Regulation of Mouse Haptoglobin Synthesis

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ABSTRACT A cloned line of mouse hepatoma cells (Hepa-1) responded to treatment with dexamethasone by a 30–80-fold increase in synthesis and secretion of functional haptoglobin. Under the same conditions, the production of albumin was only slightly elevated whereas that of α_1-fetoprotein was reduced by 50%. The hormone concentration for half-maximal stimulation of haptoglobin synthesis was between 1 and 2 × 10^{-8} M. The time course of induction is characteristic for a glucocorticoid-regulated protein. Cell-free translation of RNA indicated an increase in the amount of functional haptoglobin mRNA that can account for the change in the protein production. To correlate our findings on Hepa-1 cells with those on nontransformed liver cells, we tested the hormonal response of isolated hepatocytes in tissue culture. Haptoglobin was first synthesized and secreted by hepatocytes from 17–19-d-old fetuses. But neither prenatal nor adult hepatocytes showed a dexamethasone-dependent increase in haptoglobin synthesis. However, when several independent clones of hybrid cells formed from adult mouse hepatocytes and rat hepatoma cells were treated with dexamethasone, the synthesis of mouse haptoglobin was in all cases elevated. It appears that haptoglobin expression in mouse liver cells is potentially sensitive to glucocorticoids, but this modulation is manifested only in transformed cells and their derivatives.

The liver of the adult mouse responds to different kinds of traumas by a change in the production of several plasma proteins, the acute phase reactants (19). One of the prominently induced plasma proteins is haptoglobin (34). The rate of synthesis and secretion of haptoglobin in hepatocytes doubles during the first 24 h of acute inflammation, resulting in a two to fourfold elevation of the serum concentration of that protein[1]. Studies to define the hormones or factors modulating the production of haptoglobin and other acute phase plasma proteins are difficult because no suitable tissue culture system that represents the entire phenotype of adult liver parenchymal cells is available. Primary hepatocytes in tissue culture, even under the best culture conditions, lose the expression of specific proteins as well as responsiveness to hormones (9, 24). Despite this handicap, we were able to show that normal hepatocytes in tissue culture maintain a basal production of haptoglobin that equals 1–2% of the total secretion. The synthesis is increased two to fourfold upon treatment of the hepatocytes in tissue culture with factors derived from activated monocytes. In contrast, the presence of glucagon in the culture medium led to a specific suppression of haptoglobin production. The hepatocytes did not show any modulation of haptoglobin synthesis as a consequence of treatment with dexamethasone either in tissue culture or in vivo. Furthermore, hepatocytes from hypophysectomized animals did not show reduced expression of haptoglobin, although the cells have experienced glucocorticoid deprivation in vivo[1].

Recently, we have observed that in a subline of mouse hepatoma cells (Hepa-1) the expression of haptoglobin is regulated by dexamethasone. This prompted studies that are reported here and that illustrate that the kinetics of induction and hormone concentration dependence of haptoglobin synthesis are characteristic for a bona fide glucocorticoid-regulated sequence. Furthermore, the haptoglobin production can, as in fully differentiated adult mouse hepatocytes, be inhibited by glucagon or cAMP. To test whether haptoglobin synthesis of nontransformed adult mouse liver cells in tissue culture can be rendered sensitive to dexamethasone, we constructed hybrid cells between adult mouse hepatocytes and dexamethasone-responsive rat hepatoma cells. Several independent hybrid cell lines showed indeed a steroid-mediated induction of mouse haptoglobin, suggesting that glucocorticoid-regulation of this plasma protein is manifested only in transformed cells. Nevertheless, Hepa-1 cells represent an attractive tissue cul-

[1] H. Baumann, G. P. Jahreis, and K. C. Gaines, manuscript in preparation.

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ture system for studying the regulation of an important mouse acute phase plasma protein.

