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Rat α_1-fetoprotein messenger RNA: 5'-end sequence and glucocorticoid-suppressed liver transcription in an improved nuclear run-off assay

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ABSTRACT

Cloned cDNA fragments spanning nearly the entire coding regions of rat AFP and albumin genes were used in liver nuclear run-off assays. Under standard assay conditions, transcription signals detected with 5' probes were systematically stronger than with 3' probes. Heparin eliminated this phenomenon, which suggests that nuclear run-off assays are subject to in vitro reinitiation occurring preferentially in promoter gene regions. Transcription in the presence of heparin indicates that very few polymerases are engaged on the AFP gene in adult rat liver. Dexamethasone treatment of developing rat liver results in the loss of transcribing polymerases from all regions of the AFP gene. Albumin gene transcription is unaffected. Inhibition of liver protein synthesis with cycloheximide does not modify the AFP gene suppressive action of dexamethasone. Glucocorticoid hormone receptors may thus directly interact with the AFP locus, blocking polymerase initiation. We also report the sequence analysis of rat AFP mRNA, which reveals the existence of two potential initiation codons on this molecule.

INTRODUCTION

If immature rat liver is exposed to high doses of glucocorticoid hormones, it prematurely differentiates: a spectrum of fetal functions are repressed and among these the α_1-fetoprotein (AFP) gene is silenced (1-4). This provides a powerful system to analyse down-regulation of genes by steroid hormones. While evidence is growing that steroid-inducible loci contain DNA sequences to which steroid receptors bind, resulting in enhanced gene activity, there is little information as yet on how steroid receptors operate to inhibit transcription.

Our previous work established that liver AFP gene suppression by dexamethasone is closely correlated, temporally and quantitatively, with the accumulation of dexamethasone-receptor complexes in liver nuclei (3). Here we present further data which indicate that dexamethasone acts by preventing polymerase initiation on the AFP gene, independent of protein synthesis. This strongly supports that glucocorticoid receptors directly interact with the AFP chromatin unit to suppress transcription.

These experiments involved the differential monitoring of AFP and albumin gene transcription in 5' versus 3' regions of the loci, and this uncovered in vitro artefacts with the liver nuclear run-off system which may overestimate gene activities in vivo; we describe a modified procedure which eliminates this problem. We also report the 5'-end 291-nucleotide sequence that remained undocumented for rat AFP mRNA.

MATERIALS AND METHODS

Rat AFP and albumin cDNAs

Cloning of cDNAs in pBR322 and pAT153 plasmids was performed as described before (3). Source material was from Sprague-Dawley rats. We obtained clones covering nearly the entire AFP cDNA by successive priming of total yolk sac RNA, starting with the (3'-end) pHDQ21Ø insert (ref. 3 and Fig. 1) and following Rosbash et al. (5) for cDNA/RNA hybridization, and previously described procedures (3) for cDNA elongation, double-strand polymerization, and cloning by the homopolymeric tailing method. Near-full-length AFP cDNA (pHDQ1Ø5) was constructed by ligating restriction fragments from overlapping inserts of pHDQ21Ø, pHDQ1ØØ, pHDQ1Ø2 and pHDQ1Ø3 (Fig. 1). For albumin, we screened 2 cDNA banks from adult rat liver (6, M. Affolter, C. Parent-Vaugeois and A. Anderson, unpublished results), initially with a cDNA probe synthesized from purified rat albumin mRNA (3), and subsequently with the pHDQ83Ø insert (Fig. 1). DNA was sequenced according to Maxam and Gilbert (7) or by the RNA-primed dideoxy procedure of McReynolds et al. (8). Sequence data were analysed with the computer programmes developed by Devereux et al. (9).

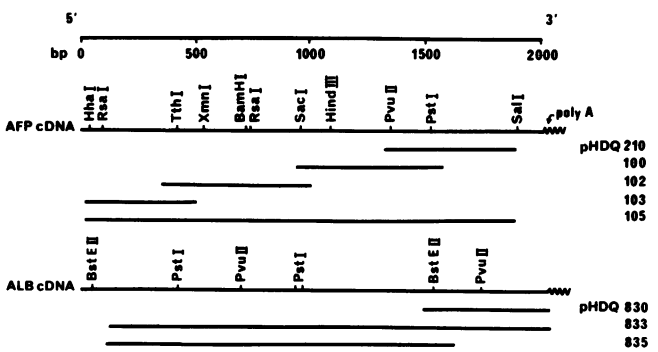

Fig. 1 Mapping of AFP and albumin cDNA fragments cloned in the PstI site of plasmids pAT153 (pHDQ1Ø5) or pBR322 (all others).

