Glucocorticosteroid Suppression of α_1-Fetoprotein Synthesis in Developing Rat Liver. Evidence for Selective Gene Repression at the Transcriptional Level†

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ABSTRACT: Glucocorticoid hormones administered to immature animals interrupt the hepatic release of α_1-fetoprotein (AFP) [Bélanger, L., Hamel, D., Lachance, L., Dufour, D., Tremblay, M., & Gagnon, P. M. (1975) *Nature (London) 256*, 657]. The molecular basis of this hormonal action was investigated in newborn rat liver and correlated with the effect on albumin expression. Dexamethasone (DEX) exerted no toxic effect on the liver as judged by cell morphology and serum glutamic-pyruvic transaminase levels. The synthesis of AFP was abruptly interrupted by the hormone whereas albumin synthesis was unchanged, as measured by immunoprecipitation of pulse-labeled liver peptides. Concomitantly, total liver polysomes lost their capacity to bind anti-AFP but not anti-albumin antibodies, polysomal RNA assayed in a cell-free system ceased to translated AFP but not albumin, and the number of polysomal mRNA sequences hybridizable to specific [^{32}P]cDNA probes was drastically suppressed for AFP while it was unchanged for albumin. These data indicate that glucocorticoids exert a selective action on AFP and operate at a pretranslational level. Hybridizations with total cellular RNA further showed a 50-fold exponential decay in the amount of AFP mRNA per hepatocyte over 6 days of hormone treatment, with no significant changes in albumin mRNA. The decay curve of AFP mRNA in total cellular RNA was parallel to that in polysomal and in postpolysomal cytoplasmic RNA, providing no evidence for changes in subcellular compartmentation of AFP mRNA or for a new metabolic steady state being reached, and it was consistent with the half-life of the message. This suggests that DEX exerts its effect at the transcriptional rather than the post-transcriptional level. The glucorcorticoid action was reversible. After DEX withdrawal, the number of AFP mRNA sequences rose again in all cellular pools, polysomes recovered their antibody-binding and translational activities, and hepatic production of AFP was reinitiated. Suppression of AFP synthesis by glucocorticoids provides a rare model of negative gene regulation exploitable at the molecular level and may create a valuable counterpart to current gene models based on steroid-activated cell functions.

A current working hypothesis in tumor biology is that the transformation of normal cells into malignant cells may result from epigenetic misprogramming of an intact genome. Several lines of evidence further suggest that such epigenetic alterations may relate to cell differentiation controls. The ultimate fact for the case is that teratocarcinoma cells injected into blastocysts can be induced to differentiate into a normal allophenic animal (Mintz & Illmensee, 1975). Other types of cancer cells can also respond to differentiation factors and loose their malignant status (Marks & Rifkind, 1978).

The control of α_1-fetoprotein (AFP)[1] expression in the mammalian liver provides a powerful model system to probe eukaryotic gene regulation in the context of differentiation and cancer biology. AFP is a major serum protein of the embryo, whose function appears to be basically that of a fetal albumin. During fetal life, AFP is produced mainly by the liver and by the yolk sac (Gitlin & Boesman, 1967). The hepatic AFP function is suppressed around the time of birth; however, it can be reactivated by malignant transformation of the hepatocyte (Abelev, 1971). Thus, in contrast to other currently used gene markers associated with fully differentiated states of the cell, AFP expression is linked instead with both fetal and cancer phenotypes. This onco-developmental nature of the AFP gene function makes its analysis likely to bear on general processes underlying and relating differentiation and cancer.

During normal development, the hepatic production of AFP can be prematurely turned off by glucocorticosteroid hormones, and this is accompanied by a suppression in liver DNA synthesis activity (Bélanger et al., 1975, 1978). This hormone action could provide a powerful regulatory signal to study the operation of the gene. It also enhances considerably the potential of the AFP model. In particular, it could permit an approach to the functioning of differentiation factors in normal vs. cancer cells, as well as to the mechanisms coordinating

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[1] Abbreviations used: AFP, α_1-fetoprotein; DEX, dexamethasone; GPT, glutamic-pyruvic transaminase; IgG, immunoglobulin G; BSA, bovine serum albumin; PB, polysome buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, and 0.1% heparin, pH 7.5); PBS, phosphate-buffered saline (0.01 M sodium phosphate and 0.15 M NaCl, pH 7.2); $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; cDNA, complementary DNA; R_0t, product of RNA concentration in moles of nucleotides per liter and the time in seconds.

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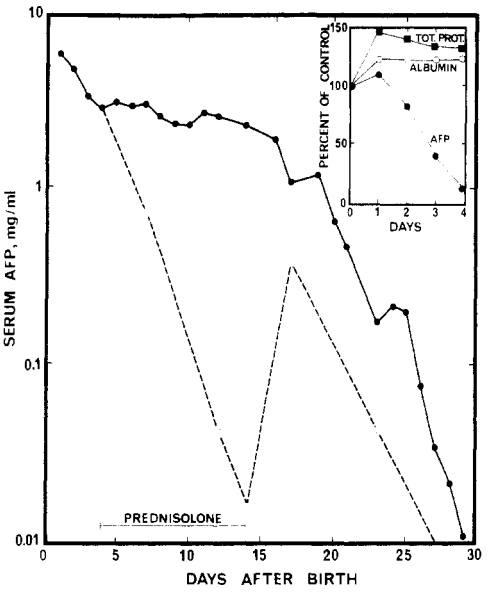

FIGURE 1: Effect of prednisolone (5 μg/g per day from day 4 to day 14) on serum AFP levels of newborn rats. Each point (○) is the mean of four to six animals from different litters. The solid line (●) is the normal disappearance curve of AFP established from 450 neonates. Inset: Relative changes in serum AFP, albumin, and total protein levels in 4-day-old rats (at day 0) under continuous DEX treatment.

differentiation and replication in the developing cells. This suppressive system could also furnish a valuable counterpart to current gene regulation models based on hormone-activated cell function.

We report here data obtained with newborn rats, indicating that glucocorticosteroids suppress liver AFP synthesis at an early step of gene expression, probably at the level of transcription. This action is reversible and selective insofar as it does not affect the expression of the albumin gene.

