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Rapid Suppression of α_1-Fetoprotein Gene Transcription by Dexamethasone in Developing Rat Liver†

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ABSTRACT: The administration of glucocorticosteroid hormones to newborn rats interrupts selectively (and reversibly, if the hormone is withdrawn) the hepatic production of α_1-fetoprotein (AFP). This results from a decreased concentration of AFP mRNA in the liver [Bélanger, L., Frain, M., Baril, P., Gingras, M. C., Bartkowiak, J., & Sala-Trepat, J. M. (1981) *Biochemistry 20*, 6665]. We have delineated further the mechanism and time course of this hormonal action in 4-day-old rats treated with dexamethasone (DEX). DNA from a recombinant plasmid containing a 578-bp insert of rat AFP cDNA was used to develop a cell-free nuclear run-off system and directly assess AFP gene transcription activity. Five minutes after DEX injection, AFP gene transcription activity is unchanged, but after 30 min, it drops to 25% that of the control; this correlates with the time required for translocation of DEX receptors to the nucleus. Dose–response curves also show that the degree of AFP gene suppression is closely correlated with the amount of DEX receptor translocated to the nucleus. The nuclear concentration of AFP mRNA, monitored by dot–blot hybridization, decreases to undetectable levels within 48 h, whereas that of albumin mRNA increases slightly, which indicates the selectivity of DEX action. These results show that DEX suppresses AFP gene expression at the transcriptional level and suggest a direct negative action of DEX–receptor complexes on the AFP chromatin transcription unit.

Studies on the regulation of eukaryotic gene expression have shown that steroid hormones can modulate the transcription of specific genes. Many model systems currently used to decipher the mechanisms of this action are based on positive modulations of gene functions. Only a few systems negatively modulated by steroids are exploitable at the molecular level. Such systems may provide valuable conceptual and experimental counterparts to the positive models.

One negatively regulated steroid-responsive gene system is the suppression of α_1-fetoprotein (AFP)[1] expression by glucocorticoid hormones in the developing liver. AFP is a fetal albumin, suppressed in adult liver but reexpressed in hepatocarcinomas. The administration of dexamethasone to developing animals suppresses prematurely the hepatic production of AFP (Bélanger et al., 1975, 1978, 1983). This action is nontoxic, selective, and reversible if hormone is withdrawn. We have previously reported (Bélanger et al., 1981) that following DEX injection to newborn rats, the levels of AFP mRNA decrease exponentially in the liver, with kinetics extrapolating to near time of injection; this suggested a rapid transcriptional effect on the AFP gene. Here, we confirm and extend this interpretation by showing, in a cell-free nuclear transcription system, that DEX suppresses AFP gene transcription within 30 min and that this action is time- and dose-related to the accumulation of DEX receptors in liver nuclei.

Experimental Procedures

Animals and Hormone Treatment. Four-day-old Sprague-Dawley rats were used. Half of each litter received intraperitoneal injections of dexamethasone (DEX) (Decadron; Merck Sharp & Dohme) at 2 μg/g twice a day in 50 μL of

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[1] Abbreviations: AFP, α_1-fetoprotein; DEX, dexamethasone; BSA, bovine serum albumin; SSC buffer, 0.15 M NaCl–0.015 M sodium citrate buffer; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TCA, trichloroacetic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate; APT-cellulose, (aminophenyl thioether)-cellulose; DPT-cellulose, (diazophenyl thioether)-cellulose.

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saline; the other half received saline.

Chemicals. [^{3}H]UTP (40 Ci/mmol), [α-^{32}P]dCTP (600 Ci/mmol), and [α-^{32}P]UTP (550 Ci/mmol) were from New England Nuclear. APT-cellulose was from Collaborative Research, dextran sulfate was from Pharmacia, and nucleotides, salmon testis DNA, and α-amanitin were from Sigma. *Escherichia coli* RNA polymerase was from Miles, yeast tRNA and pBR322 were from Bethesda Research Laboratories, and placental ribonuclease inhibitor, nucleoside-5′-diphosphate kinase, ribonuclease T_1, and creatine phosphate were from Boehringer Mannheim. Formamide (Bethesda Research Laboratories) and glyoxal (Fisher) were deionized with Bio-Rad AG50 1-X8 resin. DNase I (Worthington Biochemicals) was made RNase-free by following the technique of Brison & Chambon (1976).

Purification of Rat AFP and Albumin mRNAs. AFP mRNA was purified from yolk sacs and albumin mRNA from adult liver by double-antibody precipitation of polysomes, isolation of the immunoprecipitated 18S RNA on sucrose gradients, and isolation of the poly(A)-containing mRNA by oligo(dT)–cellulose chromatography, as previously described (Bélanger et al., 1979).