Nuclear transcription assay

Liver nuclei were isolated in the presence of spermine and spermidine, following the procedure of Marshall and Burgoyne (10). Nuclei (200 µg DNA) were incubated in a reaction mixture containing, in a final volume of 0.2 ml: 20 mM Hepes pH 7.5, 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 when specified 0.1 mg/ml of heparin (Sigma). After 30 min at 25°C, 2 mM vanadyl ribonucleoside complex, 80 µg of yeast tRNA and 6 µg of DNAseI were added, and the incubation was prolonged for 30 min at 25°C. The mix was then adjusted to 1X TES (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 1% SDS) and 40 µg of proteinase K, and incubated 1 h at 37°C. The mixture was then extracted twice with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1), once with 1 volume of chloroform:isoamyl alcohol (24:1), and the aqueous phase was ethanol-precipitated. The pellet was resuspended in 150 µl of 20 mM Hepes pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 2 mM vanadyl ribonucleoside complex, and treated with 40 µg/ml DNAseI for 1 h at room temperature. The mixture was adjusted to 0.5X TES and 0.15 M NaCl, phenol/chloroform-extracted as above, and the RNA was ethanol-precipitated three times. The final pellet was solubilized in 30 µl of 10 mM Tris pH 8, EDTA 1 mM, and used for hybridization to solid-phase plasmid DNA, as follows.

Plasmid DNA was boiled in 0.3 N NaOH for 11 min, quickly chilled, then let stand at room temperature 10-20 min, and adjusted to 2 M NH_4 acetate. DNA (3 µg) was spotted onto 8-mm nitrocellulose filters soaked in 20X SSC (1X: 0.15 M NaCl, 0.015 M sodium citrate), and the filters were vacuum-baked at 80°C for 2 h. Filters were prehybridized for 14-20 h at 42°C in 0.2 ml of 4X SSC and 50 mM $NaPO_4$, pH 7.0, containing 250 µg/ml yeast tRNA, 100 µg/ml poly(A), 0.02% each of Ficoll (Pharmacia), polyvinylpyrrolidone, and bovine serum albumin, 0.2% SDS 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 2-6x10^6 cpm of ^{32}P-labeled nuclear RNA, and 10^4 cpm of [^{3}H]cRNAs synthesized from cloned cDNA fragments according to Roop et al. (11). After hybridization, filters were washed with 2X SSC/0.1% SDS once at room temperature and once at 68°C, then once with 0.1X SSC/0.1% SDS at 68°C, then several more times at room temperature with 2X SSC. Filters were then treated with RNAse A (40 µg in 2 ml of 2X SSC, 1 h at room temperature) and washed 1 h at 68°C in 2X SSC/0.1% SDS. The radioactivity was then released with NaOH and counted (or

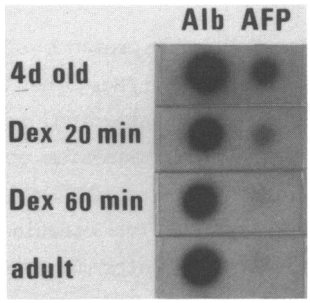

Fig. 2 Nuclear transcription assay: filter-bound albumin (pHDQ830) or AFP (pHDQ210) cDNA was hybridized with ^{32}P-labeled nuclear RNA from adult rat liver, and from 4 day old rat liver before and 20 or 60 min after one injection of dexamethasone (50 µg intraperitoneally); filters were then autoradiographed. Net cpm (counted after elution, see Table I) on albumin filters ranged from 104 to 168; they were 42, 14, 2 and 0 respectively on 4d, Dex 20, Dex 60 and adult AFP filters.

visualized by autoradiography, Fig.2). As little as 5-10 net cpm (cpm on recombinant DNA filter minus cpm on pBR322 filter) could be reliably measured with the assay. Counts from adult liver transcripts hybridized to albumin filters, and counts from fetal liver transcripts hybridized to AFP filters, increased linearly with ^{3}H-labeled-RNA inputs up to 10^{7} cpm.