Experimental Procedures

Animals and Hormone Treatments. Four-day-old Sprague-Dawley rats were used. Each litter was divided in two. One half received intraperitoneal injections of glucocorticoid in 50 μL of saline; the other half (controls) received 50 μL of saline. Except for one experiment with prednisolone (serum AFP levels, Figure 1), all data were obtained from animals treated with dexamethasone (Decadron; Merck, Sharp & Dohme). Two types of experiments were conducted. In the first protocol (6 days of continuous hormone treatment), rats received 2 μg/g DEX twice a day for 3 days and then 1 μg/g DEX twice a day for 3 more days. Animals were sacrificed by decapitation at 0, 2, 4, and 6 days. In the second protocol (hormone withdrawal), rats received 2 μg/g DEX twice a day for 2 days; they were sacrificed at 0, 27, 54, and 144 h. For each experimental point, livers from four to ten rats from different litters were pooled for either protein synthesis assays, polysome antibody binding, or RNA translation and hybridization analyses. Sera were collected individually for protein determination.

Serum Protein Assays. Total serum protein was determined by the method of Lowry et al. (1951) with the protein standard solution of Sigma. Albumin was measured by electroimmunodiffusion and AFP by radioimmunoassay with standards of pure antigen calibrated by Lowry against bovine serum albumin, as described previously (Bélanger et al., 1976, 1979b). Glutamic-pyruvic transaminases were measured on the Abbott VP analyzer.

Liver Protein Synthesis Assays. AFP and albumin synthesis activity was assayed in vivo by immunoprecipitation of pulse-labeled peptides in liver extracts. Rats were injected intraperitoneally with 1 μCi/g of a 15 tritiated amino acid mix (New England Nuclear) and sacrificed after 10 min. Livers were homogenized in a Teflon–glass homogenizer in 5 volumes of 50 mM Tris-HCl, 100 mM NaCl, and 5 mM $MgCl_2$, pH 7.6, containing 1% Triton X-100 and 1% sodium deoxycholate and centrifuged at 27000*g* for 5 min. The supernatant was measured for total albumin and AFP content. Triplicate aliquots of 0.1 mL were then adjusted to 50 μg of AFP, albumin, or rabbit IgG, using whole fetal or adult sera, and to 1% BSA, 0.1 M $NaPO_4$, pH 7.2, 0.1% NaN_3, 1% Triton X-100, and 1% sodium deoxycholate in a final volume of 0.5 mL. Equivalence amounts of sheep anti-AFP, goat anti-albumin, or sheep anti-rabbit IgG (Bélanger et al., 1979a) were then added, and the mixtures were incubated at room temperature until precipitation was complete (≃3 h). The tubes were then adjusted to 0.1 M $NaPO_4$, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.5 M sucrose, in a final volume of 1 mL, vortexed, and centrifuged at 3000 rpm for 10 min. The pellets were washed once with 0.5 mL of 0.5 M sucrose, 1% Triton, and 1% deoxycholate, in PBS, and twice with 2 mL of PBS and dissolved at 37 °C for 60 min in 0.5 mL of Protosol (New England Nuclear). The radioactivity was measured in 10 mL of Econofluor (New England Nuclear) in an LKB Rack β counter with an integrated DPM converter. Counts of rabbit IgG immunoprecipitates were taken as background and subtracted from AFP and albumin counts. The efficiency of the immunoprecipitation reactions was verified by coprecipitation of purified radioiodinated AFP, albumin, or IgG (Bélanger et al., 1979a,b). The identity of the immunoprecipitated products was confirmed by their migration in $NaDodSO_4$–urea–polyacrylamide gel electrophoresis.

Polysome Antibody Binding. Polysomes active in translation of AFP and albumin peptides were assessed by radiolabeled antibody binding, essentially as described before (Bélanger et al., 1979a). Briefly, total liver polysomes were prepared on discontinuous sucrose gradients, and 2–5 A_{260} units were incubated in 1 mL of PB at 4 °C for 60 min with 10^6 cpm of radioiodinated RNAse-free anti-AFP or anti-albumin IgGs. The mixture was overlaid on 12-mL linear (0.5–1.5 M) sucrose gradients in PB and centrifuged at 40000 rpm for 90 min in a Beckman SW40 rotor. The gradients were eluted under continuous A_{260} monitoring, and 0.6-mL fractions were collected for ^{125}I counting in a γ counter. Counts from blank gradients (^{125}I-labeled antibodies in PB) were subtracted from parallel fractions of polysome-containing gradients.

RNA Preparations. Polysomal RNA was purified by a modification of the phenol/chloroform method of Perry et al. (1972). Total liver polysomes were adjusted to 10 A_{260} units/mL in 50 mM EDTA and 1% $NaDodSO_4$, in 50 mM Tris-HCl and 100 mM NaCl, pH 7.5, and incubated at 37 °C for 1 h with 200 μg/mL RNAase-free proteinase K (Beckman). The mixture was then extracted 3 times at room temperature with 1 volume of phenol/chloroform (1:1) (freshly redistilled phenol, saturated with 50 mM Tris-HCl and 100 mM NaCl, pH 7.5). The aqueous phase was adjusted to 0.2 M NaCl and precipitated overnight at −20 °C with 2.5 volumes of 95% ethanol. The RNA was recovered by centrifugation and washed 3 times with 3 M sodium acetate, pH 6.0, once with 70% ethanol/30% sodium acetate, 0.1 M, pH 6.0, and once with 95% ethanol, to remove heparin (Palmiter, 1974). The RNA was then dissolved in water and analyzed by absorbance scanning on a Beckman 35 spectrophotometer and by sedimentation on 5–20% linear sucrose gradients

(Bélanger et al., 1979a). The preparations were pure and undegraded, judging from A_{260}/A_{280} ratios of ≥2.0 and 5S/18S/28S sedimentation profiles. Postpolysomal cytoplasmic RNA was extracted by the same method from fractions overlaying the 2.5/1 M polysome band in discontinuous sucrose gradients (Bélanger et al., 1979a).

Total cellular RNA was extracted by a modification of the phenol/*m*-cresol method described by Liarakos et al. (1973). Minced livers were washed in ice-cold 50 mM Tris-HCl, 100 mM NaCl, and 50 mM LiCl, pH 8.0, and homogenized in 20 volumes of the same buffer containing 1% $NaDodSO_4$ in a Sorvall Omni mixer for 30 s at maximum speed. One volume of Tris/NaCl/LiCl-saturated phenol containing 1% *m*-cresol and 0.1% hydroxyquinoline was added, and the mixture was extracted with 5 cycles of 30-s homogenization/2-min cooling. The mix was then filtered on gauze and centrifuged at 5000 rpm for 20 min at 0 °C in a Beckman JS7.5 rotor. The aqueous phase and interphase were adjusted to 0.5% $NaDodSO_4$ and 0.5 M LiCl, reextracted manually for 15 min at 37 °C with 1 volume of phenol/*m*-cresol/hydroxyquinoline, cooled to 0 °C, and centrifuged. After two additional extractions, the final aqueous phase was adjusted to 0.2 M NaCl and precipitated at −20 °C with 2.5 volumes of ethanol. The nucleic acid pellet was washed as described above for polysomal RNA with sodium acetate and ethanol, to solubilize DNA (Palmiter, 1974). Alternatively, RNA was separated from DNA by differential precipitation in 2.5 M LiCl at −4 °C (Steele & Busch, 1967).