Cloning of AFP cDNA in Plasmid pBR322. Single-stranded cDNA was polymerized from purified AFP mRNA with the use of reverse transcriptase (Bélanger et al., 1979, 1981). The cDNA was cloned in pBR322 by the standard poly(dC)/poly(dG) homopolymer tailing method (Bolivar et al., 1977; Otsuka, 1981). Double-stranded cDNA (Goodman & MacDonald, 1979) tailed with poly(dC) (Nelson & Brutlag, 1979) and *Pst*I-cleaved pBR322 tailed with poly(dG) (Nelson & Brutlag, 1979) were ligated (Goeddel et al., 1979) and used to transform *E. coli* RR1 (Lévesque et al., 1982). Ampicillin-sensitive tetracycline-resistant colonies were screened (Grunstein & Hogness, 1975) by hybridization with [^{32}P]-cDNA (see below). Plasmid DNA was purified according to Colman et al. (1978). Restriction mapping, blot hybridizations (Southern, 1975), and sequencing (Maxam & Gilbert, 1977) were performed on one recombinant plasmid (pHDQ210).

Cell-Free Nuclear Transcription Assay. (*a*) *Preparation of Nuclei.* Livers were homogenized at 4 °C in 5–10 volumes of 2.3 M sucrose, 10 mM $MgCl_2$, and 1 mM DTT. The homogenate was filtered through cheesecloth and centrifuged at 40000*g* for 60 min at 4 °C. The pellet was homogenized in 5 volumes of 40% glycerol in 50 mM Hepes–NaOH, 5 mM magnesium acetate, and 1 mM DTT, pH 7.5, and centrifuged at 1000*g* for 10 min at 4 °C. The nuclei were resuspended in the above Hepes–glycerol buffer and kept at −80 °C.

(*b*) *Transcription of ^{32}P-Labeled RNA in Isolated Nuclei.* Reaction mixtures contained the following in a final volume of 200 μL: 20 mM Hepes–NaOH, pH 7.6, 150 mM NaCl, 5 mM magnesium acetate, 1 mM $MnCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.4 mM each of ATP, CTP, and GTP, 4 μM [α-^{32}P]UTP, 500 units/mL placental ribonuclease inhibitor, 2 mM creatine phosphate, 6 units/mL nucleoside-5′-diphosphate kinase, 3 units/mL creatine phosphokinase, 16% glycerol, and nuclei equivalent to 200 μg of DNA. The mixtures were incubated at 25 °C for 30 min. They were then adjusted to 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% $NaDodSO_4$, and 150 μg/mL proteinase K and incubated at 40 °C for 60 min. The mix was extracted with 1 volume of phenol–chloroform–isoamyl alcohol (25:24:1) and the aqueous phase precipitated with ethanol. The precipitate was resuspended in 20 mM Hepes–NaOH, 5 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.5, and treated with 50 μg/mL of DNase I for 60 min at 25 °C. The solution was then digested with proteinase K, extracted with phenol–chloroform–isoamyl alcohol, and ethanol precipitated with 80 μg/mL yeast tRNA as carrier. The precipitate was again treated with DNase I (15 μg/mL, 20 min) and proteinase K and phenol extracted. The final aqueous phase was precipitated at 4 °C for 30 min with 10% TCA, 1% $Na_4P_2O_7$, and 0.02% uridine. The precipitate was collected by centrifugation, washed twice with cold 5% TCA, 1% $Na_4P_2O_7$, and 0.02% uridine, and then dissolved in 0.1 M Tris-HCl–100 mM NaCl, pH 8.0, and ethanol precipitated. The final pellet was dissolved in 10 mM Tris-HCl–0.1% $NaDodSO_4$, pH 8.0.

(*c*) *Hybridization of ^{32}P-Labeled Nuclear RNA to Solid-Phase AFP cDNA from Recombinant Plasmid pHDQ210.* DNA from plasmids pBR322 and pHDQ210 was linearized with *Bgl*I, denatured in 0.3 M NaOH for 30 min at 37 °C, and ethanol precipitated. The DNA was then dissolved in water, heated at 100 °C for 1 min, quickly frozen in dry ice–ethanol and stored at −80 °C. APT-cellulose sheets were converted to the DPT form according to Seed (1982) and washed with ice-cold distilled water and 0.2 M sodium acetate, pH 4.0. Ten micrograms of pBR322 or pHDQ210 DNA in 15 μL of water was applied to 8-mm DPT-cellulose disks. The disks were allowed to dry at 4 °C for 12–18 h and washed 3 times with water, 4 times with 0.4 N NaOH (37 °C), and 4 more times with water. The disks were prehybridized for 14–20 h at 42 °C in 0.2 mL of 4× SSC buffer and 50 mM $NaPO_4$, pH 7.0, containing 250 μg/mL yeast tRNA, 100 μg/mL poly(A) (Miles), 0.02% each of Ficoll (Pharmacia), polyvinylpyrrolidone, and BSA, 1% glycine, 0.2% $NaDodSO_4$, and 50% formamide. They were then hybridized in 75 μL of the same solution, under parafilm oil at 42 °C for 72–96 h, with $\simeq 5 \times 10^6$ cpm of ^{32}P-labeled nuclear RNA and 10^4 cpm of [^{3}H]cRNA AFP [[^{3}H]cRNA was synthesized with *E. coli* RNA polymerase from the 380-bp *Pst*I restriction fragment of the pHDQ210 AFP cDNA insert (see Figure 1), according to Roop et al. (1978)]. The disks were then washed at 50 °C for 1 h twice with 10 mL of 2× SSC buffer and twice with 0.2× SSC buffer–0.2% $NaDodSO_4$ and rinsed at 25 °C with 2× SSC buffer to remove $NaDodSO_4$. The disks were incubated in 1 mL of 2× SSC buffer containing 20 μg/mL pancreatic RNase A and 200 units/mL T_1 RNase for 1 h at 25 °C and washed with 2× SSC buffer–0.2% $NaDodSO_4$ for 1 h at 45 °C. The disks were incubated in 0.2 mL of 0.3 M NaOH for 30 min at 37 °C; the radioactive material released was neutralized with acetic acid and counted in xylene–Aquasol.