Counts bound to AFP and albumin filters were converted to relative transcriptional rates (cpm bound per million input) by subtracting background cpm (pBR322 filters) and by correcting for hybridization efficiency (with [^{3}H] cRNAs) and for the fraction of primary gene transcript hybridizable to recombinant plasmid cDNA [rat albumin gene data were taken from Sargent et al. (12) and we took the rat AFP gene to be organized similar to mouse (13)]. We took into account the length of introns in the gene regions covered by the cDNAs, since intron transcripts are not measured in the assay: assuming random distribution of polymerases and uniform rate of transcription throughout the loci, a region with less introns will elongate more exons, i.e. generate more cDNA-hybridizable transcripts. Correction factors thus introduced for the fraction of total gene transcription monitored by pHDQ105, pHDQ103, pHDQ210, pHDQ833, pHDQ8355 and pHDQ830 were respectively 10.4, 44.8, 24, 8.1, 47.3 and 20.5. [For example, pHDQ8355 cDNA is 330-bp long, i.e. 2.2% of the 15-kb albumin gene. It starts in exon A and finishes in exon C, i.e. it represents 12.8% of this 2584-bp region (exons constitute 13.3% of the total gene). Thus, ppm conversion for 100 net cpm bound, with 4 x 10^{6} cpm input and 25%

efficiency, is: 100 x 45.5 x 1.04 ÷ 4 x 4 = 4732.] Optimized assays yield relative transcriptional rates comparable with 5', 3', or near-full-length probes, which seems to validate the premises of ppm calculations.

RESULTS AND DISCUSSION

Sequence of rat AFP mRNA

The AFP cDNA inserts of pHDQ103 and pHDQ100 were sequenced by the Maxam-Gilbert procedure from nucleotides 17 to 341, 935 to 1083 and 1331 to 1512 (numbering from cap site), and albumin cDNA inserts were sequenced from nucleotides 103 to 164 (pHDQ835) and 1540 to 1713 (pHDQ830). The first 16 nucleotides of rat AFP mRNA were identified by primer extension on total fetal rat liver RNA using the pHDQ103 HhaI-RsaI restriction fragment [γ-^{32}P]ATP end-labeled with T_4 polynucleotide kinase: this procedure overlapped (and confirmed) the Maxam-Gilbert data for nucleotides 17 to 51.

AFP sequences 935-1083, 1331-1512 and 292-341 matched previously reported data (14). Albumin sequences matched those of Sargent et al. (15) with the exception of a G instead of an A at position 146 (we also found a G in liver albumin mRNA from rats of the Buffalo strain).

The 5'-end 291-nucleotide sequence as yet undocumented for rat AFP mRNA is presented in Fig. 3. It reveals the existence of two potential initiation codons, 5 codons apart and in phase (i.e. they would generate the same peptide except for six N-terminal amino acids), with the second (3') codon corresponding to mouse (16) and human (17) initiation codons. Whether both initiation codons on the rat message are utilized remains to be ascertained.

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                          -24                 -20                      -11
                          MET LYS GLN PRO ALA THR MET LYS TRP SER ALA SER ILE SER
TCCGGCTTCTACCACTGTCTGGG ATG AAG CAG CCA GCA ACC ATG AAG TGG AGC GCA TCC ATT TCC  (65)
-10                                1                                  10
PHE LEU LEU LEU LEU ASN PHE ALA GLU PRO ARG VAL LEU HIS THR ASN GLU PHE GLY ILE
TTC CTT CTC CTG CTA AAT TTT GCT GAA CCC AGA GTA CTG CAC ACA AAT GAG TTT GGA ATA (125)
 11                                20                                 30
GLU SER THR LEU ASP SER SER GLN CYS PRO THR GLU LYS ASN MET PHE ASN VAL ALA THR
GAA TCC ACG TTA GAC TCT TCC CAA TGC CCG ACA GAG AAA AAT ATG TTT AAC GTA GCT ACC (185)
 31                                40                                 50
ILE VAL VAL ALA GLN PHE VAL GLN ASP ALA THR LYS ALA GLU VAL ASN LYS MET SER SER
ATT GTC GTT GCC CAG TTT GTT CAG GAT GCC ACA AAG GCC GAA GTA AAC AAA ATG AGT AGC (245)
 51                                60                                 70
ASP ALA LEU ALA ALA MET LYS GLU ASN THR GLY ASP GLY CYS LEU GLU ASN GLN LEU SER
GAT GCG TTG GCT GCA ATG AAG GAA AAC ACT GGC GAT GGG TGT TTA GAA AAC CAG CTA TCT (305)
 71                                80
VAL PHE LEU ASP GLU ILE CYS HIS GLU THR GLU LEU ...
GTG TTT CTG GAT GAA ATT TGC CAC GAG ACG GAA CTC ...
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Fig. 3 5'-end sequence of rat AFP mRNA. Potential initiation codons are boxed. Amino acid 1 is the N-terminus of mature rat AFP.