Cell-Free Translation. Total polysomal RNA was assayed for translatable AFP and albumin mRNA sequences in a rabbit reticulocyte or a wheat germ cell-free system as described previously (Bélanger et al., 1979a; Sala-Trepat et al., 1978, 1979a). The conditions of assay were as described before except for pretreatment of reticulocyte lysates with micrococcal nuclease to remove endogenous mRNA activities (Pelham & Jackson, 1976). In vitro synthesis of albumin and AFP peptides was monitored by specific immunoprecipitation of postribosomal [^{3}H]leucine- or [^{35}S]methionine-labeled translation products. The efficiency of immunoprecipitation and the identity of newly synthesized AFP and albumin were confirmed as described for the in vivo protein synthesis assays (see above).

Synthesis of ^{32}P-Labeled DNAs Complementary to AFP and Albumin mRNAs. AFP and albumin cDNAs were synthesized from highly purified mRNA preparations (Sala-Trepat et al., 1979a,b). Reaction mixtures contained the following in a final volume of 20 μL: 50 mM Tris-HCl, pH 8.4, 10 mM $MgCl_2$, 50 mM NaCl, 10 mM dithiothreitol, 150 μg/mL actinomycin D (Sigma), 80 μg/mL oligo(dT)$_{12-18}$ (Miles), 500 μM each of dGTP, dATP, and dTTP (Boehringer), 50 μM [^{32}P]dCTP (250 Ci/mmol) (New England Nuclear), 100 μg/mL BSA, 10–20 μg/mL purified AFP or albumin mRNA, and 1200 units/mL avian myelobastosis virus reverse transcriptase (gift of Dr. J. Beard, Life Science Inc., St-Petersburg, FL). The mixtures were incubated at 43 °C for 60 min, and the reactions were terminated by adding 10 μL of 10% $NaDodSO_4$ and 10 μL of 0.4 M EDTA. Micrococcal DNA (30 μg) (Sigma) was then added, and the template RNA was hydrolyzed by making the mixture 0.3 M in NaOH and incubating at 70 °C for 60 min. The solution was then neutralized and chromatographed on a Sephadex G-200 column equilibrated 20 mM sodium acetate and 0.2 M LiCl, pH 5.5. The cDNA in the excluded fractions was pooled, passed over a Chelex-100 column, and precipitated with 2 volumes of 100% ethanol. The [^{32}P]cDNA preparations had a specific activity of 5×10^8 cpm/μg.

RNA Excess Hybridizations to AFP and Albumin [^{32}P]-cDNAs. RNA excess hybridizations were carried out in sealed 10-μL siliconized capillaries. Reaction mixtures contained 10 mM Tris-HCl, pH 7.4, 0.18 or 0.5 M NaCl, 1 mM EDTA, 0.1% $NaDodSO_4$, 2500–4000 cpm of AFP or albumin [^{32}P]-cDNA, and varying amounts of RNA. The mixtures were heated at 105 °C for 5 min prior to incubation at 68 °C for times up to 72 h. The reactions were terminated by instant freezing of the capillaries in dry ice/acetone. Hybrids were measured by assaying the reaction mixtures for S_1 nuclease resistance as described (Savage et al., 1978). Data were plotted as the percent [^{32}P]cDNA hybridized (S_1-resistant counts) as a function of equivalent R_0t values (observed R_0t corrected for salt concentration) (Britten et al., 1974). R_0t curves were analyzed with a computer program (Murphy et al., 1979) designed to fit the data according to

$$c/C_0 = P[1 - \exp[-\ln 2(R_0t/R_0t_{1/2})]]$$

where c/C_0 is the fraction of cDNA hybridized at time t, R_0t = mol s L^{-1} of nucleotides RNA, $R_0t_{1/2}$ = mol s L^{-1} of nucleotides RNA at 50% of maximum hybridization , and P is the fraction of cDNA hybridized at completion of the reaction.

Results

Effect of DEX Treatments on Serum Protein Levels. Serum GPT levels of DEX-treated animals, measured at 2-h intervals for the first 12 h and every 12 h thereafter during a continuous 6-day treatment, did not increase over base-line levels (<40 units/mL) of uninjected or saline-treated animals (data not shown). That DEX exerted no hepatotoxic effect was further established by the normal microscopic appearance of the liver and undegraded polysome profiles (Figure 3).

The effect of hormone treatment on total protein, albumin, and AFP serum levels is depicted in Figure 1. After a lag of 1–2 days, AFP concentration drops logarithmically at a rate consistent with its half-life of 24 h (Colquhoun et al., 1974; Sell, 1974; Bélanger et al., 1975). This, with the fact that glucocorticoids do not alter the AFP catabolic rate (Bélanger et al., 1975), indicates that secretion of AFP by producing cells stops within 2 days of hormone treatment. If the treatment is interrupted, AFP concentration rises again (though not to control levels). In contrast to AFP, glucocorticoids increase slightly the total protein and albumin serum levels over those of the controls (insets of Figures 1 and 2). Gaps between changes in serum levels and liver synthesis (below) of AFP and albumin are probably accounted for by impaired body growth and changes in fluid to tissue mass ratios during DEX treatment.

Liver Protein Synthesis Assays. DEX treatment reduced in vivo incorporation of ^{3}H-labeled amino acids into AFP to 40% of that of the control animals after 24 h and to undetectable levels within 3 days (Figures 2 and 8A). Interrupting DEX treatment after 2 days resulted in the resumption of tracer incorporation into AFP (Figure 8A). ^{3}H-labeled amino acid labeling of albumin, as well as total protein (measured as 10% Cl_3CCOOH-precipitable material, not shown), was unchanged or slighly increased after glucocorticoid treatment. The pulse-labeling time of the assay (10 min) being within transit time (≃15 min) of AFP and albumin peptides in the producing cells (Peters & Peters, 1972; Bélanger et al., 1975), the data are independent of secretion processes and therefore demonstrate that glucocorticoids interrupt hepatic release of AFP by suppressing, in a reversible manner, synthesis of the protein (that secretion is not involved was also shown by

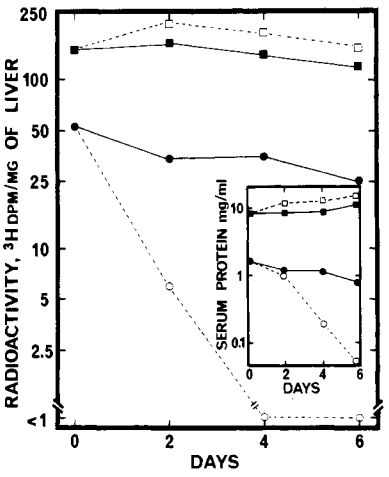

FIGURE 2: Effect of continuous DEX treatment of 4-day-old rats on liver AFP (○, ●) and albumin (□, ■) synthesis activity. Closed symbols, controls; open symbols, DEX treatment. Liver proteins were radiolabeled in vivo, and newly synthesized peptides were measured in liver homogenates by specific immunoprecipitation, as described under Experimental Procedures. Inset: Changes in serum AFP and albumin levels.

