5 or more rats were killed at specified intervals and serum and total liver RNA isolated (A) Serum AFP was assayed by rocket immunoelectrophoresis. (B) AFP mRNA concentration in RNA samples were determined by dot hybridization and quantitated by scintillation counting. Arrow indicates point of dexamethasone withdrawal.

reduction of AFP mRNA by dexamethasone administration agrees with values obtained for the measured half-life of AFP mRNA (32; J.R. Cook and J-F. Chiu, manuscript in preparation). This agreement suggests that dexamethasone acts directly at the transcriptional level.

The effect of dexamethasone on AFP gene expression appears to be irreversible. As shown in Figure 3, when 4 day old rats were treated for 2 days with dexamethasone (1 μ g/g), and then the hormone withdrawn, neither AFP serum or mRNA levels returned to those of controls. In fact AFP serum and mRNA levels continued to decrease, but not to the same extent as seen in continuously treated animals. This may result from the mechanism involved in the intrinsic decline in serum AFP levels after birth. During this period normal differentiation of AFP-producing to non-AFP-producing hepatocytes occurs. Dexamethasone may be inducing a premature differentiation of AFP-producing hepatocytes resulting in what appears to be an irreversible inhibition of AFP mRNA transcription.

We have observed high molecular weight bands in preparations of total RNA that hybridize to AFP cDNA. Particularly intense hybridization occurred with one band at about 4800 bp. Although not conclusive, this band is presumably a precursor to mature AFP mRNA. The fact that this band could never be observed

Figure 4: Northern blot of nuclear RNA from control and dexamethasone treated samples. 1) control, 2) RNA isolated 6 hours after a single injection (2 µg/g) of dexamethasone, 3) RNA isolated 16 hours after hormone treatment. Fifteen µg of each RNA sample was applied to the gel. Major precursor band is at about 4800. Exposure was for one week with an intensifying screen. Standards were lambda Hind III fragments and expressed in kilobases.

in RNA from hormone treated animals prompted us to investigate the effect of dexamethasone on transcription of the putative precursor. As shown in Figure 4, the 4800 base pair band hybridized quite strongly in nuclear RNA isolated from the livers of four day old rats. However in nuclear RNA isolated six hours following a single (2 µg/g) injection of dexamethasone the putative precursor band disappeared, although the concentration of mature AFP mRNA was essentially the same as control. At sixteen hours no high molecular weight band was visible and the concentration of mature AFP mRNA was reduced relative to controls.

Recently we have established an *in vitro* nuclear transcription system with HeLa whole cell extract (HWCE) (26). We added HWCE in a rat liver nuclei transcription system to study the faithful transcription of α -fetoprotein and albumin genes. We found that HWCE selectively and faithfully enhances the reinitiation of transcriptionally active genes in a rat liver nuclear transcription system (26). This *in vitro* transcription system was employed to assess the effect of dexamethasone on AFP mRNA transcription. Nuclei were isolated from liver of 8-day old rats which had been treated with either dexamethasone or saline for four days. Nascent RNA chains were pulse labeled with ^{32}P -UTP for 30 minutes and isolated for hybridization analysis. RNA

Figure 5: AFP mRNA synthesis in isolated liver nuclei of 8 day old or adult rats treated with saline (control) or dexamethasone for four days. Nuclei were isolated from 8 day old rats liver (●), liver of 8 day old rats treated with 0.1 $\mu\text{g/g}$ body weight of dexamethasone (▲), liver of 8 day old rats treated with 1 $\mu\text{g/g}$ body weight of dexamethasone (△), or adult rat liver (○) and spleen (○). The detailed method for *in vitro* transcription experiments is described in Methods.

labeled *in vitro* was hybridized to excess (5 μg) cloned AFP cDNA bound to nitrocellulose disks. Another filter disk containing an equivalent amount of pBR322 plasmid DNA was used as an background control and all radioactivity bound to this filter was subtracted from the radioactivity bound to the cloned AFP cDNA filter to obtain the specific hybridization.