Rat AFP mRNA translated in cell-free systems yields a single translation product by analysis in 10% polyacrylamide gel electrophoresis (18 and personal communication of S. Nishi): the use of both initiation codons would generate primary translation products with molecular weights of 68650 and 67886, a difference that could have been missed. Nevertheless, it seems likely that translation of rat AFP mRNA would proceed from the first (5') codon, on the basis that both potential initiation codons are in a sequence context equally favourable to initiation (particularly a purine at position -3) (19): according to the ribosome scanning model (20), the first initiation codon encountered should be preferentially utilized. Compared to mouse and human pre-AFP's, rat pre-AFP may thus be expected to contain 6 extra N-terminal amino acids. On the other hand, counting from the initiation codon common to the three species, the rat signal sequence contains one less amino acid, which corresponds to the C-terminus residue, based on optimal nucleotide alignment (41/54 homology with both human and mouse) and the known N-terminus residue of secreted rat AFP (21). Secreted rat AFP shows no N-terminal heterogeneity [the 18-amino-acid N-terminal sequence reported by Peters et al. (21) is in agreement with our cDNA sequence], which further indicates that even if both initiation codons are in fact utilized, the two signal peptides are processed similarly to yield a single mature peptide.

Computer-assisted analysis of mRNA sequences, in reference to rat AFP cDNA from cap site to polyA junction (13 and this report), indicates 86% nucleotide homology of rat AFP mRNA with mouse AFP mRNA (16), 72% with human AFP mRNA (17) and 46% with rat albumin mRNA (15), and 83%, 65% and 31% amino acid homologies in the corresponding mature peptides. The mature rat AFP peptide is 587 amino acid long and its molecular weight is 65679.

Liver nuclear transcription assay

Nuclear "run-off" assays are gaining widespread acceptance as a mean to directly assess transcriptional activities of genes in vivo, independent of post-transcriptional processes. In this technique, nascent RNA chains of isolated nuclei are elongated in vitro in the presence of radioactive RNA precursors and the amount of newly synthesized radiolabeled RNA is taken to reflect the activity of RNA polymerases engaged in transcription in vivo. One assumption of this system is that reinitiation of transcription in vitro is negligible. When cDNA fragments spanning either 5' or 3' coding regions of rat AFP and albumin genes were tested in our standard liver nuclear run-off system (3), we systematically detected 5' transcription signals stronger than 3' signals (Table I, A); in adult rat liver, 3' probes indicated null AFP gene

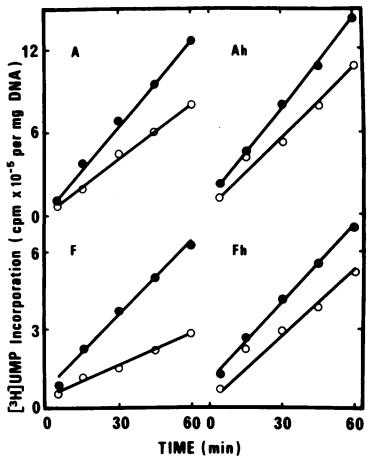

Fig. 4 RNA polymerase activities of isolated nuclei from adult (A) and fetal (20 day) (F) rat livers. Transcription was conducted as described in the text, without or with 0.1 mg/ml of heparin (h). Polymerase II (O) and polymerases I + III (●) activities were distinguished with α-amanitin (2 µg/ml).

activity, whereas relative transcriptional rates in the order of 1000 ppm were consistently recorded with 5' probes. One possible explanation for this phenomenon was that reinitiation was occurring in vitro in the 5' region of both AFP and albumin genes. To test this, we analysed the transcriptional effects of heparin in the run-off assay: heparin has been shown to inactivate free RNA polymerases and to inhibit initiation but not elongation on chromatin or DNA templates (22-23 and references therein).