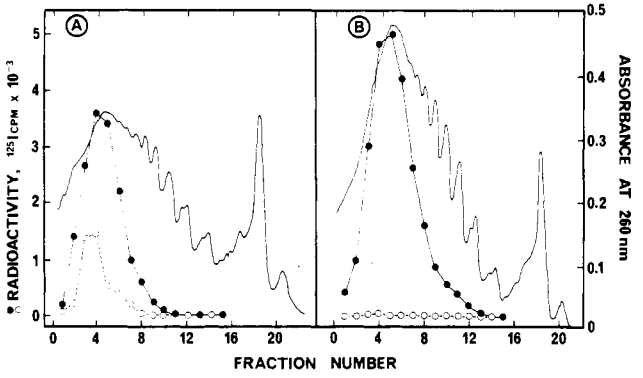

FIGURE 3: Sedimentation profiles on linear sucrose gradients and radiolabeled anti-AFP (○) and anti-albumin (●) antibody binding activity of liver polysomes from 8-day-old rats after 4 days of saline (A) or DEX (B) treatment.

parallel fluctuations in the amount of AFP in serum and in whole liver extracts; not shown). DEX action occurs with a high degree of selectivity, leaving unaffected the synthesis of albumin and of the bulk of liver proteins.

Polysome Antibody Binding Activity during DEX Treatment. The capacity of liver polysomes to bind radiolabeled anti-AFP antibodies was also reversibly suppressed by DEX treatment, in parallel with the suppression of AFP synthesis assayed in vivo; anti-albumin antibody binding was unchanged (Figures 3 and 8B). The loss of immunoreactive AFP on polysomes indicates a suppression of AFP mRNA translation before any significant peptide elongation (however, it does not strictly rule out ribosome initiation of AFP messages).

Translation of Polysomal RNA. Total polysomal RNA extracted from DEX-treated livers lost its ability to direct the in vitro translation of AFP with kinetics similar to polysome anti-AFP binding activity; albumin translation was unchanged (Figures 4 and 8C). Since the method used for polysome isolation yields the full spectrum of ribosomal translation complexes, down to monosomes, the loss of translatable AFP mRNA therefore suggests that no functional message has formed initiation complexes, i.e., that glucortocoids suppress AFP synthesis at a pretranslational level.

Effect of DEX Treatments on cDNA/mRNA Hybridization Kinetics. RNA excess hybridizations with the specific cDNA

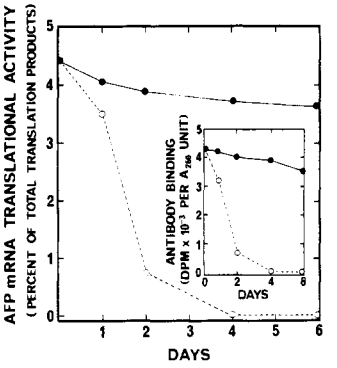

FIGURE 4: Effect of continuous DEX treatment of 4-day-old rats on AFP translational activity of liver polysomal RNA in a rabbit reticulocyte cell-free system. (●) Control; (○) DEX treatment. Results are expressed as the percent of anti-AFP immunoprecipitable translation products over total Cl_3CCOOH-precipitable products. Inset: Changes in polysome anti-AFP binding capacity (^{125}I counts bound per A_{260} unit, see Figure 3).

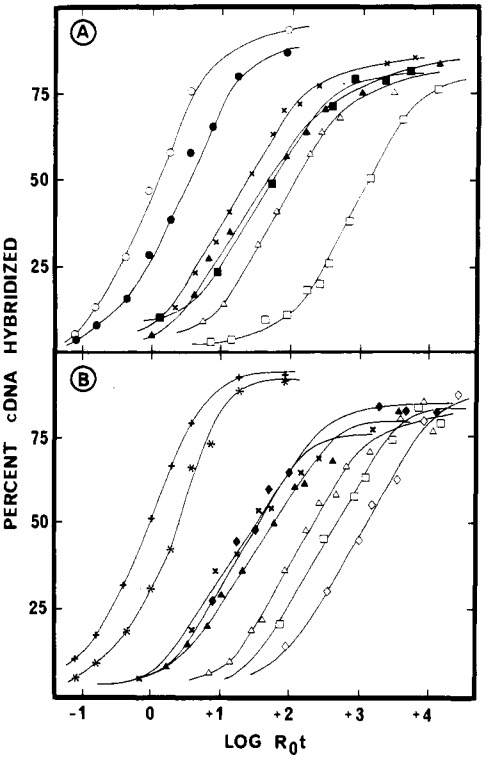

FIGURE 5: Hybridization kinetics of AFP [^{32}P]cDNA and albumin [^{32}P]cDNA with liver polysomal RNA (A) or whole-cell RNA (B) from 4-day-old rats treated with saline or DEX during 6 consecutive days. (×) AFP saline, 0 h; (▲) AFP saline, 48 h; (△) AFP DEX, 48 h; (■) AFP saline, 96 h; (□) AFP DEX, 96 h; (♦) AFP saline, 144 h; (◇) AFP DEX, 144 h; (●) albumin saline, 96 h; (○) albumin DEX, 96 h; (+) albumin saline, 144 h; (*) albumin DEX, 144 h.

probes provided further insight into the effect of DEX on AFP and albumin mRNA levels in developing liver. A few among many R_0t curves obtained with polysomal and total cell RNA preparations are presented in Figures 5 and 6. The hybridization kinetics are consistent with the cDNA probes reacting with a single species of RNA. $R_0t_{1/2}$ values obtained with pure mRNAs (1.55×10^{-3} for albumin and 1.33×10^{-3} for AFP, not shown) were consistent with the sequence complexity of these mRNA species (Sala-Trepat et al., 1979a).

