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Glucocorticoid-mediated induction of α_1-acid glycoprotein: Evidence for hormone-regulated RNA processing

(*in vitro* transcription/gene expression/glucocorticoid action)

JAMES L. VANNICE*, JOHN M. TAYLOR†, AND GORDON M. RINGOLD*

*Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305; and †Gladstone Foundation Laboratories, University of California at San Francisco, 2550 23rd Street, P.O. Box 40608, San Francisco, CA 94140

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ABSTRACT **We have studied the glucocorticoid-mediated accumulation of α_1-acid glycoprotein (AGP) mRNA in HTC rat hepatoma cells. In contrast to the well-characterized primary response of mouse mammary tumor virus, *in vitro* transcription assays in isolated nuclei show that the rate of transcription of the *AGP* gene is high even in the absence of hormone. Despite the constitutive transcription of the *AGP* gene, no detectable AGP RNA can be found in either the cytoplasm or the nuclei of untreated cells. Previous experiments have shown that the glucocorticoid induction of AGP RNA requires ongoing protein synthesis. In conjunction with the present study, our data suggest that glucocorticoids stimulate accumulation of AGP RNA by inducing an RNA processing factor that allows production of stable transcripts.**

The acute phase reactant α_1-acid glycoprotein (AGP), also called orosomucoid, is one of several plasma proteins synthesized by the liver in response to various stressful stimuli. Physical trauma such as surgery or wounding, bacterial infection, or nonspecific inflammatory stimuli such as subcutaneous injection of turpentine elicit the so-called acute-phase response (for review, see ref. 1). In addition to AGP, whose physiological function remains obscure, a variety of protease inhibitors (e.g., α_1-anti-trypsin) and iron scavengers (e.g., haptoglobin and hemopexin) are also induced. However, the mechanisms by which acute-phase reactants are induced are poorly understood. Macrophage factors, in particular interleukin I (2), and glucocorticoids (3, 4) have both been implicated in induction of some acute-phase reactant proteins. We (5), Baumann *et al.* (6), and Feinberg *et al.* (7) have recently shown that AGP and its mRNA are induced several hundred-fold by glucocorticoids in the rat hepatoma cell line HTC.

Glucocorticoids, and steroid hormones in general, act by altering the rates of transcription of specific genes. The model for steroid hormone action, originally proposed by Jensen *et al.* (8) and Gorski *et al.* (9), entails hormone binding to a soluble cytoplasmic receptor protein, resulting in a structural alteration called activation, followed by accumulation of steroid–receptor complexes in the nucleus. The nuclear complexes bind with high affinity to specific DNA sites adjacent to regulated genes (10–12) and, by as yet unidentified action(s) at these regulatory loci, stimulate gene transcription (13, 14). A limited number of genes exhibiting this kind of regulation by steroid–receptor complexes have recently been characterized. In the case of glucocorticoids, mouse mammary tumor virus (MMTV) DNA has been shown to contain regions of DNA that exhibit specific binding of the glucocorticoid–receptor complex and that are necessary and sufficient for hormone-regulated expression (for review, see ref. 15).

The gene encoding AGP, however, appears to be regulated in quite a different manner. The several hundred-fold increase in AGP RNA observed in glucocorticoid-treated HTC cells is dependent on ongoing protein synthesis (5–7); this is in contrast to the induction of MMTV RNA, which proceeds normally in the absence of protein synthesis (16, 17). Thus, the induction of AGP RNA by glucocorticoids is a secondary action of the hormone, presumably mediated by a protein that is induced by direct action of the glucocorticoid–receptor complex. In this manuscript, we describe experiments showing that the induction of AGP RNA occurs at a post-transcriptional level. Our data implicate a glucocorticoid-regulated RNA processing system in the production of AGP RNA.

MATERIALS AND METHODS

Cells and Cell Culture. The cell lines used in these studies were derived from the rat hepatoma cell line HTC (18). JZ.1 and MSC1 are HTC-derived cell lines containing 1 and ≈10 MMTV proviral copies, respectively. They are described in more detail elsewhere (19). These cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% newborn calf serum and 3% fetal calf serum (Irvine Scientific) at 37°C in 5% CO_2/95% air when grown in monolayer culture. When grown in suspension culture at 37°C, Swims S-77 medium (GIBCO) supplemented with newborn calf serum and fetal calf serum, as described above, was used. Dexamethasone (Sigma) was added to cell cultures from a 10 mM stock in 95% ethanol, to a final concentration of 1 μM, except when noted otherwise.