MATERIALS AND METHODS

Cells: All cells used in these studies were maintained as monolayer cultures under a 5% CO_2-95% air atmosphere in Dulbecco's modified Eagle's medium containing 25 mM HEPES, 2 mM glutamine, 4.5 g/l glucose, and 10% heat-inactivated fetal calf serum. A subline of the mouse hepatoma cells, Hepa-1 (7) (kindly provided by Dr. G. Darlington, Cornell Medical College, New York), was derived from a selected clonal monolayer. Hypoxanthine guanine phosphoribosyl transferase-negative ($HPRT^-$)[2] rat hepatoma tissue culture hepatoma tissue culture (HTC) cells were selected from a cloned cell line by culturing in medium containing increasing concentrations of 8-aza-guanine (maximal concentration was 20 μg/ml). The absence of HPRT activity was verified by enzymatic measurements and by the hypoxanthine aminopterine thymidine-sensitivity of the cells. Adult mouse primary hepatocytes were prepared by collagenase perfusion of the liver of C57BL/6J male according to the procedure described in detail elsewhere (27). To obtain fetal hepatocytes, livers from 15- to 20-d-old fetuses of Ha/ICR mice were minced and digested with collagenase as outlined by Guguen-Guillouzo et al. (16). The cells were cultured in selective medium as described for fetal rat hepatocytes (1). Hybrid cells between primary mouse hepatocytes and HTC cells were generated by essentially following the method outlined by Widman et al. (33). Briefly, freshly prepared primary mouse hepatocytes (3×10^6) were mixed with trypsinized $HPRT^-$-HTC cells (3×10^6) and plated onto a collagen-coated culture dish (75 cm^2). After 16 h, cell fusion was initiated by exposure of the mixed monolayer to 50% polyethylene glycol (Carbowax 1000; J. T. Baker Chemical Co., Phillipsburg, NJ) in serum-free medium for 1 min at room temperature. Hybrid cells were selected using arginine-free Dulbecco's modified Eagle's medium containing 2 mM ornithine, hypoxanthine aminopterine thymidine, and dialyzed fetal calf serum. The medium was changed every third day. After 5 wk, 20 individual primary clones (designated HH-1 to HH-20) were picked and amplified. From the initially selected clones, 10 lines could be maintained for more than five passages, and these were therefore included in the analysis.

Hormonal treatments of the cells were carried out as follows: dexamethasone (Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol (1 mg/ml) and aliquots were added to the medium yielding concentrations indicated in the particular experiments. Glucagon (Calbiochem-Behring Corp., San Diego, CA) was dissolved in 1×10^{-4} N HCl (2 mg/ml) and added to the medium to a concentration of 10 μM. Control cells were treated with medium containing only the solvents.

Radioactive Labeling and Cell-free Translation of RNA: Cell monolayers were washed three times with serum-free Dulbecco's modified Eagle's medium containing 1/10 of the normal methionine concentration. To 10 cm^2 monolayer, 1 ml of the same medium plus 30-150 μCi [^{35}S]methionine (1,000-1,200 Ci/mmol) was added. After 3- or 6-h incubation, the medium was removed and centrifuged for 5 min at 1,000 *g*. The cell monolayer was washed three times with PBS and then used to measure protein amounts and specific incorporation of the label. In all cell cultures, the incorporation of [^{35}S]-methionine into the combined acid-insoluble cellular and medium proteins was found to be linear with time (tested between 15 min and 12 h of continuous labeling). After 30-min labeling period, the rate of extracellular accumulation of all medium proteins, including haptoglobin, was constant for at least up to 12 h. RNA were extracted from monolayers and translated in a cell-free system as described previously (4).

Analyses of Radiolabeled Products: Haptoglobin was purified from [^{35}S]methionine-labeled tissue culture medium by affinity chromatography on immobilized hemoglobin as described (17). The chromatographic conditions employed were such that in all cases the recovery of haptoglobin was quantitative. The separation into α and β subunits was achieved by electrophoresis of the affinity-purified fractions on a 12% SDS polyacrylamide gel (20). The radioactivity present in the bands corresponding to the haptoglobin subunits was determined by scintillation counting (5).

Two-dimensional gel electrophoresis of cellular and cell-free products was carried out according to the technique of O'Farrell (23). The second dimension consisted of uniform 11% polyacrylamide gels. The radioactive pattern was visualized by fluorography (10). The protein spots corresponding to albumin, α_1-fetoprotein and transferrin were identified by co-migration with immunoprecipitated material. Cell-free synthesized haptoglobin was identified by precipitation with monospecific rabbit anti-mouse $\alpha_2\beta_2$ haptoglobin (17) and by proteolytic mapping (4, 12) using nonglycosylated haptoglobin forms for comparison. The relative incorporation of [^{35}S]methionine into individual protein spots separated on two-dimensional gels was determined as described (4, 5).

[2] *Abbreviations used in this paper:* HPRT, hypoxanthine guanine phosphoribosyl transferase; HTC, hepatoma tissue culture.

RESULTS

Effect of Dexamethasone on the Secretion Program of Hepa-1 Cells

Treatment of Hepa-1 cells with dexamethasone had a remarkable effect on the composition of the synthesized and