AFP $R_0t_{1/2}$ values of both polysomal and total cellular RNA preparations from DEX-treated livers were increased over those of control animals, indicating a lower abundance of AFP mRNA sequences. Albumin $R_0t_{1/2}$ values did not show con-

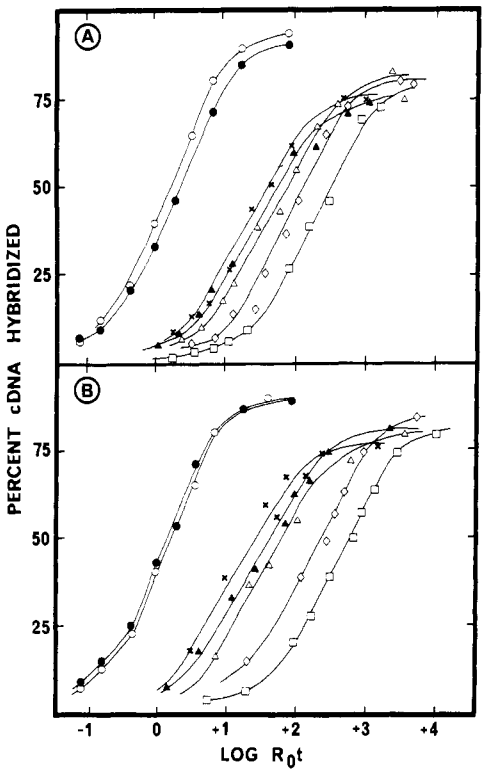

FIGURE 6: Hybridization kinetics of AFP [^{32}P]cDNA and albumin [^{32}P]cDNA with liver polysomal (A) or whole-cell (B) RNA from 4-day-old rats treated for 2 days with DEX or saline (hormone-withdrawal protocol). (×) AFP saline, 0 h; (▲) AFP saline, 27 h; (△) AFP DEX, 27 h; (□) AFP DEX, 54 h; (◇) AFP DEX, 144 h; (●) albumin saline, 54 h; (○) albumin DEX, 54 h.

sistent variations. In hormone-treated as well as control livers, the mass fraction of AFP and albumin mRNAs (obtained from the ratio of the $R_0t_{1/2}$ value of pure mRNA and the $R_0t_{1/2}$ value of the RNA preparation) was always higher in polysomal RNA than in total cell RNA. Since polysomal RNA contributes a very large fraction of total cell RNA (Ramsey & Steele, 1976), this indicates that the vast majority of liver AFP and albumin mRNA sequences is concentrated in the polysomes. This was further substantiated by the very low concentration of hybridizable AFP and albumin sequences in postpolysomal cytoplasmic RNA (data not shown). Ratios of albumin to AFP mRNA mass fractions were similar in total cell RNA and in polysomal RNA and reasonably consistent though significantly higher than ratios of albumin to AFP synthesis in vivo (Figure 2). This result suggests that there is no gross difference in compartmentation but perhaps better utilization of the AFP than the albumin message in the developing hepatocyte.

Changes in the number of AFP and albumin mRNA sequences per liver cell, calculated from the mass fraction of mRNA in total cell RNA preparations and the amount of RNA per cell (Sala-Trepat et al., 1979a), are presented in Figure 7. Under continuous DEX treatment, the amount of AFP mRNA per liver cell decreased exponentially, down to a 50-fold lower level after 6 days (Figure 7A). The AFP mRNA decay curves were parallel in whole-cell, polysomal, and postpolysomal cytoplasmic RNA preparations. In control animals, the amount of AFP mRNA per cell remained relatively stable ($\simeq$1800 copies per cell) [since liver grows during the fourth to tenth postnatal days covered by these experiments, AFP mRNA sequences per liver thus increased in control animals; this is consistent with the increasing liver production of AFP during the first 10 days after birth (Bélanger et al., 1978, 1979b)]. The effect of DEX on AFP

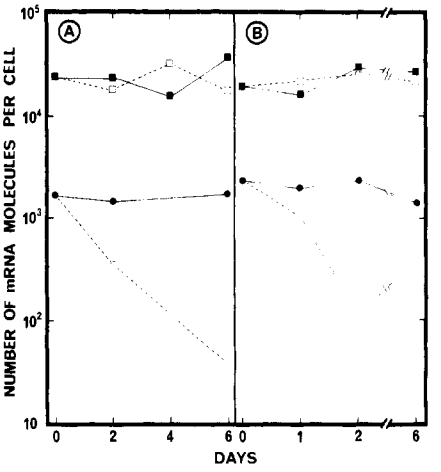

FIGURE 7: Changes in the number of albumin (□, ■) and AFP (○, ●) mRNA molecules per liver cell during continuous (A) or withdrawn (B) DEX treatment. (●, ■) Saline; (○, □) DEX. The number of mRNA molecules was calculated as the mass fraction of mRNA times the amount of RNA per cell times the Avogadro number divided by the molecular weight of the mRNA. The mRNA mass fraction was measured as $R_0t_{1/2}$ purified mRNA/$R_0t_{1/2}$ whole-cell RNA, assuming the amount of RNA per cell to be a constant 37 pg, with the molecular weight of albumin mRNA taken as 770 000 and that of AFP mRNA as 760 000 (Sala-Trepat et al., 1979a).

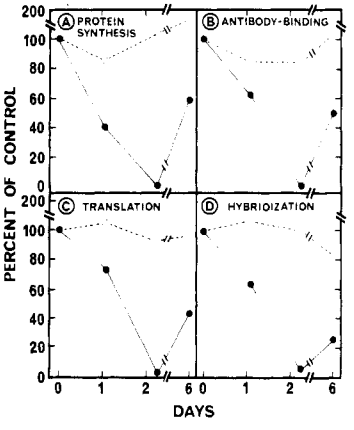

FIGURE 8: Comparative changes in AFP and albumin liver synthesis activity, polysome antibody binding capacity, polysomal RNA translational activity, and cDNA-hybridizable polysomal RNA sequences in 4-day-old rats treated for 2 days with DEX or saline (hormone-withdrawal protocol). Results are ratios of DEX- over saline-treated animals. (●) AFP; (○) albumin. (A) Liver protein synthesis measured by incorporation of radiolabeled amino acids into immunoprecipitable cytoplasmic liver protein (see Figure 2). (B) Capacity of polysomes to bind radiolabeled anti-AFP and anti-albumin IgG (calculated as counts bound per A_{260} unit; see Figure 3). (C) Translational activity of polysomal RNA (see Figure 4). (D) Number of cDNA-hybridizable sequences in polysomal RNA (levels compared at $R_0t_{1/2}$ values).

mRNA levels was reversible; after the hormone was withdrawn, the number of AFP sequences increased again in all RNA preparations (Figure 7B). The number of albumin mRNA sequences per liver cell was not significantly affected by DEX treatments.