The addition of heparin at 0.1 mg/ml in the nuclear transcription mixture resulted in significant changes in transcriptional activities of isolated rat liver nuclei. It increased total RNA synthesis by 20-30%: both α-amanitin-sensitive (polymerase II) and α-amanitin-resistant (polymerases I + III) activities were stimulated, proportionnally more so for polymerase II, whose relative contribution increased as much as 10-15% in fetal liver nuclei (Fig. 4). These results are consistent with similar analyses conducted with rat liver nuclei at higher concentration of heparin (23). Probing liver transcription with AFP and albumin cDNAs (Table I, B) provided further information. Heparin roughly equalized the relative transcriptional rates measured with 5', 3' or full-length cDNAs: they remained in the ranges recorded with 3' probes in the absence of heparin. A 5' AFP signal was no longer detected in adult rat liver. These data seem to rule out that 5'/3' differences (in the absence of heparin) might relate to 5' clustering of

Table I: AFP and Albumin Gene Transcription in Nuclear Cell-free System

Experiment	Liver[a]	Heparin[b]	UTP in RNA	Input	RNA hybridized[c]							AFP transcription[d]			Alb transcription[d]		
					pBR322	AFP recombinants			Albumin recombinants			Total	5'	3'	Total	5'	3'
						pHDQ105 (Total)	pHDQ103 (5')	pHDQ210 (3')	pHDQ833 (Total)	pHDQ8355 (5')	pHDQ830 (3')						
			cpm/mg DNA x 10^{-7}	cpm x 10^{-6}	cpm	cpm	cpm	cpm	cpm	cpm	cpm	ppm	ppm	ppm	ppm	ppm	ppm
A	20-day fetal	-	21	4	62 56		149 155	103 106		216 242	220 205		4170	1092		8041	3146
	5-day old	-	12	2	36 38		78 79	60 56		137 128	144 135		3718	1382		9034	4203
	22-day-old	-	33	4	52 47		69 70	46 46		296 281	266 260		896	0		11305	4377
	adult	-	26	4	48 50		71 69	49 46		253 250	238 248		941	0		9578	3977
B	4-day-old	+	15	3.5	7 8	144 143	46 47	66 66	503 463			1617	1997	1605	4402		
	4-day-old DEX 20 min	+	16	3.5	11 13	60 61	27 26	36 27	510 439			577	742	535	4281		
	4-day-old DEX 1 h	+	15	3.5	13 13	18 23	15 15	17 15	452 431			89	102	82	3967		
	adult	+	28	6	19 18	17 14	20 17	16 19	863 794			0	0	0	4374		
C	4-day-old																
	saline 25 min	+	29	3.8	35 35	157 164						1374					
	cyclo 25 min	+	18	3.8	35 40	172 165						1434					
	saline 25/DEX 20 min	+	24	3.8	29 33	92 93						673					
	cyclo 25/DEX 20 min	+	21	3.8	46 49	108 117						712					

[a] Liver pools were obtained from 30-40 fetuses from 3-4 litters, 3-6 newborns from 3 litters, or 3 adults; saline, dexamethasone (DEX) (50 µg), and cycloheximide (cyclo) (10 µg/g) were injected intraperitoneally at the indicated times before sacrifice. [b] Nuclear transcription assays were conducted without (-) or with (+) 0.1 mg/ml of heparin. [c] Nuclear cell-free transcribed [^{32}P]UTP-labeled RNA ("UTP in RNA") was hybridized ("Input") to filter-bound DNA (3 µg) from pBR322 plasmid or from recombinant pBR322 or pAT153 plasmids containing (see Fig. 1) near-full-length AFP cDNA (pHDQ105), near-full-length albumin cDNA (pHDQ833), a 5'-end fragment of AFP cDNA (pHDQ103), a 3'-end AFP fragment (pHDQ210), or a 3'-end albumin fragment (pHDQ830); pHDQ8355 filters were loaded with the 5'-end PstI/plasmid albumin restriction fragment mixed with pBR322 DNA at a ratio comparable to that of recombinant plasmids DNA; data are counts from duplicate filters, with instrument background (≈20 cpm) subtracted. [d] Relative transcriptional rates were calculated from average cpm on AFP and albumin filters, after subtraction of pBR322 cpm, converted in parts per million (cpm hybridized per million input) with correction for hybridization efficiency and fraction of primary gene transcript hybridizable to cDNA (see under Materials and Methods).

polymerases in vivo (and premature termination of transcription in vitro could not explain the results since 5' and 3' regions are >9 kb apart and RNA is only elongated a few hundred nucleotides in the assay).