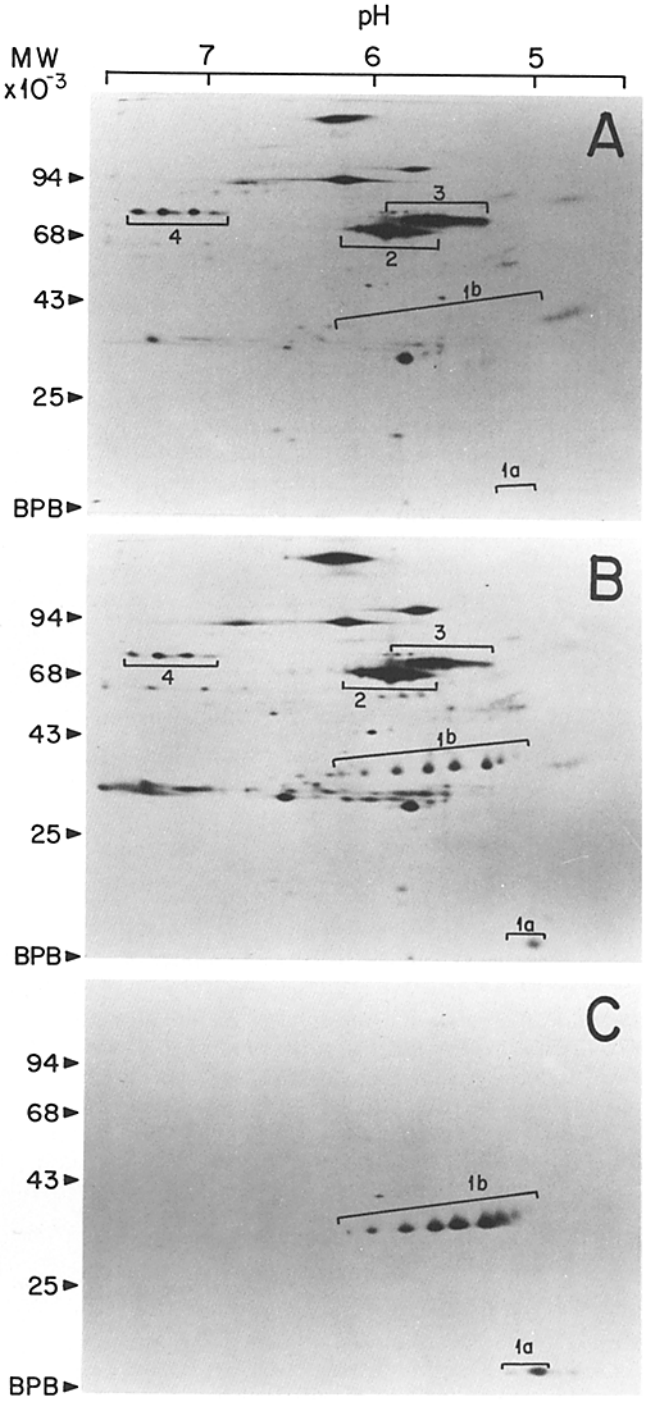

FIGURE 1 Effect of dexamethasone on the production of secretory proteins by Hepa-1 cells. Confluent monolayers of Hepa-1 cells were treated for 24 h without (*A*) or with (*B*) 1 μM dexamethasone. The cells were labeled for 6 h with 150 μCi/ml [^{35}S]methionine. 50 μl of each culture medium was separated by two-dimensional gel electrophoresis. The fluorograms were exposed for 3 d. Numbers indicate the spots corresponding to *1a*, α-haptoglobin; *1b*, β-haptoglobin; *2*, albumin; *3*, α_1-fetoprotein; *4*, transferrin. *BPB*, bromphenol blue. In *C*, haptoglobin was purified by affinity chromatography from 0.5 ml culture medium shown in *B*. Fluorogram was exposed for 3 h.

secreted proteins (Fig. 1, *A* and *B*). One of the most prominent changes was the steroid-mediated appearance of α and β haptoglobin (marked with *1a* and *1b* in Fig. 1). The identity of these spots was verified by their co-migration with biologically active haptoglobin affinity purified from medium of dexamethasone-treated hepatoma cells (Fig. 1 *C*). The overall charge of the hepatoma β-haptoglobin was more basic than that of normal mouse haptoglobin (see below Figs. 4 and 5). This difference is probably due to incomplete sialylation of the protein in Hepa-1 cells because when haptoglobin from mouse hepatocytes was briefly treated with neuramidase, a β-haptoglobin electrophoretically identical to that shown in Fig. 1 *C* was obtained.

Besides affecting secretion, dexamethasone is known to affect a variety of other processes in hepatic cells (18, 29). We measured, therefore, to what extent cell growth, protein synthesis, and secretion in the employed Hepa-1 cell line are influenced by the steroid (Table I). We included in our analysis the two plasma proteins albumin and α_1-fetoprotein, as both are reported to be regulated by glucocorticoids (6, 11, 13, 15). Transferrin served as plasma protein marker for which no glucocorticoid regulation has been described. As Table I indicates, dexamethasone did not significantly influence either cell growth, label uptake, or total cell protein synthesis. The production of total secretory proteins, however, appears to be slightly elevated. Increased synthesis and secretion of albumin and, of course, haptoglobin are primarily responsible for this elevation. At the same time, α_1-fetoprotein production is reduced by half. We have corroborated these results by immunoelectrophoretic analysis of culture media (data not presented).