Changes in hybridizable polysomal AFP mRNA sequences in hormone-treated animals were parallel to changes in protein synthesis, polysome antibody binding and in vitro translation activities (Figure 8). This further demonstrates that glucocorticoids regulate the supply of the AFP message to polysomes. The parallel fluctuations of AFP mRNA in polysomal and postpolysomal cytoplasmic RNA exclude cytoplasmic sequestration of the message in the form of ribonucleoprotein particles as a mode of hormone action. The coordinated decay

of AFP mRNA in whole-cell and cytoplasmic RNA provides no evidence for changes in compartmentation of the message (e.g., accumulation in the nucleus), and the exponential clearance rate shows no indication for a new steady state being established.

Discussion

This work confirms and extends our previous observations on the effect of hormones on AFP production in developing rat liver (Bélanger et al., 1975, 1978). The combination of experimental approaches used here demonstrates that glucocorticoids suppress AFP synthesis in a nontoxic, selective, and reversible manner through mechanisms that act at a pretranslational level to modulate the number of AFP mRNA molecules in the hepatocyte.

The specificity of DEX action on AFP, principally referred here to albumin, was further demonstrated by more extensive analyses of liver-secreted peptides, total mRNA translation products, and hepatic enzyme profiles (unpublished experiments). These analyses showed that the pattern of liver proteins is modulated in a highly selective and coordinated manner. In particular, DEX produces a synchronized shift toward an adult phenotype in a number of carbohydrate-related liver enzymes and isozymes; this is in line with previous observations on precocious activation of enzymatic functions in developing rat liver by glucocorticoids, such as tyrosine aminotransferase and tryptophan 2,3-dioxygenase (Franz & Knox, 1967). AFP repression is thus part of a global scheme of liver differentiation prematurely activated by glucocorticoids.

Albumin was a most significant gene product to compare with AFP. Albumin and AFP are presumed to function as fetal/adult counterparts, and their genes are phylogenically related (Ruoslahti & Terry, 1976; Liao et al., 1980b; Gorin et al., 1981; Kioussis et al., 1981) and possibly closely linked in the genome [in the mouse, AFP and albumin genes are located on the same chromosome (D'Eustachio et al., 1981)]. That DEX suppresses AFP without affecting albumin expression suggests that these two liver functions are not coordinated in a manner that could resemble fetal/adult globin switches. It further suggests that neonatal hepatocytes are already in a fully differentiated state with respect to albumin function. This is consistent with the relatively stable population of the albumin message throughout postnatal liver development (Figure 7; Sala-Trepat et al., 1979a, and unpublished experiments) and with quantitatively identical subcellular distribution of albumin peptides in newborn and adult rat livers (Bélanger et al., 1979b).

The results obtained here delimit sharply the level of DEX action in suppressing AFP. Clearly, DEX operates by modulating the supply of the AFP message to polysomes; i.e., its effect is pretranslational. There is no evidence for compartmentation or accumulation of the message to suggest cytoplasmic sequestration or impaired nuclear release. The reversible hormone action eliminates alterations at the genomic level such as deletion or rearrangement of gene sequences. This leaves the alternative of decreased synthesis (transcription) or increased degradation (posttranscriptional destabilization) of the message. The data do not permit to rule out destabilization of the AFP mRNA, but a primarily transcriptional effect appears much more likely. The 50-fold decrease in the number of AFP mRNA sequences after a few days of DEX exposure, if it were posttranscriptional, would require an exquisitely sensitive and selective catabolic process of which, to our knowledge, no comparable instance has been documented [generally, hormones including glucocorticoids have been found to stabilize mRNAs (Palmiter & Carey, 1974; Cox, 1977; Houdebine et al., 1978)]. On the other hand, the disappearance rate of the message, assuming transcription arrest, yields a calculated AFP mRNA half-life of 27 h, consistent with the 38–40-h half-life estimated in Morris 7777 hepatoma cells (Innis & Miller, 1979) [the slight discrepancy might indicate DEX-enhanced catabolism of the message in newborn liver, but perhaps more likely stabilization of the message in the hepatoma, as 7777 cells have optimized AFP production mechanisms (Innis & Miller, 1979)]. The rapidity of DEX action also favors transcription. The exponential AFP mRNA decay curve, which extrapolates to near time zero (Figures 7 and 8), indicates a very short lag phase in hormone action, referable, for instance, to the rapid induction of tyrosine aminotransferase mRNA by glucocorticoids in rat liver or in hepatoma tissue culture cells (Nickol et al., 1978; Olson et al., 1980) or to the fast transcriptional effect of DEX on mouse mammary tumor virus gene expression (Ringold et al., 1977; Young et al., 1977). A transcriptional mechanism would be in line with considerable experimental evidence which indicates that glucocorticoids exert many of their multiple physiological actions in a manner analogous to other classes of steroids, i.e., by changing template restriction states of the genome (Johnson et al., 1979).

Like other steroids, glucocorticoid action upon AFP is also likely to be mediated by a receptor mechanism. Glucocorticoid receptors are present in rat liver from early fetal life (Feldman, 1974; Giannopoulos, 1975) and can account for DEX suppressing AFP synthesis at all times throughout the pre- and postnatal period (Bélanger et al., 1978). It remains more speculative whether the putative receptor mechanism would suppress AFP by direct action on chromatin or operate through or in conjunction with other factors. A primary action on chromatin seems favored by the fast and developmental stage-independent effect of DEX. On the other hand, other compounds, such as thyroid hormones or cyclic AMP, were also found to depress AFP levels significantly in newborn rats (Bélanger et al., 1975, 1978), and furthermore, DEX suppressed AFP less efficiently in isolated liver cells than in intact animals (Bélanger et al., 1978). Thus, glucocorticoid–receptor complexes might perhaps interact with other factors in suppressing AFP. There are many examples of glucocorticoids cooperating with other factors to act on gene products [e.g., with cyclic AMP on tyrosine aminotransferase in isolated rat hepatocytes (Ernest et al., 1977), with prolactin on casein in rat mammary gland (Matuzik & Rosen, 1978; Houdebine et al., 1978), with thyroid hormones on somatotropin in rat pituitary cells (Martial et al., 1977), with estrogens on ovalbumin and conalbumin in chick oviduct (Hager et al., 1980), and with thyroid and sex hormones on α_{2u}-globulin in rat liver (Feigelson & Kurtz, 1978)].