RNA Isolation and Hybridization. Total cellular RNA was isolated by lysing cells in a 4 M guanidine thiocyanate solution and spinning the cell lysate through a 5.7 M CsCl cushion as described by Chirgwin *et al.* (20). RNA was then blotted onto nitrocellulose using a Hybri-dot manifold (Bethesda Research Laboratories) at the concentrations indicated. Filters were baked *in vacuo* for 2 hr at 80°C and hybridized essentially as described by Thomas (21). The plasmids pAGP663 (22) and pJM7.1a (a plasmid containing most of the MMTV genome) were nick-translated (23) to a specific activity of 2×10^8 dpm/μg, and the hybridizations were done at a concentration of 2×10^6 dpm/ml.

Nuclear Transcription Assays. Cells were grown in suspension cultures at a density of $\approx 3 \times 10^5$/ml as described above and then incubated with 1 μM dexamethasone for the indicated times prior to isolation of nuclei. Preparation of nuclei for RNA polymerase elongation reactions was essentially as described by Stallcup and Washington (24). Cells were washed once with phosphate-buffered saline (P_i/NaCl) and then scraped into P_i/NaCl at 0°C, using a rubber policeman. All subsequent steps were carried out at 0–4°C. Cells were centrifuged for 5 min at 600 × *g*, washed once with ice-cold

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Abbreviations: AGP, α_1-acid glycoprotein; MMTV, mouse mammary tumor virus; P_i/NaCl, phosphate-buffered saline; SSPE, 180 mM NaCl/10 mM $NaPO_4$, pH 7.7/1 mM EDTA.

P_i/NaCl, centrifuged again, and resuspended in hypotonic buffer (20 mM Tris/HCl, pH 8.0/4 mM $MgCl_2$/6 mM $CaCl_2$/0.5 mM dithiothreitol) and held on ice for 5 min. An equal volume of lysis buffer (0.6 M sucrose/0.2% Nonidet P-40/0.5 mM dithiothreitol) was added and the cells were homogenized in a tight-fitting Dounce homogenizer. Nuclei were pelleted by centrifugation at 1000 × *g*, washed once in resuspension buffer (0.25 M sucrose/10 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/1 mM dithiothreitol), repelleted, and finally resuspended in reaction buffer [50 mM Hepes, pH 8.0/90 mM NH_4Cl/5 mM $MgCl_2$/0.5 mM $MnCl_2$/2 mM dithiothreitol/0.1 mM EDTA/0.4 mM each of ATP, CTP, and GTP/10% (vol/vol) glycerol/bovine serum albumin (10 μg/ml)] at a concentration of ≈10^8 nuclei per ml. [α-^{32}P]UTP (400–600 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) was added to a concentration of 300 μCi/ml, and the nuclei were incubated with vigorous shaking for 40 min at 26°C. Preliminary experiments indicated that incorporation was maximal by 30–40 min, and no degradation of newly synthesized RNA occurred during a chase period of equal duration in the presence of excess unlabeled UTP (data not shown).

RNA was prepared from the nuclei as described by Smith *et al.* (25). Briefly, the reaction was diluted with 5 vol of H_2O containing 100 μg of yeast RNA as carrier. $NaDodSO_4$ was added to 0.4%, and after gentle mixing an equal volume of 100 mM Na acetate, pH 5.0/20 mM EDTA, was added. Proteins and high molecular weight DNA were removed by extraction with H_2O-saturated phenol, and the RNA was collected by two successive precipitations with EtOH.

The ^{32}P-labeled products (10^7 dpm) from each reaction were hybridized to nitrocellulose filters containing 3 μg of denatured linear plasmid DNA (prepared by boiling in 0.1 M NaOH for 5 min, followed by addition of 1.5 vol of ice-cold 20× SSPE) in 200 μl of 50% formamide/5× SSPE (1× SSPE = 180 mM NaCl/10 mM $NaPO_4$, pH 7.7/ 1 mM EDTA)/2× Denhardt's solution (26)/yeast RNA (200 μg/ml) at 37°C for 4 days with gentle mixing. The DNA-bearing filters were pre-hybridized in the same buffer (without ^{32}P-labeled RNA) for >4 hr. Filters were washed once with 2× SSPE/0.1% $NaDodSO_4$ at room temperature for 15 min, and then with 0.1× SSPE/0.1% $NaDodSO_4$ twice at 50°C for 15 min and twice at 60°C for 15 min. The filters were then exposed to film at −70°C, with a Cronex intensifying screen. Preliminary experiments indicated a linear relationship between the amount of probe used per hybridization and the signal detected by this assay; hybridizations were carried out in DNA excess.