As presented in Figure 5 nuclei isolated from adult rat liver and 8 day old rat spleen did not detectably transcribe AFP mRNA. AFP mRNA transcribed in nuclei from 8 day control liver constituted approximately 0.03% of the total RNA input. Relative to control, nuclei isolated from dexamethasone treated rats (0.1 $\mu\text{g/g/day}$) showed a 5-6 fold lower transcriptional activity for AFP mRNA synthesis. A ten times higher dosage (1 $\mu\text{g/g}$ body weight/day) of dexamethasone reduced hybridizable AFP mRNA to 0.001% of the total RNA input.

By using the same *in vitro* transcriptional system, we have also demonstrated the suppression of dexamethasone on AFP gene transcription *in vitro*. Partially purified steroid hormone-receptor complexes were obtained from cytosol of dexamethasone treated adrenalectomized Morris hepatoma 7777. The cytosol fraction of untreated adrenalectomized Morris hepatoma 7777 was used as control. The reason for not using adult liver cytosol for the source of hormone-receptor complexes was that adult liver cytosol may contain an AFP gene repressor which suppresses AFP gene transcription in adult rat livers (33). The AFP gene is actively transcribed in Morris hepatoma 7777 (5).

Figure 6: Hybridization of [32 P]labeled RNA to nitrocellulose filters which were immobilized with 5 μ g of either, cloned AFP cDNA (pRAF87), or cloned albumin cDNA (pRSA57) respectively. (A) [32 P]RNAs were transcribed in *in vitro* nuclear transcription conditions without adding Morris hepatoma 7777 partially purified cytosol. (B) [32 P]RNAs were transcribed in transcription conditions with untreated Morris hepatoma 7777 cytosol. (C) [32 P]RNAs were transcribed in *in vitro* nuclear transcription conditions with cytosols isolated from Morris hepatoma 7777 which have been treated with 2 μ g/g body weight of dexamethasone for 10 days. In each case values have been corrected by the subtraction of the appropriate pBR322 control hybridization.

Nuclei were isolated from 3 day old rat liver and used an *in vitro* transcription assay after the addition of partially purified cytosol fraction of dexamethasone-treated Morris hepatoma 7777. Matched control samples were assayed in parallel with cytosol isolated from Morris hepatoma 7777 growing in an adrenalectomized rat. As described earlier, [32 P]RNA synthesized in nuclei was extracted, purified and hybridized to nitrocellulose filters containing either plasmid DNA pBR322 or cloned AFP cDNA pRAF87, or cloned albumin cDNA pRSA57. The [32 P]RNA which specifically hybridized to nitrocellulose filters containing AFP or albumin cloned cDNA represented AFP or albumin mRNA sequences transcribed *in vitro* respectively. The results are shown in Figure 6. The data clearly indicate that cytosol fraction isolated from Morris hepatoma 7777 in dexamethasone treated rats suppressed AFP gene transcription. The cytosol of Morris hepatoma 7777 which has not been treated with dexamethasone did not affect the transcription. There is a slight enhancement of dexamethasone-treated Morris hepatoma 7777 cytosol on albumin gene transcription.

Table 1. [^3H] Dexamethasone Binding of Liver and 7777 Cytosols

Sample	No Pretreatment	pHMB Pretreatment
Control rats		
Liver	2598 $\pm$ 62	2125 $\pm$ 310
7777	5947 $\pm$ 15	5480 $\pm$ 187
Treated rats		
Liver	50 $\pm$ 14	374 $\pm$ 20
7777	51 $\pm$ 23	438 $\pm$ 30

Adrenalectomized rats were injected i.m. with Morris₃ hepatoma 7777 tumor cells as described in Methods. When tumors reached $\sim 1 \text{ cm}^3$ mass, animals were injected i.p. daily for 10 days with saline (control) or 2 $\mu\text{g/g}$ dexamethasone (treated). Values are expressed as counts/min/mg protein minus appropriate controls $\pm$ standard error.