The most likely interpretation of these combined observations seems to be that in the absence of heparin, reinitiation occurs in isolated nuclei, preferentially on polymerases I + III genes and in the 5' (presumably promoter) region of polymerase II genes; in the presence of heparin, reinitiation is inhibited and elongation is enhanced, resulting in a ≈25% net increase in RNA synthesis. For the specific genes studied here, even if it were to exert differential elongation effects on liver polymerases classes, the use of heparin resulted in an important gain of accuracy: in its absence, the 5' nuclear assay strongly overestimated the relative transcriptional rates of both AFP and albumin genes, particularly AFP gene activity in adult liver [reinitiation on the AFP promoter in adult liver seems fit with the observation that AFP chromatin, although nearly silenced in the adult state, remains in an extended (nuclease-sensitive) configuration (24), presumably readily accessible to free RNA polymerases]. In the light of these observations, it would appear that nuclear run-off systems should take into account possible reinitiation artefacts, especially in low ionic conditions and with probes proximal to gene promoters.

AFP gene control in developing and glucocorticoid-treated livers

The initial objective of this work was to further define the mechanisms by which the AFP gene is turned off during normal liver development and in response to glucocorticoid hormones. Specifically, we wanted to distinguish between initiation and elongation of RNA polymerases on the AFP gene, and to assess the possible induction of an AFP gene repressor by glucocorticoids.

The results presented above clearly indicate that, in the conditions of our transcription assay, all polymerases engaged on AFP or albumin genes in vivo freely elongate RNA chains in vitro: this is shown, in particular, by the fact that in adult rat liver, RNA synthesis is detected on the AFP gene in the absence of heparin. The data obtained in the presence of heparin, which stimulates elongation, can thus safely be taken, within the sensitivity limit of the assay, as a reflection of the maximum number of polymerases having formed initiation complexes in vivo.

The lack of any detectable transcription signal in adult liver nuclei in the presence of heparin thus points to the developmental control of the AFP gene being at the level of polymerase initiation, i.e. in spite of the "open" conformation of AFP chromatin in adult liver (24), very few polymerases

appear to form transcriptional complexes with the AFP gene. This is in contrast with another developmentally regulated system, the globin system, in which clustering of polymerases was found at the 5' end of the β-globin genes in mature (transcriptionally inert) hen erythrocytes (25): this finding suggested that developmentally suppressed genes might be controled at an elongation step, analogous to attenuation in prokaryotes. The AFP data would indicate that such a mechanism is not general.

Dexamethasone administered to immature rats abruptly curtails liver AFP gene transcription and the decline is the same whether monitored with 5', 3' or full-length cDNAs (Table I, B, and Fig. 2). This again strongly suggests that polymerases are disengaged from all regions of the AFP locus, i.e. glucocorticoid hormones would act by preventing the formation of transcriptional complexes on the AFP gene. The relative transcriptional rate of albumin, the adult member of the AFP/albumin gene family (13-14), is unchanged by dexamethasone (we monitored up to 6 h after one injection of 50 μg). This extends our previous data showing that dexamethasone does not change the relative amount of albumin mRNA in total cellular or nuclear RNA of newborn rat liver (3,26). In adult rat liver treated with dexamethasone, Danesh et al. (27) also found no change in the accumulation of albumin mRNA, but they did observe a two-fold increase in the relative rate of albumin gene transcription. The reason for this difference is unclear; there is perhaps a transient stimulation of RNA polymerase II by dexamethasone in adult but not in neonatal liver (27).

To assess whether ongoing protein synthesis is required for the suppressive action of dexamethasone on the AFP gene, newborn rats were treated with cycloheximide, then challenged with dexamethasone (Table I, C). Cycloheximide suppressed liver protein synthesis by >85% [as monitored by radioactive amino acid incorporation into trichloroacetic acid-precipitable material (26) from the time of dexamethasone injection to the time of liver processing] but it had no significant effect on the AFP gene-suppressive action of dexamethasone. These results seem to eliminate the possibility that glucocorticoid hormones might act by inducing an AFP gene repressor.

These combined observations strongly reinforce the view that glucocorticoid hormones turn off the AFP gene by direct down-interaction of their receptors with AFP chromatin, plausibly by binding to 5'-flanking receptor recognition sites (28) to block polymerase access to the gene. The exact mechanism by which receptor interaction might result in the activation

of certain genes and the extinction of others, remains to be elucidated. Glucocorticoid receptors perhaps dislodge an activator from the AFP gene.

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