Regulation of Haptoglobin Synthesis in Hepa-1 Cells by Dexamethasone

To characterize more precisely the effect of dexamethasone on the production of haptoglobin, we followed its hormonal concentration dependence and the time course of stimulation by measuring the rate of synthesis and secretion of the functional protein (Fig. 2). The haptoglobin synthesis is half-maximally induced at 1–2 × 10^{-8} M dexamethasone, at a concentration close to the equilibrium dissociation constant (K_d) of murine glucocorticoid receptors (25). As far as the sensitivity and time resolution of the measurements in Fig. 2 allowed, we did not detect any lag period in the onset of increased haptoglobin synthesis. We cannot, however, rule out, that there was a lag period within the first 3 h. The time course of haptoglobin induction agrees with that of other known glucocorticoid-regulated proteins (2, 21, 26, 30, 32).

In several experiments we have found that the haptoglobin synthesis in Hepa-1 cells is stimulated between 30- and 80-fold (see also Table I and Table II). To determine whether the measured rates of haptoglobin synthesis are a reflection of functional mRNA concentration, we analyzed RNA from control and dexamethasone-treated Hepa-1 cells by cell-free translation (Fig. 3). The most prominent change we could observe was the dexamethasone-mediated appearance of a spot with M_r = 40,000 (Fig. 3 *B*). This spot co-migrated with cell-free synthesized and immunoprecipitated haptoglobin precursor of the mouse liver (Fig. 3 *C*). To prove the identity of haptoglobin, we compared the cell-free precursor forms by

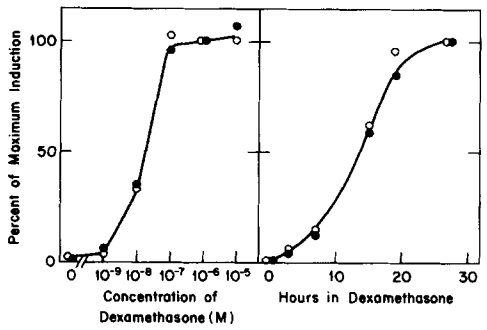

FIGURE 2 Stimulation of haptoglobin synthesis by dexamethasone. Hepa-1 cells were grown to confluency in 10-cm² wells of cluster plates. To determine the hormone concentration dependence of haptoglobin synthesis (*left*), the cells were incubated for 24 h in medium containing the indicated concentrations of dexamethasone. To assess the time course of stimulation (*right*), cells were treated for various lengths of time with 1 μM dexamethasone. Times indicated represent the sum of hormone pretreatment and labeling; the 0 h value represents control cells. In both experiments, the synthesis of haptoglobin was probed by labeling the cells with 50 μCi/ml [^{35}S]methionine for 3 h. Haptoglobin present in the entire medium was isolated by affinity chromatography and separated into its subunits by polyacrylamide gel electrophoresis. The radioactivity present in the α- (●) and β- (○) subunits were measured (values ranged from 25–6,000 cpm for α-haptoglobin and 200–40,000 cpm for β-haptoglobin). The ratio of β:α was in all cases between 6.5–7.5. The values were related to the amount of total cell protein present and expressed as percent of maximum induction. Maximum induction was arbitrarily defined as the synthesis of haptoglobin by cells treated for 24 h with 1 μM dexamethasone.

TABLE I

Effect of Dexamethasone on Protein Synthesis and Secretion of Hepa-1 Cells

Treatment	Amount of cell protein (μg/monolayer)	Incorporation (cpm/μg of total cell protein)					
		Total cell protein	Total medium protein	β-Haptoglobin	Albumin	α_1-Fetoprotein	Transferrin
− Dexamethasone	1,885 ± 40	15,400 ± 200	830 ± 36	0.7 ± 0.1	180 ± 9	145 ± 6	28 ± 2
+ Dexamethasone	1,975 ± 13	14,400 ± 100	1,040 ± 24	25.2 ± 4	212 ± 27	79 ± 12	23 ± 5

Hepa-1 cells were grown to confluence in eight 10-cm² dishes. Four dishes were treated for 24 h with 1 μM dexamethasone. All dishes were labeled with [^{35}S]methionine (150 μCi/ml) for 6 h. Then, the amount of total cell protein and the specific activity in cell and medium protein were determined. 50 μl of each medium was separated on two-dimensional gels as in Fig. 1 and the radioactivities in spots representing β-haptoglobin, albumin, α_1-fetoprotein, and transferrin were measured. The values were corrected for background contribution, and expressed relative to the cell protein amount. The values represent the mean and standard deviation of the four identically treated cultures. To assess uptake of methionine, total radioactivity associated with similarly treated cell monolayers, but incubated for only 15 min in tracer containing medium, was determined. Control cells yielded a value of 806 ± 35 cpm/μg cell protein and dexamethasone-treated cells a value of 760 ± 7 cpm/μg cell protein. To measure growth rates, 1 × 10^6 Hepa-1 cells (1/15 of a confluent monolayer) were plated into 75-cm² flasks and cultured for 72 h in medium with or without 1 μM dexamethasone. The increase in cell numbers yielded generation times of 23.5 h for control cells and 23.9 h for cells in dexamethasone.