Analysis of RNA from Nuclei and Cytoplasm. Cells were lysed and nuclei were isolated exactly as described for nuclear transcription assays. After Dounce homogenization and centrifugation, the cytosol was saved and centrifuged once more; the nuclei were washed and centrifuged twice with resuspension buffer. Three volumes of 4 M guanidine thiocyanate/10 mM EDTA was added to the cytosol solution; the nuclear pellet was resuspended directly in the guanidine thiocyanate and the RNA was isolated by centrifugation through CsCl (20). RNA was electrophoresed through a 1.4% formaldehyde agarose gel as described (27) and transferred to ATP paper (28). The filter was probed with nick-translated pAGP663 (22) and then with the actin probe pHFβA-1 (29).

Southern Analysis of JZ.1 Genomic DNA. Cellular DNA was isolated as described (30), digested with various restriction enzymes, and analyzed essentially as described by Southern (31).

RESULTS

We have recently found that glucocorticoids induce a several hundred-fold accumulation of AGP RNA in HTC cells (5). Furthermore, the hormonal effect on AGP RNA levels is dependent on ongoing protein synthesis. The increase in abundance of a specific mRNA species in a cell may be due to an increase in the rate of synthesis, a decrease in the rate of degradation, or a combination of these processes. To determine whether the rate of synthesis of AGP mRNA changes in response to glucocorticoids, we carried out *in vitro* transcription assays in isolated nuclei from treated and untreated cells. In this assay, nuclei are isolated at 0°C and the nascent transcripts in these nuclei are allowed to elongate in the presence of [α-^{32}P]UTP. Elongated transcripts are then isolated and hybridized to nitrocellulose filters containing DNA from specific genes. The amount of radioactive RNA hybridized to each dot is, therefore, a reflection of the relative number of transcripts being synthesized when the nuclei were isolated. Fig. 1 shows the results of such an assay after autoradiography of these dots. JZ.1 cells exhibit an increase in transcription rate of MMTV RNA in response to 1 μM dexamethasone, while both actin and AGP message synthesis remain nearly constant. Furthermore, MSC1 cells, which contain 10 MMTV proviral copies (as opposed to the single copy in JZ.1 cells), show a proportional increase in MMTV mRNA synthesis over JZ.1 cells in both the presence and absence of dexamethasone, while AGP transcription rate is constant and equal to that seen in JZ.1 cells. The time course of the effect of dexamethasone on the transcription rate of these two genes is shown in Fig. 2*A*. The synthetic rate of MMTV mRNA is maximal between 30 and 60 min after addition of hormone [this is slightly longer than the time required in mouse mammary tumor cells (32, 33)]. However, AGP mRNA shows little, if any, increase in synthesis for times up to 4 hr. Induction of mature AGP mRNA has occurred in the hormone-treated cells, however, because cytoplasmic RNA isolated from these same cells at the time of preparing the nuclei exhibits a dramatic increase in AGP mRNA abundance (Fig. 2*B*).

Since it appears that the *AGP* gene is being constitutively transcribed, we analyzed RNA isolated from the cytoplasm and nuclei of dexamethasone-treated and untreated cells to see if we could detect accumulation of short-lived, but sta-

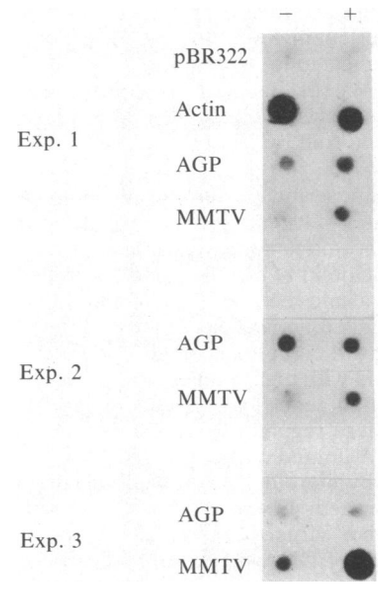

FIG. 1. Nuclear transcription assays of AGP. JZ.1 (Exp. 1 and 2) or MSC1 cells (Exp. 3) were grown in suspension culture and treated without (−) or with (+) 1 μM dexamethasone for 1 hr, after which cells were isolated and *in vitro* transcription assays were done. α-^{32}P-labeled RNA (10^7 dpm) was incubated with filters containing denatured DNA plasmids, as indicated, for 4 days at 37°C, washed, and autoradiographed.