Dot-Blot Hybridization of Nuclear RNA. (*a*) *Preparation of Nuclear RNA.* Nuclei were isolated by a modification of the citric acid procedure described by Roop et al. (1978). Nuclear RNA was extracted as follows: nuclei were lysed at room temperature in 10 mM Tris-HCl, 0.35 M NaCl, 1 mM EDTA, 2% $NaDodSO_4$, and 7 M urea, pH 8.0. The lysate was extracted with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1). The organic phase was reextracted with lysis buffer. The aqueous phases were combined and extracted with 1 volume of phenol–chloroform–isoamyl alcohol. The aqueous phase was adjusted to 0.2 M sodium acetate, pH 5.0, and ethanol precipitated. The pellet was dissolved in 10 mM Tris-HCl, 3 mM $MgCl_2$, and 0.1 M NaCl, pH 8.0, and treated with 50 μg/mL of DNase I at 25 °C for 60 min. The solution was then adjusted to 1% $NaDodSO_4$, 200 μg/mL proteinase K, and 5 mM EDTA and incubated at 37 °C for 30 min. The mixture was extracted twice with phenol–chloroform–isoamyl alcohol and precipitated with ethanol. The precipitate was again treated with DNase, phenol extracted,

and ethanol precipitated. The RNA was then separated from DNA by precipitation in 2.5 M LiCl at 4 °C (Steele & Bush, 1967).

(*b*) *Probes.* pHDQ210 DNA was labeled with [^{32}P]dCTP by nick translation (Rigby et al., 1977). The labeled DNA [(1–2) × 10^8 cpm/μg] was extracted with phenol–chloroform, chromatographed on Sephadex G-50, and heat denatured before use. ^{32}P-Labeled albumin cDNA was synthesized from purified mRNA in reaction mixtures containing the following in a final volume of 20 μL: 50 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 20 mM DTT, 150 μg/mL actinomycin D, 80 μg/mL oligo(dT)$_{12-18}$ (Miles), 250 μM each of dGTP, dATP, and dTTP, 12.5 μM [^{32}P]dCTP, 1000 units/mL reverse transcriptase (provided by Dr. J. Beard, Life Sciences Inc., St. Petersburg, FL), and 20 μg/mL albumin mRNA. The mix was incubated at 43 °C for 60 min, extracted with phenol–chloroform, treated with NaOH, neutralized, and chromatographed on Sephadex G-50. The specific activity of the cDNA was ≃10^8 cpm/μg.

(*c*) *Hybridization.* Nuclear RNA was dotted (Thomas, 1980) in 5-μg amounts onto nitrocellulose filters saturated with 20× SSC buffer. The filters were dried at room temperature, baked at 80 °C for 2 h, and prehybridized at 42 °C for 18–24 h in 4× SSC buffer and 50 mM $NaPO_4$, pH 7.0, containing 50% formamide, 0.4% $NaDodSO_4$, 50 μg/mL heat-denatured salmon sperm DNA, and 0.1% each of Ficoll, polyvinylpyrrolidone, and BSA. They were then hybridized for 24 h at 42 °C in 4× SSC buffer and 50 mM $NaPO_4$, pH 7.0, containing 50% formamide, 0.02% each of Ficoll, polyvinylpyrrolidone, and BSA, 50 μg/mL heat-denatured salmon sperm DNA, 100 μg/mL poly(A), 10% dextran sulfate, and 15–20 ng/mL heat-denatured AFP or albumin [^{32}P]cDNA. After hybridization, the filters were washed twice with 100 mL of 2× SSC buffer and 0.2% $NaDodSO_4$ at 63 °C for 2 h, then washed twice with 100 mL of 0.1× SSC buffer and 15 mM Tris-HCl, pH 7.5, containing 0.05% pyrophosphate and 0.2% $NaDodSO_4$, for 1 h at 63 °C, and then dried and autoradiographed at −80 °C on a Fuji RX film with a Du Pont Hi-Plus intensifier screen.

Nuclear Translocation of Dexamethasone Receptors. Four-day-old rats were injected intraperitoneally with 10^7 cpm of [^{3}H]dexamethasone (50 Ci/mmol; New England Nuclear), with or without cold dexamethasone at doses up to 50 μg/g. The radioactivity accumulated into liver nuclei was measured at times up to 60 min after injection. Livers were homogenized at 4 °C in 4 volumes of 10 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$, containing 0.25 M sucrose and 0.5% (v/v) Triton X-100. The homogenate was filtered through cheesecloth; nuclei were collected by low-speed centrifugation, further purified by sedimentation though 2.3 and 1 M sucrose pads in 10 mM Tris-HCl, pH 7.5, and 3 mM $MgCl_2$, resuspended in 25% glycerol in Tris–$MgCl_2$, and counted in xylene–Aquasol.