TABLE II

Modulation of Haptoglobin Synthesis by Glucagon or cAMP

First treatment	Second treatment	Incorporation (cpm/μg total cell protein)			
		β-Haptoglobin	Albumin	α_1-Fetoprotein	Transferrin
Experiment I					
—	—	0.4	121	89	21
—	Dex	28.8	183	64	12
—	Dex + glucagon	8.9	135	40	17
—	Dex + cAMP	10.3	188	51	11
Dex	—	20.4	186	44	14
Dex	Glucagon	6.9	158	90	20
Dex	cAMP	3.8	191	94	14
Experiment II					
—	—	0.2			
—	Dex	21.1			
—	Dex + Glucagon	4.6			
Dex	—	16.3			
Dex	Glucagon	2.1			
Dex	Dex	22.5			
Dex	Dex + Glucagon	9.0			

Experiment I. Hepa-1 cells were grown to confluence in 10-cm² dishes. 48 h prior to labeling, the same cells were cultured for 24 h in 1 μM dexamethasone (*first treatment*). During the subsequent 24 h (*second treatment*) the cells were treated as indicated with 1 μM dexamethasone, 10 μM glucagon, or 3 mM 8-bromo-cAMP. The cells were labeled for 6 h with [^{35}S]methionine (150 μCi/ml). The secretory glycoproteins were separated by two-dimensional gel electrophoresis and the radioactivity present in β-haptoglobin, albumin, α_1-fetoprotein, and transferrin was determined as in Table I.
Experiment II. Hepa-1 cells were treated as indicated and labeled as in Experiment I. The synthesized and secreted haptoglobin was affinity-purified and separated on one-dimensional polyacrylamide gel. The radioactivity present in the β-haptoglobin band was measured.

proteolytic mapping with nonglycosylated haptoglobin that had been purified by affinity-chromatography from tunicamycin-treated primary cultures of mouse hepatocytes or Hepa-1 cells. As illustrated in Fig. 3*D*, cell-free synthesized precursor of Hepa-1 cells and of liver yielded same fragmentation patterns. Furthermore, the proteolytic mapping of cellular haptoglobin showed fragments with identical electrophoretic mobilities. To estimate the magnitude of induction of functional haptoglobin mRNA in Hepa-1 cells, we determined the relative incorporation of [^{35}S]methionine into the cell-free synthesized precursor after separation on two-dimensional gels. Using two independent preparations of RNA from control cells, we could not detect any significant radioactivity above background in the region of the haptoglobin spot (<15 ppm). Using RNA from dexamethasone-treated cells, the radioactivity in the haptoglobin spot represented 520 and 660 ppm. This result suggests that haptoglobin production in Hepa-1 cells is regulated on the level of functional mRNA, although contribution of posttranslational modulation of the synthesis rate cannot be ruled out.

As mentioned above, haptoglobin production in adult hepatocytes is selectively inhibited by glucagon or cAMP analog. The question arises as to whether Hepa-1 cells respond to that hormone in a comparable manner. Because Hepa-1 cells have a very low basal expression of haptoglobin, the effect of glucagon or 8-bromo-cAMP can only be efficiently studied on cells during or after stimulation with dexamethasone. The experiments described in Table II revealed that glucagon and 8-bromo-cAMP indeed influenced preferentially the synthesis and secretion of haptoglobin. Both components could not only suppress dexamethasone stimulation but could also accelerate the return to basal level after dexamethasone withdrawal. Glucagon or 8-bromo-cAMP appears also to have a modulating effect on albumin or α_1-fetoprotein production. However, the results in Table II and those of other analyses (not shown) do not yet allow a definitive statement as done elsewhere (11).

Dexamethasone-regulated Haptoglobin Synthesis in Mouse Hepatocytes-Rat Hepatoma Hybrid Cells

Hepatocytes prepared from adult mouse livers and maintained in tissue culture exhibited an appreciable and constant basal level of haptoglobin synthesis, ranging from 1 to 2% of total secretion. The relative haptoglobin synthesis was not significantly changed when cells were treated in vivo or in tissue culture with dexamethasone[1]. The question arises as to whether the dexamethasone regulation of haptoglobin synthesis is a unique feature of Hepa-1 cells or whether haptoglobin expression in normal hepatocytes would be responsive to dexamethasone if the steroid action was not prevented by an unknown mechanism(s).