The in vitro suppression of AFP gene transcription is in agreement with in vivo results obtained in our laboratory (34). To correlate this suppression to dexamethasone treatment, glucocorticoid receptor assays were performed on cytosols prepared from liver and 7777 tumor tissues of control and dexamethasone treated (2 $\mu\text{g/g/day}$) animals. The suppression of AFP transcription is based on the existence of steroid occupied receptors in the 7777 cytosol of dexamethasone treated animals and their absence in the 7777 cytosol of control animals. Steroid binding assays utilizing [^3H] dexamethasone only measure unoccupied receptors. For this reason an exchange assay must be utilized which removes endogenously bound steroid from receptors in order to measure the total receptor number. We have employed an exchange assay described by Banerji and Kalimi (29) for this purpose. In this procedure endogenous steroid is removed by pretreatment of the cytosol with p-hydroxymercuribenzoate (pHMB), followed by absorption of steroid with dextran-charcoal, and finally regeneration of receptor binding studies by the addition of DTT.

Table 1 shows the results of [^3H] dexamethasone binding in cytosols of liver and 7777 tumor tissue of dexamethasone treated and control animals. The [^3H] dexamethasone binding of these cytosols was measured in the presence (for measuring total receptor) or absence (for measuring unoccupied receptor) of pHMB. In control cytosols, all receptors exist in an unoccupied state as

endogenous steroid has been eliminated by adrenalectomy. Treatment with pHMB results in an 82% regeneration of [^3H] dexamethasone binding in liver and a 92% regeneration in 7777. The incomplete regeneration of [^3H] dexamethasone results from incomplete removal of pHMB from the receptor(29). The cytosols prepared from dexamethasone treated animals show a dramatic decrease in total [^3H] dexamethasone binding. Two factors could contribute to this finding, receptor occupancy by steroid, as well as down regulation of receptor due to prolonged steroid exposure (35,36). The preincubation of these cytosols with pHMB results in a greater than 7-fold increase in [^3H] dexamethasone binding. The total binding however is still less than that seen in pHMB treated controls. Control cytosols incubated with nonlabeled dexamethasone followed by pHMB treatment show complete regeneration of [^3H] dexamethasone binding (data not shown). This indicates that pHMB treatment effectively removes endogenous steroid and also suggests that down regulation of receptor occurs. These results confirm the presence of steroid occupied receptor in the 7777 cytosol of dexamethasone treated rats and the absence of occupied receptor in untreated rats.

DISCUSSION

Much research on the mechanisms underlying glucocorticoid hormone action is presently focused on the regulation of transcription. In a number of model systems glucocorticoids have been shown to increase the levels of specific proteins. Tyrosine aminotransferase, $\alpha_2\text{U}$ -globulin, and growth hormone are examples (37-39). In each case glucocorticoid treatment causes an increased level of the mRNA.

Thymus cells are known to be extraordinarily sensitive to glucocorticoids, indeed glucocorticoid treatment usually causes cell death (40). The negative effect of glucocorticoid on skin has been recognized since the introduction of glucocorticoid therapy (41). The immediate cause of skin atrophy appears to be an inhibition of collagen biosynthesis which is probably the result of a decreased level of procollagen mRNA available for translation (42). Glucocorticoids are able to inhibit the synthesis of corticotropin mRNA *in vivo* and *in vitro*. Nakanishi *et al.*, (38) observed that the level of ACTH mRNA activity in rat pituitary is increased dramatically by adrenalectomy and, conversely, decreased by glucocorticoids. In mouse pituitary tumor cells, glucocorticoids decrease by 50-60% the levels of ACTH, β -endorphin and the amino-terminal fragment of the large ACTH precursor (14).

In previous publications (17) we have shown that dexamethasone is a

potent supressor of AFP expression in vivb. The observation that dexamethasone strikingly reduced rat AFP serum levels prompted us to further investigate the phenomenon at the molecular level. Molecular hybridization studies have proved that AFP mRNA is markedly reduced in livers of glucocorticoid treated rats. The reduction in AFP is dose related both in terms of the serum level of AFP and the cellular concentration of AFP mRNA. Newly synthesized RNA transcribed in vitro in isolated nuclei was shown to be depleted in AFP coding RNA sequences. A dose of 0.1 µg/g of dexamethasone decreased AFP mRNA at least 6-fold while a ten times higher dose reduced AFP mRNA transcription about 30 times.