Results

AFP and Albumin mRNAs and cDNAs. Purified AFP mRNA migrated in formamide–polyacrylamide gel as a homogeneous band, 2230 nucleotides long, in agreement with previous figures (Sala-Trepat et al., 1979; Liao et al., 1980b). Purified albumin mRNA sedimented as a single sharp peak in the 17S position in linear 5–20% sucrose gradients. The AFP cDNA was 43% full-length, and the albumin cDNA was 450–1150 nucleotides long. In liquid hybridization (Bélanger et al., 1981), the AFP cDNA reannealed with purified AFP mRNA with an $R_0t_{1/2}$ value of 1.67 × 10^{-3}, consistent with the mRNA complexity and in agreement with previous data (Sala-Trepat et al., 1979; Liao et al., 1980a). In dot–blot hybridization, both AFP and albumin cDNAs hybridized with nuclear RNA from newborn rat liver, neither hybridized significantly with nuclear RNA from brain or kidney (up to 5 μg), and albumin cDNA did not hybridize with purified AFP mRNA (up to 3 ng). These criteria indicate a high degree of purity of both AFP and albumin mRNAs and their corresponding cDNAs.

AFP cDNA Recombinant Plasmid. Restriction endonuclease mapping, Southern blot analysis, and partial DNA sequence of the pHDQ210 recombinant plasmid are presented in Figure 1. The cloned AFP cDNA fragment is some 580 bp in length and corresponds to a region adjacent to the poly(A) tract of the AFP mRNA. It contains the *Pst*I and *Pvu*II sites but not the *Sal*I site, known to be present in rat AFP cDNA (Liao et al., 1980b; Jagodzinski et al., 1981; Innis & Miller, 1980). Its 5′-end begins 32-bp upstream of the *Pvu*II cleavage site, and its 3′-end is within the *Sal*I site. The *Pst*I site at the 5′-end was not reconstituted in the cloning process, due to a single base pair deletion. The 30-bp sequence at the 5′-end of the cDNA insert of pHDQ210 matches exactly with the corresponding sequence reported by Jagodzinski et al. (1981). However, when the 80-bp sequence at the 3′-end is compared with the corresponding sequence from three other reports (Liao et al., 1980b; Jagodzinski et al., 1981; Innis & Miller, 1980), there are several differences. These do not correlate with the rat strain or tissue of origin of the mRNA; our sequence agrees better (four mismatches) with that of Jagodzinski et al. (1981), who used Morris hepatoma 7777 grown in Buffalo rats as AFP mRNA source material, than with that of Liao et al. (1980b) (nine mismatches), who, like us, used yolk sacs from Sprague-Dawley rats.

Cell-Free Nuclear Transcription of the AFP Gene. In the nuclear run-off assay, incorporation of [^{3}H]UMP into nascent RNA chains proceeded near linearly for 30 min, reaching ≃100 pmol/mg DNA (Figure 2); there was no significant degradation of newly synthesized transcripts and no RNA-dependent RNA synthesis, as evidenced by the stability of TCA-precipitable counts after inhibition of DNA-dependent RNA synthesis by actinomycin D (Figure 2). UMP incorporation into 4-day-old rat liver nuclear transcripts was 68% inhibited by 2 μg/mL α-amanitin (data not shown), indicating 68% polymerase II directed RNA synthesis; this is in agreement with previous estimates of polymerase II activity in newborn rat liver (Köhler, 1972; C. Dauphinais and L. Bélanger, unpublished results).

The specific transcriptional activity of the AFP gene was assessed by hybridizing the radiolabeled nuclear transcripts to solid-phase AFP cDNA. Transcripts from newborn rat liver, but not from adult brain or liver, gave significant hybridization signals (Table I), consistent with only the newborn liver being an active producer of AFP. The synthesis of AFP cDNA hybridizable transcripts was completely inhibited by 2 μg/mL α-amanitin, indicative of polymerase II directed transcription, and hybridization of newborn liver transcripts to AFP cDNA was competed by purified AFP mRNA but not by 18S poly(A)-containing polysomal RNA from adult rat liver (this fraction is enriched in albumin mRNA) (Table I). These criteria indicate the specificity of the hybridization assay and the fidelity of the nuclear run-off in reflecting AFP gene transcription in vivo.