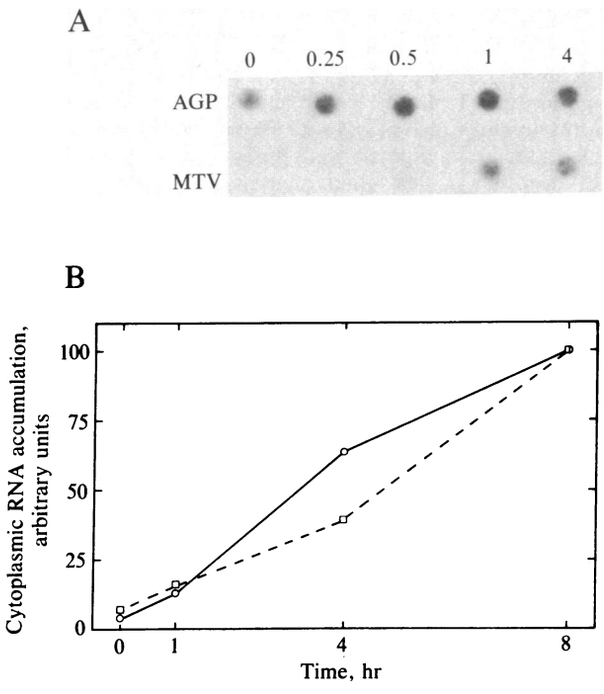

FIG. 2. Time course of alterations in transcription rate and cytoplasmic accumulation of AGP and MMTV sequences in response to dexamethasone treatment. (*A*) Nuclear transcription assays were carried out using nuclei from JZ.1 cells collected at various times (in hr) after addition of 1 μM dexamethasone; 5×10^6 dpm were incubated with each pair of filters. (*B*) Cytoplasmic RNA was isolated from the same cells and analyzed for AGP (○) and MMTV (□) RNA accumulation. Autoradiograms were scanned with an optical densitometer, and the densities were plotted relative to the 8-hr time point.

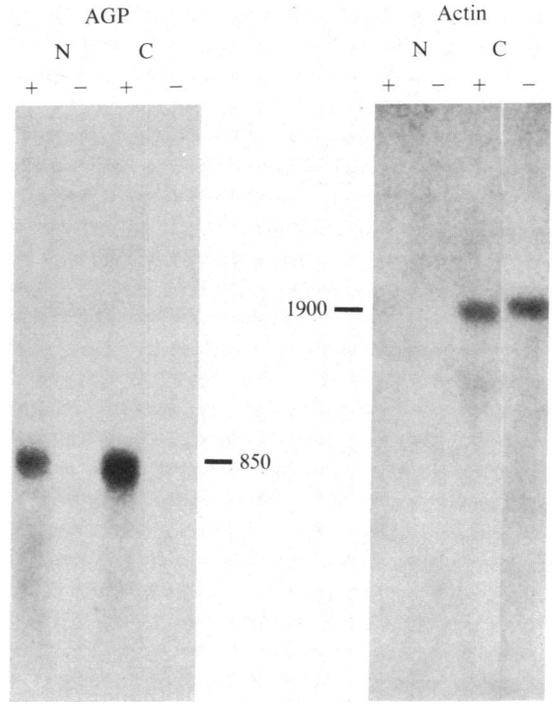

FIG. 3. RNA blot analysis of nuclear and cytoplasmic RNA from dexamethasone-treated and control cells. Ten micrograms of nuclear (N) and cytoplasmic (C) RNA from dexamethasone-treated (+) and control (−) cells were electrophoresed on a 1.4% formaldehyde agarose gel and transferred to ATP paper. The filter was probed sequentially with AGP nick-translated probe and then with β-actin nick-translated probe (after removal of all AGP probe by washing twice in 50% formamide/10 mM EDTA at 70°C for 30 min) at a concentration of 2×10^6 dpm per ml of hybridization mix. Molecular sizes are given in bases.

ble, nuclear transcripts in the nuclei of untreated cells. Fig. 3 shows that, although there is a large amount of AGP RNA present in the nuclei of induced cells, there are no detectable AGP transcripts in the nuclei of untreated cells. The same filter probed with actin cDNA shows nearly identical levels of actin mRNA in the cytosol of both control and hormone-treated cells and, under these conditions, undetectable nuclear RNA levels. Since only total rather than polyadenylylated nuclear RNA was analyzed in this experiment, it is possible that rare precursors might have been missed. Nevertheless, it is clear that a large pool of AGP transcripts does not accumulate in the nuclei of induced HTC cells.