Since Hepa-1 cells produce substantial quantities of α_1-fetoprotein (Table I), they may be considered more fetal-like in phenotype. Therefore, we first determined how the effect of dexamethasone on fetal liver cells compared with that on Hepa-1 cells. Analyzing the proteins secreted by different-age fetal hepatocytes immediately after preparation (see selected examples in Fig. 4), we found that the onset of detectable haptoglobin synthesis takes place between day 17 and 19 of gestation. The developmental appearance of haptoglobin roughly coincided with that of α_1-antichymotrypsin (protein spot 5 in Fig. 4). Interestingly, hepatocytes, either in early (day 16) or late (day 19) states of development, started or continued to produce increasing amounts of haptoglobin when maintained in tissue culture (in Fig. 4, only the results from hepatocytes of 19-day fetuses are shown). A similar behavior was already described for fetal rat hepatocytes (28). The presence of dexamethasone in the medium had, however, no significant promoting activity on haptoglobin production, although the steroid influenced other plasma proteins, such as apolipoprotein A-1.

Finding that haptoglobin production could not be modulated by dexamethasone in fetal or adult hepatocytes, we

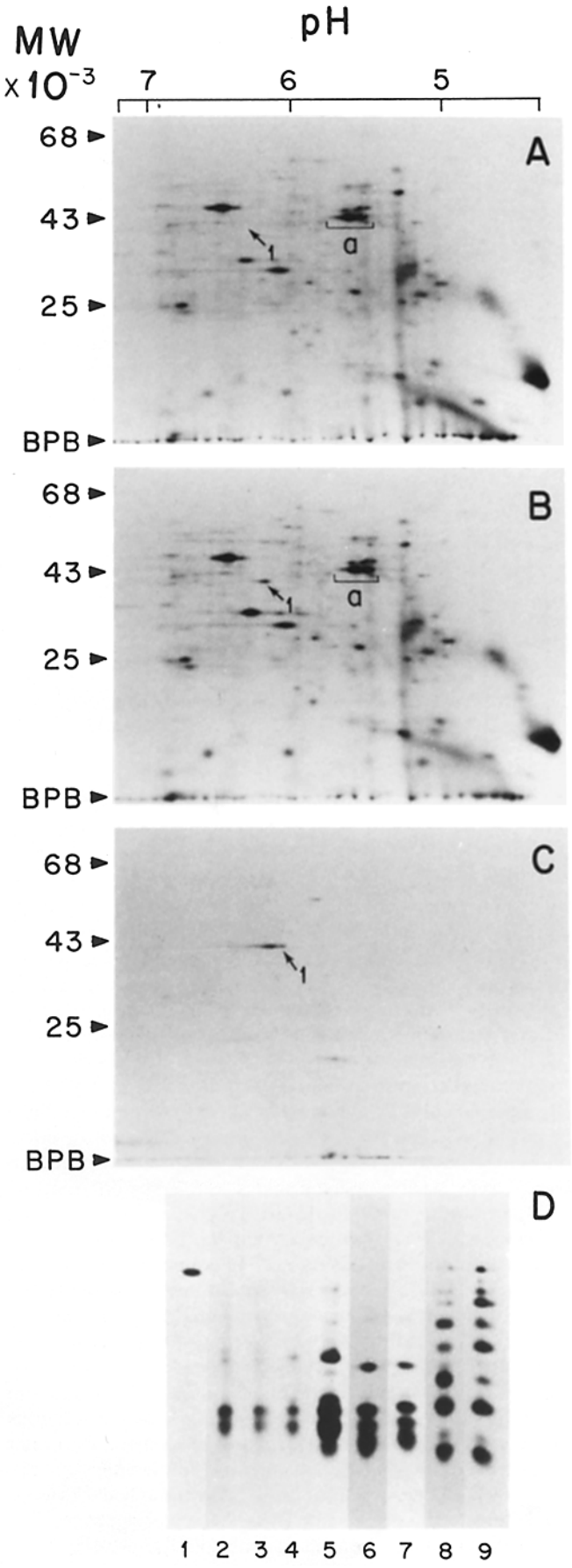

FIGURE 3 Dexamethasone-mediated change in functional mRNA in Hepa-1 cells. (*A* and *B*) Two confluent monolayers (175 cm^2) of Hepa-1 cells were cultured for 24 h either without (*A*) or with 1 μM dexamethasone (*B*). RNA was extracted and translated in a cell-free system. The products in 5 μl of translation mixture, containing 400,000 acid-insoluble cpm, were separated by two-dimensional gel electrophoresis. The fluorograms were exposed for 3 d. Arrow with number *1* points to the spot representing the haptoglobin precursor. *a* indicates the position of actin. (*C*) Polyadenylated RNA, which was isolated from a mouse liver 24 h after an experimentally induced inflammation, was translated in a 50-μl reaction mixture. The cell-free translation products were incubated first with monospecific rabbit immunoglobins against mouse $\alpha_2\beta_2$ haptoglobin followed with immobilized goat anti-rabbit immunoglobins. The extensively washed immunoprecipitate was solubilized and separated on a two-dimensional gel. The fluorogram was exposed for 10 d. (*D*) To compare in vitro synthesized precursor forms with appropriate forms synthesized by cells, we treated monolayers (25 cm^2) of primary cultures of mouse hepatocytes and dexamethasone-stimulated Hepa-1 cells for 2 h with tunicamycin (2 μg/ml). Then, the cells were labeled for 30 min with [^{35}S]methionine (250 μCi/ml) and the cellular haptoglobin was purified by affinity chromatography on hemoglobin-Sepharose. After separation on