Effect of Dexamethasone on Synthesis of AFP mRNA in Newborn Rat Liver. Relation to Nuclear Accumulation of Glucocorticoid Receptors. Four-day-old rats were treated with 2 μg/g dexamethasone twice a day, a regimen that suppresses

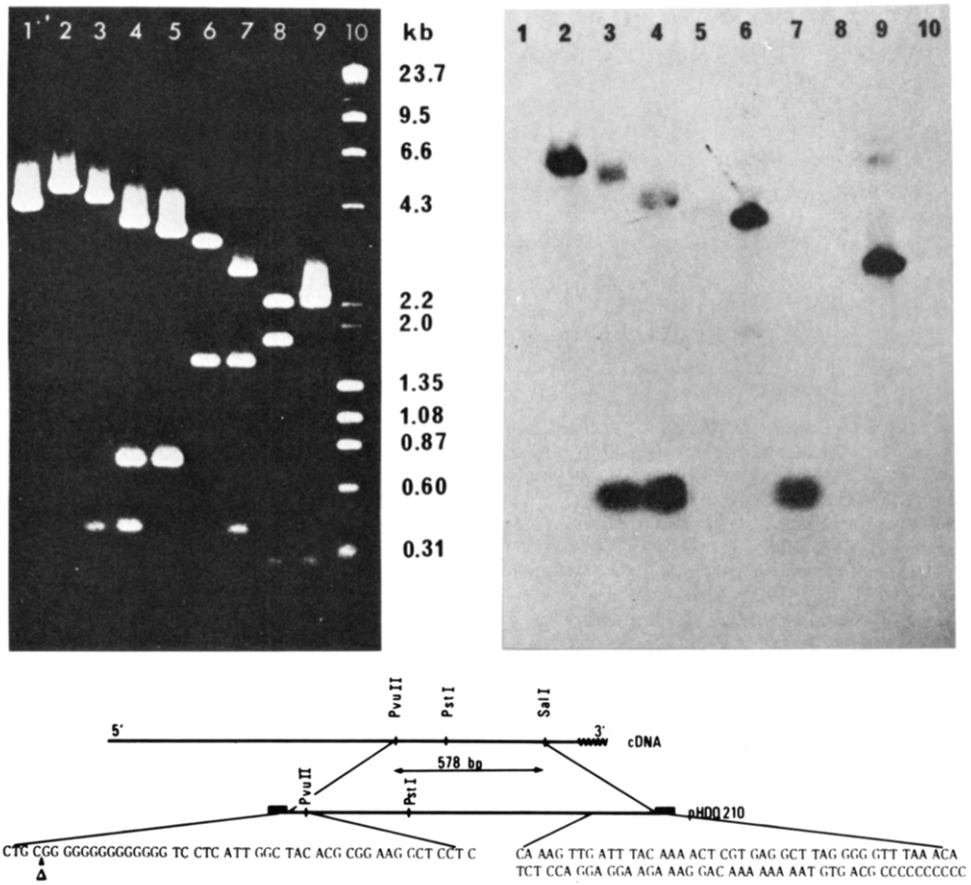

FIGURE 1: Restriction endonuclease mapping, Southern blot analysis, and partial sequence of the pHDQ210 recombinant plasmid. Purified plasmid DNA was cleaved with restriction endonucleases, electrophoresed in 1% agarose gels, and stained with ethidium bromide (left photograph) and then blotted on a nitrocellulose filter and hybridized with rat AFP [^{32}P]cDNA (right fluorogram): (lane 1) pBR322 + *Pst*I; (lane 2) pHDQ210 + *Eco*RI; (lane 3) pHDQ210 + *Pst*I; (lane 4) pHDQ210 + *Pst*I and *Eco*RI; (lane 5) pBR322 + *Pst*I and *Eco*RI; (lane 6) pHDQ210 + *Pvu*II; (lane 7) pHDQ210 + *Pst*I and *Pvu*II; (lane 8) pBR322 + *Bgl*I; (lane 9) pHDQ210 + *Bgl*I; (lane 10) *Hin*dIII fragments of λ DNA and *Hae*III fragments of ϕX174 RF DNA. The orientation of the insert is given by its 3′-end being closer to the *Eco*RI site of pBR322 than its 5′-end. Note that a *Pst*I site was not reconstituted at one end of the cloned fragment (lanes 3 and 4); this was due to a single base pair deletion. (Drawing) The upper line represents full-length rat AFP cDNA [the wavy line represents the mRNA poly(A) tract]. Thick bars at the ends of the pHDQ210 insert represent pBR322 DNA. (Δ) A base deletion explaining the nonreconstitution of the 5′ *Pst*I site.

Table I: Transcriptional Activity of AFP Gene in Nuclear Run-Off System[a]

source of rat nuclei	^{32}P cpm of nuclear transcripts hybridized to cellulose-bound plasmid DNA	
	pBR322	pHDQ210
experiment A		
adult brain	11, 12	10, 11 (<5)[b]
adult liver	11, 12	12, 13 (<5)
4-day-old liver	12, 13	48, 52 (185)
+2 μg/mL α-amanitin[c]	12, 12	12, 13 (<5)
+10 μg/mL AFP mRNA[d]	11, 12	26, 28 (77)
+10 μg/mL 18S mRNA adult liver[d]	13, 13	51, 55 (192)
4-day-old liver 30 min after DEX	11, 12	20, 22 (46)
4-day-old liver 24 h after DEX	12, 12	13, 14 (7)
experiment B (4-day-old liver)		
control	25, 25	120, 130 (333)
30 min after DEX	24, 26	46, 49 (79)
3 h after DEX	22, 23	40, 46 (69)
12 h after DEX	21, 23	33, 35 (32)