The possibility that the *in vitro* transcription assay is measuring the production of a constitutively expressed repetitive sequence present in the AGP cDNA was tested in several ways. First, Table 1 shows the results of hybridization of radioactive transcription products to different parts of the AGP cDNA insert in pAGP663. Little, if any, induction of AGP transcription is observed using either the 3′ or 5′ segments of the cDNA. It seems unlikely that both halves of the cDNA would contain repetitive sequences. Second, Southern analysis of JZ.1 DNA cut with several restriction enzymes reveals, at most, four restriction fragments after *Bam*HI digestion and two after *Eco*RI or *Sal* I digestion (Fig. 4). Since this filter was hybridized and washed at relatively high stringency, we hybridized identical filters containing *Eco*RI-digested JZ.1 DNA at decreasing stringency (from 50% to 20% formamide) and washed at low stringency (3 times for 30 min each in 1× SSPE/0.1% $NaDodSO_4$ at 40°C) and saw virtually no change in the banding pattern (data not shown). We conclude from these results that the AGP cDNA is homologous to a single or low copy gene. Thus this DNA is not detecting a highly expressed repetitive sequence but rather *bona fide* AGP RNA in our nuclear transcription assays.

DISCUSSION

Glucocorticoid treatment of HTC cells leads to a dramatic increase in the production of AGP and its mRNA (5–7). Since the increased accumulation of AGP mRNA is blocked by cycloheximide (5–7), we performed experiments to examine the mechanism by which this indirect (secondary) effect of glucocorticoids occurs. The hormone-stimulated production of AGP mRNA in these cells could be due to an increase in the rate of its synthesis and/or a decrease in the rate of its degradation. We have shown in this report that the transcription rate of the *AGP* gene, as measured by *in vitro* nuclear

Table 1. Analysis of the transcription rates of MMTV and AGP

DNA	-fold induction
MMTV (pJM7.1a)	≥12
AGP (pAGP663)	1.2
AGP (pAGP663, fragment A)	1.3
AGP (pAGP663, fragment B)	0.8

Nuclear transcription assays were performed using nuclei from control cells and cells treated with 1 μM dexamethasone for 1 hr. Filters containing the various DNAs were incubated with these probes. The autoradiograms were scanned and the ratio of the amount hybridized in the treated sample to the amount hybridized from the control probe is listed as the -fold induction. pAGP663 was cut with *Sal* I, yielding 2 fragments—one containing the 3′ 250 bases (fragment A) and the other containing the 5′ 450 bases (fragment B) of the AGP cDNA insert of pAGP663 (22). AGP mRNA is ≈850 bases long (Fig. 4).