Total cellular RNA was reduced in AFP mRNA after 12 hours as evidenced by RNA hybridization analysis (Figure 2). The disappearance of the putative AFP mRNA precursor following hormone administration and the observation that the time course of dexamethasone inhibition parallels AFP mRNA half-life indicates that dexamethasone acts directly to inhibit AFP transcription (Figure 4). The observation that the level of AFP mRNA was affected more than serum AFP protein level by dexamethasone (Figure 1), also suggests that dexamethasone suppresses AFP gene expression at the transcriptional steps. It is curious that only one "precursor" was observed considering that processing of the primary AFP transcript would produce numerous intermediates. Possibly these intermediates are very close in molecular weight or are rather short-lived.

Although liver is without question a target organ for glucocorticoids we cannot prove a direct cause and effect relationship in vivo. Obviously numerous organ systems and target sites are involved and the possibility that the ultimate effect of dexamethasone is mediated by another factor is real. Data on dexamethasone treatment of cultured cells is contradictory. Fetal rat hepatocytes treated with hydrocortisone exhibit prolonged expression of AFP during in vitro differentiation (43). Results from our (44) and other (45) laboratories have demonstrated, however, that dexamethasone treatment of cultured Morris hepatoma 7777 cells causes decreased production of AFP.

Early attempts to directly investigate the effects of steroid hormone-receptor complexes on RNA synthesis in reconstituted cell-free systems demonstrated stimulation of transcriptional capacity in target tissue nuclei by the addition of crude receptor preparations. For instance, Raynaud-Jammet and Baulieu (46) showed a 3-fold stimulation of [³H]RNA synthesis in isolated calf uterus nuclei following the addition of calf uterus cytosol and 1 nM estradiol. Recently Taylor and Smith (47,48) have also demonstrated that the capacity of chick oviduct nuclei to synthesize [³H]RNA in vitro was enhanced

by the addition of pure nuclear estrogen-receptor complexes. Using a similar reconstituted cell-free transcriptional system, the experiments reported here have directly addressed the inhibitory effect of glucocorticoids on AFP gene transcription.

We have shown that reconstitution with partially purified hepatoma cytosol (containing hormone-receptor complexes) decreases AFP coding mRNA synthesis in isolated 3-day old rat liver nuclei in a specific manner (Figure 6). The observation that in vitro AFP mRNA synthesis was suppressed by the addition of partially purified hepatoma cytosol cannot be ascribed to contamination of the cytosol fraction by nuclease, if one considers the fact that albumin mRNA synthesis was enhanced slightly by the addition of cytosol. We postulate that the glucocorticoid-receptor may inhibit AFP mRNA synthesis by decreasing the affinity of RNA polymerase for AFP DNA template and/or by decreasing its rate of elongation. The detailed mechanism is not known.

In the same experiment (figure 6) we also demonstrate that dexamethasone slightly induces albumin mRNA synthesis in the in vitro nuclei transcription system. This inverse relationship between albumin and AFP synthesis has been extensively documented in vitro and in vivo in the literature (1,49-51). However, recently Chiu et al. (17) and Belanger et al. (52) demonstrated that there is no direct reciprocal relationship between AFP and albumin gene expression. Both authors have shown that dexamethasone suppress AFP gene expression in newborn-rat liver, but there is no significant effect of dexamethasone on albumin gene expression. The opposing effects of dexamethasone on albumin and AFP gene expression is very interesting in view of the high degree of homology between gene and protein structures of albumin and AFP (53,54). How these regulatory mechanisms evolved is curious, since both albumin and AFP seem to share a common ancestry (53,54).

Our observation that AFP is irreversibly suppressed by dexamethasone suggests that the hormone acts by accelerating liver cell differentiation. Other examples are known in which glucocorticoids accelerate normal developmental processes. For example, cortisol withdrawal of cultured embryonic chick retinas causes glutamine synthetase activity to plateau, but the enzyme does not return to the preinduction level (55). We must also state that this finding of irreversible AFP transcription is not in agreement with previously reported data (52).

The question remains as to the possible physiological significance of the glucocorticoid effect on AFP synthesis. In the rat, AFP levels are very high (~5 mg/ml) just prior to parturition and decline after birth to a very

low adult level (~20 ng/ml). Interestingly, serum glucocorticoid levels in a number of mammalian species increase during late gestation and throughout the early postnatal period (56,57). Corticosterone, the major glucocorticoid in rodents, has been shown to rise significantly on day 14 until peaking on day 24 (49).

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