[a] Purified nuclei were incubated with [^{32}P]UTP for 30 min, the nuclear RNA was isolated, and 5 × 10^6 cpm of ^{32}P-labeled nuclear transcripts, together with 10^4 cpm of [^{3}H]cRNA$_{AFP}$, was hybridized to DPT-cellulose disks loaded with DNA from pBR322 or from the AFP cDNA recombinant plasmid pHDQ210, as described under Experimental Procedures. Data are counts from duplicate disks. [b] Number in parentheses is net counts (pHDQ210 − pBR322 cpm) corrected for hybridization efficiency (^{3}H cpm bound/10^4). [c] Added to transcription mix. [d] Added to hybridization mix.

liver AFP synthesis in about 2 days (Bélanger et al., 1975, 1981). AFP gene transcription activity was assayed in the nuclear run-off system. The data in Table I and Figure 3 indicate that as early as 30 min after the first DEX injection, transcription of the AFP gene has dropped by ≃75%; after 24 h, it is down to a residual level of ≃10% of control animals. Total RNA synthesis and the relative activity of polymerase II were similar in control and 24-h DEX-treated liver nuclei (9.5 vs. 9.3 pmol of UMP incorporated/mg of DNA, and 68% vs. 63% polymerase II directed RNA synthesis), and therefore,

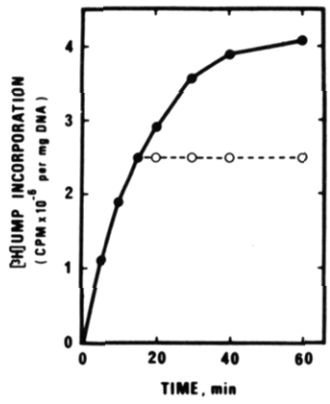

FIGURE 2: Time course of RNA synthesis in isolated rat liver nuclei. Nuclei from 4-day-old rats were incubated at 1 mg/mL DNA at 25 °C in the presence of 4 μM [α-^{32}P]UTP in the cell-free transcription mixture. At the times indicated, the incorporation of UMP into TCA-precipitable material was measured. 10 μL of transcription mix was spotted on 3-mm Millipore filters and incubated for 30 min at 4 °C in 5% TCA–0.02% uridine–1% sodium pyrophosphate; the filters were washed successively with 5% TCA, 95% ethanol, and ether; they were then dried, incubated 30 min at 60 °C in 0.5 mL of Protosol, neutralized with acetic acid, and counted in Econofluor. (O) Actinomycin D, 40 μg/mL, added after 15 min of incubation.

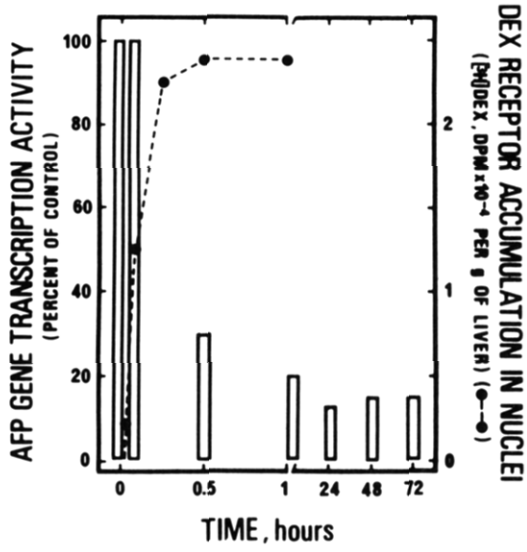

FIGURE 3: Changes in AFP gene transcription activity of newborn rat liver following administration of dexamethasone. Four-day-old rats were injected twice a day with 2 μg/g DEX; controls received saline. After 5 min, 30 min, 3 h, 24 h, 48 h, and 72 h, liver nuclei were isolated from DEX- and saline-treated animals and assayed for AFP mRNA synthesis in the nuclear run-off system. Results (histogram) are expressed as net corrected counts of AFP cDNA hybridized transcripts (see Table I) from DEX-treated over saline-treated livers. Nuclear translocation of DEX receptors (curve) was monitored in a parallel experiment; 4-day-old rats received 10^7 cpm of [^{3}H]DEX, and the accumulation of radioactivity into purified liver nuclei was determined as described under Experimental Procedures.

the decline in hybridizable ^{32}P-labeled AFP mRNA sequences reflects an absolute and selective decrease in AFP gene transcription activity.