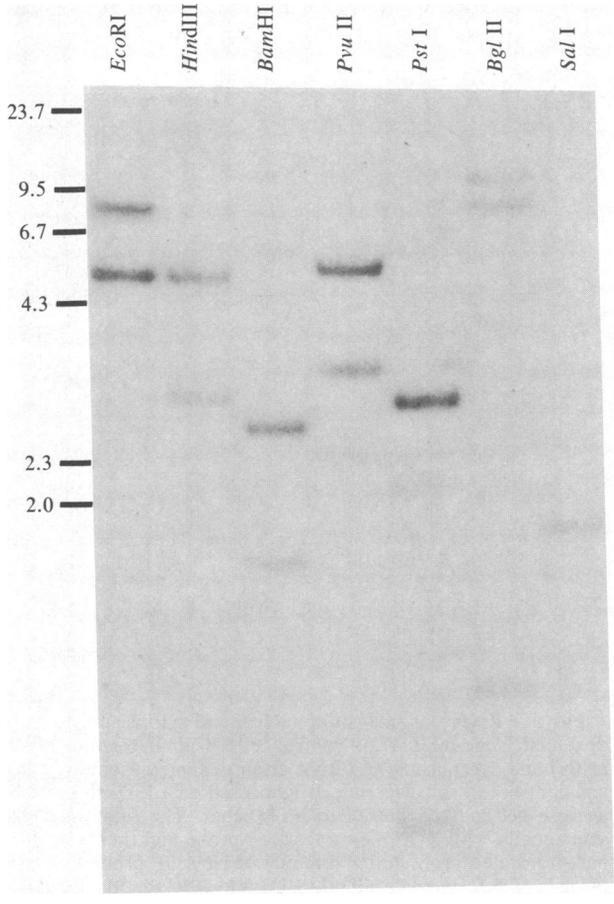

FIG. 4. Southern analysis of JZ.1 DNA. Ten micrograms of total genomic JZ.1 DNA was cut with various restriction enzymes, electrophoresed, and transferred to nitrocellulose paper as described (31). The digests were monitored by adding phage λ DNA to a small amount of the reaction mixture and checking completion of the phage λ digest. The filter was probed with 2×10^6 dpm per ml of nick-translated AGP cDNA insert [obtained as the *Pst* I fragment from pAGP663 (22)] and autoradiographed. Size markers were phage λ DNA cut with *Hin*dIII, run on the same gel, stained with ethidium bromide, and are expressed as kilobase pairs.

transcription assays, does not seem to be affected by the hormonal status of the cells. Moreover, since there is no detectable steady-state level of AGP mRNA in nuclei of untreated HTC cells (Fig. 3), there must be very rapid processing or degradation of nascent or newly synthesized AGP transcripts. We suggest that this step is the point at which glucocorticoids affect the production of mature AGP mRNA.

Glucocorticoids and other steroid hormones appear to regulate gene expression by forming a hormone–receptor complex that interacts directly with regulatory regions residing on inducible genes. The most thoroughly studied example of this type of regulation is that of MMTV, in which transcription is directly stimulated by an apparent high-affinity interaction of the glucocorticoid–receptor complex with DNA sequences adjacent to the promoter (15). The induction of AGP, in contrast, seems to involve the maturation of nuclear AGP transcripts. Although there are several examples of hormone effects on turnover rates of specific RNAs (34–36), to our knowledge, this is the first description of nuclear RNA processing or stabilization that is regulated by steroid hormones. Since inhibitors of protein synthesis abrogate the induction of AGP RNA, we surmise that the product of a gene that is induced directly by the glucocorticoid–receptor complex (or the decay of a very labile protein) is responsible for altering the processing of AGP transcripts. Indeed, this mediator may facilitate the coordinated expression of several acute-phase proteins that appear to be under glucocorticoid control (1, 5, 6). This mechanism of regulating gene expression may also play a role in other systems [e.g., ecdysone-inducible middle and late puffs in *Drosophila* polytene chromosomes (37)] in which ongoing protein synthesis is required for induction.

There are several possible models that can be used to explain the experimental results presented here. The first is that a nuclease specific for AGP RNA is present in the nuclei of uninduced cells; the product of a glucocorticoid-inducible gene might simply inhibit this enzyme. Alternatively, glucocorticoids may induce a factor that stabilizes nuclear AGP transcripts, perhaps by binding directly to them. Lastly, the hormone may cause the production of an RNA processing protein that is required for maturation of the primary transcript. In this vein, several examples of tissue-specific processing of transcripts from the same gene have recently been described. For example, vimentin (38) and fibronectin (39) transcripts are processed differentially in various tissues, yet they give rise to indistinguishable or very similar proteins. Perhaps more germane, the calcitonin gene gives rise to a message that encodes calcitonin in the thyroid and another message that encodes a related peptide hormone or neurotransmitter in the brain (40). An apparently similar RNA processing mechanism gives rise to membrane vs. secreted forms of immunoglobulins (41, 42). Analysis of genomic clones of AGP and further studies on the AGP transcription unit will be required to clarify the details of the hormonally regulated process described here.

The concerted physiological actions of glucocorticoids appear to use multiple modes of regulating gene expression. In addition to direct stimulation of gene transcription (32, 33), the products of glucocorticoid-inducible genes themselves may have profound effects on subsequent regulatory events. Firestone *et al.* (43) have recently reported the induction of a glycoprotein processing system by glucocorticoids that could have marked effects on membrane function. The results of our experiments on regulation of AGP RNA implicate the existence of a glucocorticoid–regulated RNA processing or stabilizing system that may be responsible for altering the expression of a large set of genes.

Note Added in Proof. We have recently analyzed the products of nuclear transcription reactions with a single stranded AGP DNA (cloned in the phage M13) that only detects AGP RNA of the same polarity as the mRNA strand. As observed with double-stranded AGP DNA, there is no effect of dexamethasone on synthesis of AGP "sense"-strand RNA.

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