two-dimensional gels, in vitro synthesized and cellular haptoglobin forms were cut out from the gels and subjected to digestion with *S. aureus* V8 protease within a 15% polyacrylamide gel (12). The lanes represent: *1*, nondigested cell-free precursor of Hepa-1 cell; *2*, cell-free precursor of mouse liver (immunoprecipitated as in *C*); *3*, cell-free precursor of Hepa-1 cells; *4*, nonglycosylated, intact haptoglobin of mouse hepatocytes (prior cleavage into α and β); *5*, nonglycosylated, intact haptoglobin of Hepa-1 cells; *6*, nonglycosylated β-haptoglobin of mouse hepatocytes; *7*, nonglycosylated β-haptoglobin in Hepa-1 cells; *8*, mature β-haptoglobin of mouse hepatocytes (most basic spot of the medium form); *9*, mature β-haptoglobin of Hepa-1 cells (third most acidic spot of the medium form). Because the samples contained different amounts of radioactivities, the fluorograph of the digestion gel required exposure times between 3 h and 1 mo to obtain lanes with comparable intensity. *BPB*, bromphenol blue.

resorted to the analysis of hybrid cells between primary adult mouse hepatocytes and rat hepatoma tissue culture cells. We have constructed such hybrid cells with the intention of immortalizing the functions of fully differentiated adult mouse liver cells. The combination mouse-rat was chosen because the hybrid nature of the resulting cells could be conveniently assessed by analysis of karyotype, isoenzyme pattern, and physico-chemical differences of most of the plasma proteins. In addition, we expected that the known glucocorticoid responsiveness of the rat hepatoma cells (2, 3) would be retained by the hybrid cells. Ten separate hybrid cell clones, each having undergone five to eight passages, were tested to determine to what extent the synthesis of secretory proteins was influenced by dexamethasone. The results of the two cell lines HH-13 and HH-19 are reproduced in Fig. 5. The response patterns of these examples represent the two extreme cases; other hybrid cell clones were found to have intermediate reactivity. Although all hybrid cells have lost the production of many mouse plasma proteins, each clone showed nevertheless at least the production of mouse transferrin. To our surprise, *all* clones responded to dexamethasone by an increase in mouse haptoglobin synthesis. (Mouse haptoglobin can readily be distinguished from that of the rat because of its higher molecular weight and the presence of a methionine-containing α-subunit; [17]). Quantitation of the relative radioactivity incorporated into haptoglobin, as was done for Hepa-1 cells in Table I, indicated that, in the absence of dexamethasone, the amount of haptoglobin produced by all clones was <0.1% of the total secretion, and that treatment of the cells for 24 h with dexamethasone yielded a 10- to 50-