Another observation made here was that AFP suppression is only partly reversible after DEX withdrawal, suggesting that the hormone evokes stable changes in the hepatocyte. One plausible view, that would also account for the multiple and coordinated differentiation effects brought about by DEX, would be that the basis for AFP turnoff resides in a common catalytic process switching chromatin to a new conformational state. This idea is consistent with current concepts on the molecular basis of cell differentiation and glucocorticoid receptor function (Johnson et al., 1979).

A further relevant aspect of DEX action on AFP relates to the suppression of liver DNA synthesis induced by the hormone. There is evidence in many experimental systems that an alteration in the cell cycle is required for the expression

of differentiation programs [such as in the chemically induced erythroid transition of the Friend murine erythroleukemia cell (Marks & Rifkind, 1978)]. Suppression of DNA synthesis evoked by DEX in newborn rat liver may be a prerequisite to the implementation of a developmental program that includes extinction of the AFP function.

A number of hormone-inducible gene models, including several glucocorticoid-responsive systems mentioned above, are currently exploited for eukaryotic gene regulation analysis. DEX-induced rat AFP suppression is a rare example of a negative system exploitable at the molecular level.[2] It illustrates the fact that models of steroid hormone action must take into account repression as well as activation of gene functions. Whether or not suppression of AFP synthesis turns out to be mediated by the activation of a gene repressor, a negative system will provide conceptual and methodological advantages in many experimental approaches to gene control. Whether the model is valid in species or in cell types other than developing rat hepatocytes (Becker et all., 1976; Bélanger et al., 1975, 1978; Commer et al., 1979) remains to be documented with respect to glucocorticoid cytotoxicity, level, and selectivity of action.

AFP has evoked considerable interest as a marker protein for eukaryotic gene regulation studies (Tamaoki et al., 1974; Miura et al., 1979; Atrysek et al., 1980; Innis & Miller, 1979; Commer et al., 1979; Tilghman et al., 1979; Liao et al., 1980a,b; Sala-Trepat et al., 1979a,b; Bélanger et al., 1979a). With glucocorticoids providing a sharp regulatory signal to turn off AFP, the use of in vivo and in vitro transcription systems and the availability of the AFP gene in purified form should permit a highly refined analysis of steroid hormone action at the genome level.

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References

Abelev, G. I. (1971) *Adv. Cancer Res. 14*, 295.

Ashburner, M., Chihara, C., Meltzer, P., & Richard, G. (1973) *Cold Spring Harbor Symp. Quant. Biol. 38*, 655.

Atryzek, V., Tamaoki, T., & Fausto, N. (1980) *Cancer Res. 40*, 3713.

Becker, J., de Nêchaud, B., & Potter, V. R. (1976) in *Onco-Developmental Gene Expression* (Fishman, W. H., & Sell, S., Eds.) p 259, Academic Press, New York.

Bélanger, L., Hamel, D., Lachance, L., Dufour, D., Tremblay, M., & Gagnon, P. M. (1975) *Nature (London) 256*, 657.

Bélanger, L., Hamel, D., Dufour, D., & Pouliot, M. (1976) *Clin. Chem. (Winston-Salem, N.C.) 22*, 198.

Bélanger, L., Hamel, D., Dufour, D., Guillouzo, A., & Chiu, J. F. (1978) *Scand. J. Immunol. 8*, 239.

Bélanger, L., Commer, C., & Chiu, J. F. (1979a) *Cancer Res. 39*, 2141.

Bélanger, L., Fleischer, B., Fleischer, S., Guillouzo, A., Lemonnier, M., & Chiu, J. F. (1979b) *Biochemistry 18*, 1962.

Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29E*, 363.

Colquhoun, B., Gold, P., Barzell, W., Oakes, D. D., & Shuster, J. (1974) *Cancer Res. 34*, 2296.

Commer, P., Schwartz, C., Tracy, S., Tamaoki, T., & Chiu, J. F. (1979) *Biochem. Biophys. Res. Commun. 89*, 1294.

Cox, R. F. (1977) *Biochemistry 16*, 3433.

D'Eustachio, P., Ingram, R. S., Tilghman, S. M., & Ruddle, F. H. (1981) *Somatic Cell Genet. 7*, 289.

Dougherty, T. F., & White, A. (1945) *Am. J. Anat. 77*, 81.

Ernest, M. J., Chen, C. L., & Feigelson, P. (1977) *J. Biol. Chem. 252*, 6783.

Feigelson, P., & Kurtz, D. T. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47*, 275.

Feldman, D. (1974) *Endocrinology (Philadelphia) 95*, 1219.

Franz, J. M., & Knox, W. E. (1967) *Biochemistry 6*, 3464.

Giannopoulos, G. (1975) *J. Biol. Chem. 250*, 5847.

Gitlin, D., & Boesman, M. (1967) *J. Clin. Invest. 46*, 1010.

Gorin, M. B., Cooper, D. L., Eiferman, F., van de Rijn, P., & Tilghman, S. M. (1981) *J. Biol. Chem. 256*, 1954.

Hager, L. J., McKnight, G. S., & Palmiter, R. D. (1980) *J. Biol. Chem. 255*, 7796.

Houdebine, L. M. (1976) *Eur. J. Biochem. 68*, 219.

Houdebine, L. M., Devinoy, E., & Delouis, C. (1978) *Biochimie 60*, 57.

Innis, M. A., & Miller, D. L. (1979) *J. Biol. Chem. 254*, 9148.

Johnson, L. K., Baxter, J. D., & Rousseau, G. G. (1979) in *Glucocorticoid Hormone Action* (Baxter, J. D., & Rousseau, G. G., Eds.) p 305, Springer-Verlag, Berlin, Heidelberg, and New York.

Kioussis, D., Eiferman, F., van de Rijn, P., Gorin, M. B., Ingram, R. S., & Tilghman, S. M. (1981) *J. Biol. Chem. 256*, 1960.

Kurtz, D. T., Sippel, A. E., Ansah-Yiadom, R., & Feigelson, P. (1976) *J. Biol. Chem. 251*, 3194.

Liao, W. S. L., Conn, A. R., & Taylor, J. M. (1980a) *J. Biol. Chem. 255*, 10036.

Liao, W. S. L., Hamilton, R. W., & Taylor, J. M. (1980b) *J. Biol. Chem. 255*, 8046.

Liarakos, C. D., Rosen, J. M., & O'Malley, B. W. (1973) *Biochemistry 12*, 2809.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Marks, P. A., & Rifkind, R. A. (1978) *Annu. Rev. Biochem. 47*, 419.

Martial, J. A., Seeburg, P. H., Guenzi, D., Goodman, H. M., & Baxter, J. D. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4293.