The AFP gene suppression activity of dexamethasone was closely related, temporally and quantitatively, to the accumulation of DEX receptors in liver nuclei (we infer that our [^{3}H]DEX nuclear uptake assay monitors receptor translocation because nuclear uptake of injected [^{3}H]DEX was totally prevented by concomitant injection of 50 μg/g of cold DEX and because the radioactivity extracted from nuclei with 0.4 M KCl was $\simeq$80% bound by hydroxylapatite). Figure 3 shows that AFP gene activity is unchanged 5 min after DEX injection

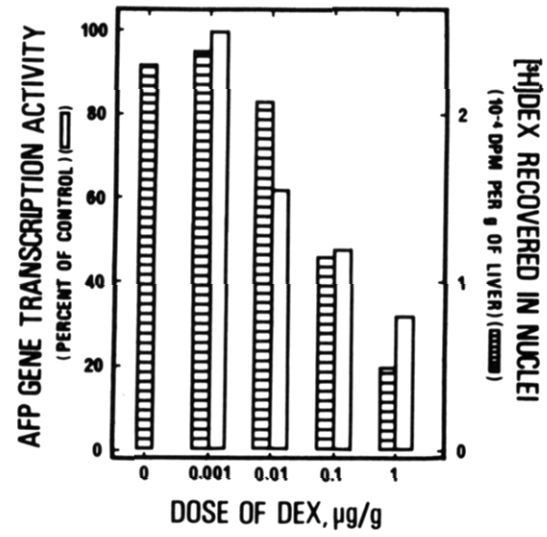

FIGURE 4: Dose effect of dexamethasone on AFP gene transcription and nuclear translocation of DEX receptors in newborn rat liver. Four-day-old rats were injected with saline (controls) or with the indicated doses of DEX. After 1 h, liver nuclei were isolated and assayed for AFP mRNA synthesis in the nuclear run-off system. Results (open columns) are expressed as net corrected counts of AFP cDNA hybridized transcripts (see Table I) from DEX-treated over saline-treated livers. In a parallel experiment, 4-day-old rats received 10^7 cpm of [^{3}H]DEX and the indicated doses of cold DEX, and after 1 h, the radioactivity accumulated into liver nuclei was measured (barred columns); the displacement of ^{3}H counts reflects the amount of DEX receptors translocated to the nucleus.

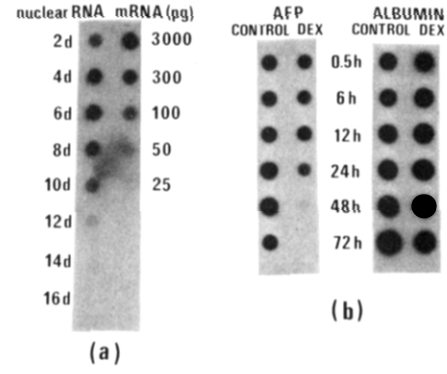

FIGURE 5: (a) Dot hybridization of AFP [^{32}P]cDNA to purified AFP mRNA and liver nuclear RNA (5 μg) from 2- to 16-day-old rats. (b) Hybridization of AFP and albumin [^{32}P]cDNA to liver nuclear RNA (5 μg) from 4- to 7-day-old rats, 0.5–72 h through DEX treatment (2 μg/g twice a day).

but sharply shut down between 5 and 30 min, and this correlates with the time required for maximal translocation of DEX receptors to the nucleus. Figure 4 shows that the degree of AFP gene suppression 1 h after administration of increasing doses of DEX is also closely correlated with the amount of DEX receptor translocated to the nuclei, as measured by the degree of displacement of tracer [^{3}H]DEX.

Effect of Dexamethasone on Nuclear Levels of AFP and Albumin mRNAs in Newborn Rat Liver. The amounts of AFP and albumin mRNA sequences in liver nuclei were monitored by dot–blot hybridization for 72 h through DEX treatment of 4-day-old rats (Figure 5). Nuclei were free of cytoplasmic contamination as judged by electron microscopy. In control rats, nuclear AFP mRNA increased slightly between 0 and 48 h and decreased at 72 h and albumin mRNA increased from 0 to 72 h; this is consistent with the observations that both AFP and albumin synthesis increase in rat liver during the first postnatal week and that liver AFP synthesis declines, with decreasing mRNA levels, in the second postnatal

week (Bélanger et al., 1983; Figure 5). In DEX-treated rat livers, the concentration of nuclear AFP mRNA declined slightly after 0.5 h and remained fairly stable through 12 h, and then, it dropped sharply to undetectable levels at 48 h. In contrast, the concentration of albumin mRNA increased slightly at 0.5 h and remained higher than that of controls through the next 48 h.

Discussion

The cell-free nuclear transcription system that we have established here measures the elongation in vitro of nascent RNA chains initiated in vivo. It thus provides a faithful estimate of gene transcription activities in vivo. Our results show that the administration of dexamethasone to newborn rats causes an abrupt shutdown, within 30 min, in liver AFP gene transcription. This is the time required for the accumulation of DEX receptors in the nucleus and thus it strongly indicates a direct negative action of DEX–receptor complexes on the AFP chromatin transcription unit (as opposed to the induction of a gene repressor). A similar fast ($\simeq$30 min) action of DEX in modulating transcription has been documented in inductive models and shown not to require protein synthesis (Spindler et al., 1982; Mayo & Palmiter, 1981; Ringold et al., 1977). Another rapid negative action of DEX has also recently been documented: Mierendorf & Mueller (1982) have shown that the exposure of Friend erythroleukemia cells to DEX interrupts, within 30 min, the transcription of β-globin induced by 1-methyl-2-pyrrolidone (in both AFP and globin systems, the kinetics of DEX action are closely similar: no effect after 5 min, $\simeq$70% suppression after 30 min, and then a decline to $\simeq$10–15% of controls after 12–16 h). Models of gene regulation by steroid hormones must thus take into account suppressions as well as inductions of gene functions.