Matuzik, R. J., & Rosen, J. M. (1978) *J. Biol. Chem. 253*, 2343.

Mintz, B., & Illmensee, K. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3585.

Miura, K., Law, S. W. T., Nishi, S., & Tamaoki, T. (1979) *J. Biol. Chem. 254*, 5515.

Murphy, R. F., Pearson, W. R., & Bonner, J. (1979) *Nucleic Acids Res. 6*, 3911.

Nickol, J., Lee, K. L., & Kenney, F. (1978) *J. Biol. Chem. 253*, 4009.

[2] Although the profound growth-inhibitory and cytotoxic action of glucocorticoids on lymphoid tissues has long been appreciated (Dougherty & White, 1945) and the suppressive effects of steroids on the induction of differentiated cell functions have been described [e.g., estrogen inhibits testosterone-mediated expression of α_{2u}-globulin in rat liver (Kurtz et al., 1979), progesterone blocks prolactin-induced casein in the mammary gland (Houdebine, 1976; Matuzik & Rosen, 1978), and DEX suppresses erythroid differentiation of erythroleukemia cells induced by dimethyl sulfoxide (Tsiftsoglou et al., 1979)], to our knowledge negative control over transcription by steroid hormones has been postulated only for ecdysone on certain genetic loci in insects (Ashburner et al., 1973).

Olson, P. S., Thompson, E. B., & Granner, D. K. (1980) *Biochemistry 19*, 1705.
Palmiter, R. D. (1974) *Biochemistry 13*, 3606.
Palmiter, R. D., & Carey, N. H. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 2357.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.
Perry, R. P., La Torre, J., Kelley, O. E., & Greenberg, J. R. (1972) *Biochim. Biophys. Acta 262*, 220.
Peters, T., Jr., & Peters, J. C. (1972) *J. Biol. Chem. 247*, 3858.
Ramsey, J. C., & Steele, W. J. (1976) *Biochemistry 8*, 1704.
Ringold, G. M., Yamamoto, K. R., Bishop, J. M., & Varmux, H. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2879.
Ruoslahti, E., & Terry, W. D. (1976) *Nature (London) 260*, 804.
Sala-Trepat, J. M., Savage, M., & Bonner, J. (1978) *Biochim. Biophys. Acta 519*, 173.
Sala-Trepat, J. M., Dever, J., Sargent, T. D., Thomas, K., Sell, S., & Bonner, J. (1979a) *Biochemistry 18*, 2167.
Sala-Trepat, J. M., Sargent, T. D., Sell, S., & Bonner, J. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 695.
Savage, M. J., Sala-Trepat, J. M., & Bonner, J. (1978) *Biochemistry 17*, 462.
Sell, S. (1974) *Cancer Res. 34*, 1608.
Steele, W. J., & Bush, M. (1967) *Methods Cancer Res. 31*, 61.
Tamaoki, T., Thomas, K., & Schindler, I. (1974) *Nature (London) 249*, 269.
Tilghman, S. M., Kioussis, D., Gorin, M. B., Ruiz, J., & Ingram, R. S. (1979) *J. Biol. Chem. 254*, 7393.
Tsiftsoglou, A. S., Gusella, J. F., Volloch, V., & Housman, D. E. (1979) *Cancer Res. 39*, 3849.
Young, H. A., Shih, T. Y., Scolnick, E. M., & Parks, W. P. (1977) *J. Virol. 21*, 139.

Reduction of Prostatic Binding Protein-Messenger Ribonucleic Acid Sequences in Rat Prostate by Castration†

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ABSTRACT: Messenger RNA coding for the three subunits of prostatic binding protein was isolated from polysomal RNA of rat ventral prostate by oligo(dT)-cellulose affinity chromatography and purified by repeated sedimentations through sucrose gradients under denaturing conditions. The purified mRNA migrated as a 9S peak in sucrose gradient centrifugation and hybridized with its cDNA within 2 log R_0t units. In a cell-free reticulocyte lysate system, the mRNA directed the synthesis of three polypeptides of 12 000, 9000, and 8000 daltons. These translation products were identified as the subunits of prostatic binding protein by immunoreaction with antibodies to this protein. Quantitation of prostatic binding protein-mRNA sequences in normal and castrated rats by hybridization with the cDNA probe showed that 3-day castration reduced the prostatic binding protein-mRNA sequences to less than 2% of the normal level. Similar hybridization was performed by using the cDNA to determine the level of prostatic binding protein coding sequences in polysomal poly(A) RNA following castration. The results showed a first-order rate constant of 3.92×10^{-2} h^{-1} for reduction of prostatic binding protein-mRNA sequences in polysomes. The period of castration required to reduce the level of these sequences to 50% of the normal level was calculated to be 17.6 h.

Testosterone regulates the levels of several mRNAs in rat prostate coding for androgen-specific proteins (Heyns & DeMoor, 1977; Parker et al., 1978). Among these, the only known protein is the prostatic binding protein (PBP),[1] which is probably the same protein as the α-protein (Fang & Liao, 1971; Chen et al., 1979), the estramustine binding protein (Forsgren et al., 1979), and prostatein (Lea et al., 1977, 1979). PBP is the most abundant protein synthesized and secreted by rat ventral prostate (Heyns et al., 1977). It consists of three subunits with molecular weights of 13 000, 11 000, and 8000 (Peeters et al., 1980). It is encoded by mRNAs in the abundant class of prostatic poly(A) RNA regulated by androgens (Parker & Scrace, 1978). Three-day castration results in a dramatic decrease in PBP-mRNA as well as cytosolic PBP (Heyns et al., 1977; Hiremath et al., 1981). In vitro translation of rat prostatic poly(A) RNA and immunoprecipitation of the translational product by anti-PBP serum have shown that synthesis of PBP-mRNA occurs early following androgen treatment, detectable 1 h after injection of testosterone to castrated rat (Hiremath et al., 1981).

In the present work, we have examined the androgen regulation of PBP-mRNA sequences in rat prostate by quantitation of PBP-coding sequences following androgen withdrawal. To this end, we have purified the PBP-mRNA from polysomal poly(A) RNA of normal rat prostate and synthesized cDNA complementary to the purified PBP-mRNA to determine the level of PBP-mRNA sequences by RNA excess hybridization. The results are reported herein.

Materials and Methods

Materials. Male Sprague-Dawley rats (300 g) were purchased from Harlan-Sprague Dawley, Madison, WI. Guan-

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[1] Abbreviations used: PBP, prostatic binding protein; TKM, 50 mM Tris-HCl, pH 7.9, 25 mM KCl, and 5 mM $MgCl_2$; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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