Recent studies have indicated that steroid–receptor complexes can bind in vitro to gene fragments amplified by molecular cloning. A consensus sequence has been derived (Mulvihill et al., 1982) to which receptors seem to bind preferentially and which is located in the 5′-adjacent region of inducible genes, 100–500 bp upstream from the mRNA initiation site (Tata, 1982; Cochet et al., 1982; Govidan et al., 1982). Since DEX suppresses AFP without affecting albumin and considering that AFP and albumin genes have been found, in the mouse, to be located in tandem in the genome, separated from each other by a 13.5-kb DNA segment (Ingram et al., 1981), it is tempting to speculate that DEX-receptors modulate AFP gene transcription by interacting with DNA sequences intercalated between the albumin and AFP genes. On the other hand, the fact that DEX exerts pleiotropic (inductive and suppressive) differentiation actions in the newborn rat liver (Bélanger et al., 1983) may also suggest a global change in chromatin structure, and this may or may not result from multiple binding to (positive and negative) regulatory sites adjacent to structural genes.

Although a transcriptional level of AFP gene suppression by DEX was clearly established here, we found a residual level of AFP gene transcription activity (10–15% of controls) from $\simeq$12 h onward. In the same system, Chiu et al. (1981) suggested that, after 4 days of DEX treatment, transcription of the AFP gene is suppressed $\simeq$70%. These combined observations suggest that a residual level of AFP gene transcription may persist in DEX-treated animals. One simple explanation may be that the dose of DEX utilized is not sufficient to saturate the receptor mechanism. And indeed, in the dose–response curve shown in Figure 4, it is apparent that the dose of DEX used in our time-course experiments (2 μg/g twice a day) is not sufficient to completely displace [^{3}H]DEX accumulation in the nuclei. It is thus plausible that a borderline dose of DEX was used, allowing the AFP gene to partly escape hormone action. These pharmacological considerations notwithstanding, it is quite possible that the apparent residual transcription activity merely reflects technical limitations. In the mercuri–uridine system (Chiu et al., 1981), contamination by endogenous AFP mRNA sequences, a possible artifact with this approach, may have led to overestimates of transcriptional activity. In our run-off system, nicks generated by endogenous nucleases (Vanderbilt et al., 1982), a degree of reinitiation, or perhaps some antisense transcription may all lead to irrelevant polymerization of hybridizable transcripts. That this might well be the case is suggested by the observation that the concentration of nuclear AFP mRNA rapidly decreases to undetectable levels during the course of DEX treatment; a residual production of 10–15% AFP transcripts should have been detected by dot hybridization. It may be argued that an increased turnover rate of the AFP mRNA in the nucleus could explain a residual level of run-off transcription with negative dot hybridization. However, with regard to the nuclear content of AFP mRNA during DEX treatment, it is interesting to note that it remained fairly stable for at least 12 h, in spite of the drastic suppression in gene transcription activity [a similar phenomenon has been observed in differentiating Friend cells, in which continuous exposure to DEX for 16 h caused a 10-fold decrease in β-globin gene transcription but only 2-fold reduction in the concentration of nuclear β-globin mRNA (Mierendorf & Mueller, 1982)]. These data thus seem to argue that DEX not only would not increase the turnover rate of the AFP message in the nucleus but perhaps may tend to stabilize it. Stabilizing actions of steroids have been documented for other mRNAs (McKnight & Palmiter, 1979). In the globin system (Mierendorf & Mueller, 1982), DEX was also found to have no important effect on globin mRNA degradation. On the other hand, the turnover rate of the AFP mRNA in the cytoplasm of DEX-treated liver (Bélanger et al., 1981, 1983) seemed significantly higher than the calculated AFP mRNA half-life in hepatoma cells. The exact effect of DEX on posttranscriptional metabolism of AFP mRNA remains to be clarified.

It also remains to be seen whether DEX–receptor complexes interrupt transcription of the AFP gene at the level of elongation or initiation. Our probe covers the 3′-region of the cDNA, and thus it could have missed prematurely terminated transcripts. However, for the above considerations regarding DNA binding sites of steroid–receptor complexes, it seems more plausible at this point that DEX would act by preventing access of polymerase II to the AFP gene promoter. In the globin gene system (Mierendorf & Mueller, 1982), DEX action was shown to be at the initiation step.

Liver AFP suppression by DEX is a rare model of negative hormonal gene regulation exploitable at the molecular level. Unless one postulates that the expression of AFP is under the control of a gene inducer, the AFP–DEX model is in fact unique in not being a deinductive system. The fact that AFP is an oncodevelopmental gene product also adds to the interest of the AFP–DEX system. While the AFP gene in newborn rat liver responds to DEX, in malignant hepatocytes it partially or totally escapes DEX action (Bélanger et al., 1983). This provides a powerful approach to the functioning of differentiation factors in normally developing vs. malignant cells.

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