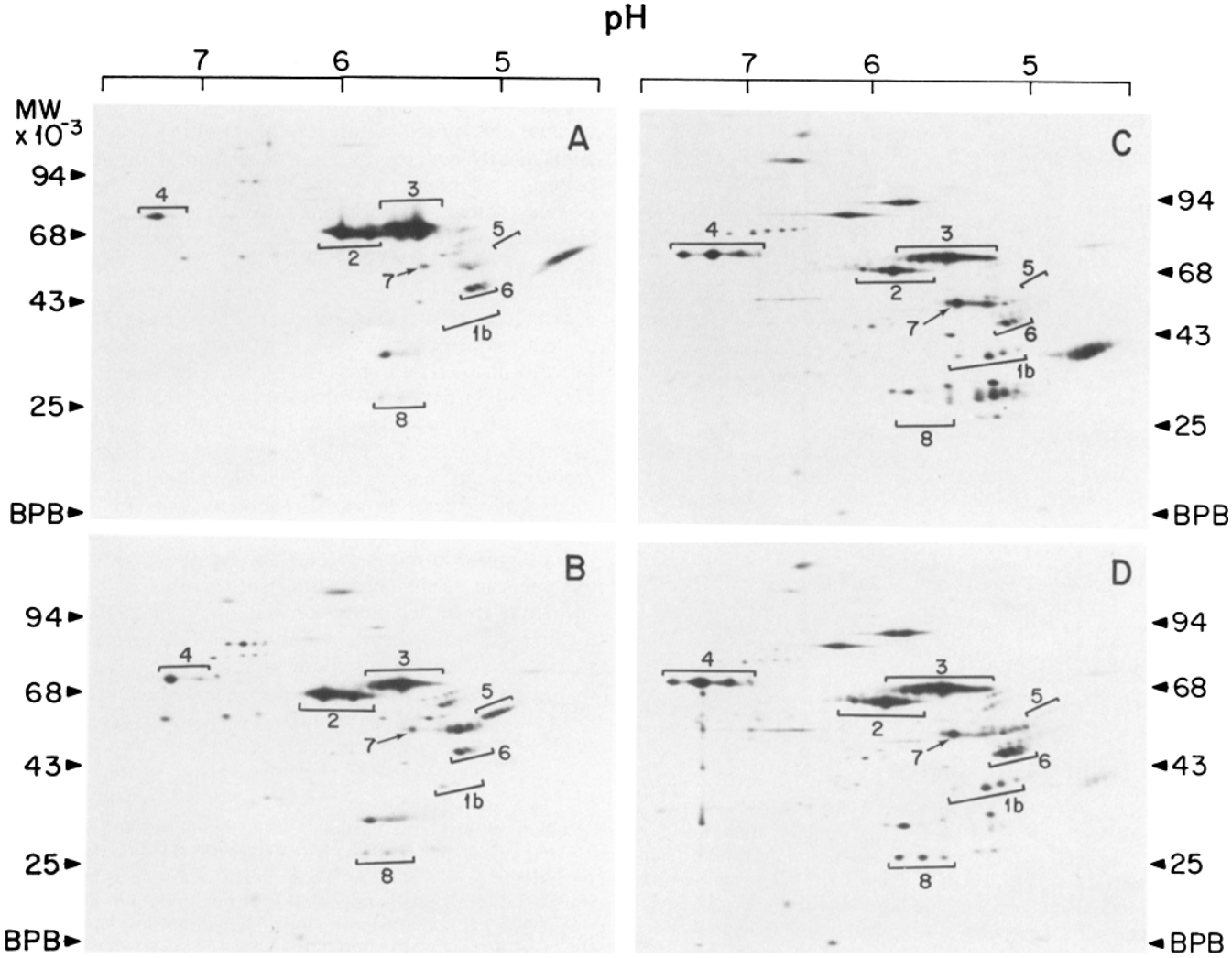

FIGURE 4 Synthesis and secretion of proteins by fetal mouse hepatocytes. Hepatocytes were prepared from 16-d- *(A)* or 19-d- *(B–D)* old fetuses. For each preparation, 15–18 livers were combined. Some of the hepatocytes from 19-d-old fetuses were maintained for 4 d in tissue culture using medium without *(C)* or with 1 μM dexamethasone *(D)*. The cells (in *A* and *B*, immediately after preparation; in *C* and *D*, after 4 d in culture) were labeled for 6 h with [^{35}S]methionine (150 μCi/ml). Comparative aliquots of labeled medium were separated by two-dimensional gel electrophoresis. The fluorograms are exposed for 18 h. The indicated spots represent following proteins: *1*, β-haptoglobin; *2*, albumin; *3*, α_1-fetoprotein; *4*, transferrin; *5*, α_1-antichymotrypsin; *6*, α_1-antitrypsin; *7*, antithrombin III; *8*, apolipoprotein A-1. *BPB*, bromphenol blue.

fold increase. Although all hybrid clones, like the parental HTC cells, synthesized trace amounts of rat haptoglobin, this production was never found to be significantly affected by dexamethasone treatment.

Beside haptoglobin, α_1-acid glycoprotein of the rat hepatoma cells was also normally regulated by dexamethasone in all hybrid clones tested. The second prominent hepatoma product, a glycoprotein with M_r 50,000, however, was only inducible by the steroid in seven of the clones. In four clones, the synthesis of mouse albumin can be stimulated by dexamethasone (see *HH-19* in Fig. 5).

These results suggest that the expression of haptoglobin gene or genes in developing and adult mouse hepatocytes is not modulated by dexamethasone. When, however, the gene(s) are transferred into an environment of transformed cells, as existing in Hepa-1 cells or hybrid cells, the basal level of its expression is reduced and is now regulatable by dexamethasone.

DISCUSSION

The major finding of the studies reported in this paper is that mouse haptoglobin production, while not normally glucocorticoid-regulated, becomes sensitive to dexamethasone in transformed cells and their derivatives. Hormonal dependence and kinetics of stimulated haptoglobin synthesis in Hepa-1 cells follow properties described for other glucocorticoid-regulated systems (21, 26, 30, 32). Results from cell-free translation analyses have indicated that the increased haptoglobin synthesis is most likely a reflection of a change in the concentration of functional mRNA (Fig. 3). In all respects, dexamethasone modulation of haptoglobin synthesis in Hepa-1 cells is comparable with that of α_1-acid glycoprotein synthesis in rat hepatoma tissue culture cells (2). Remarkably, both proteins represent major acute phase plasma proteins (4, 17). Although in the tumor cells the production of both proteins is regulated by dexamethasone, the same proteins were found

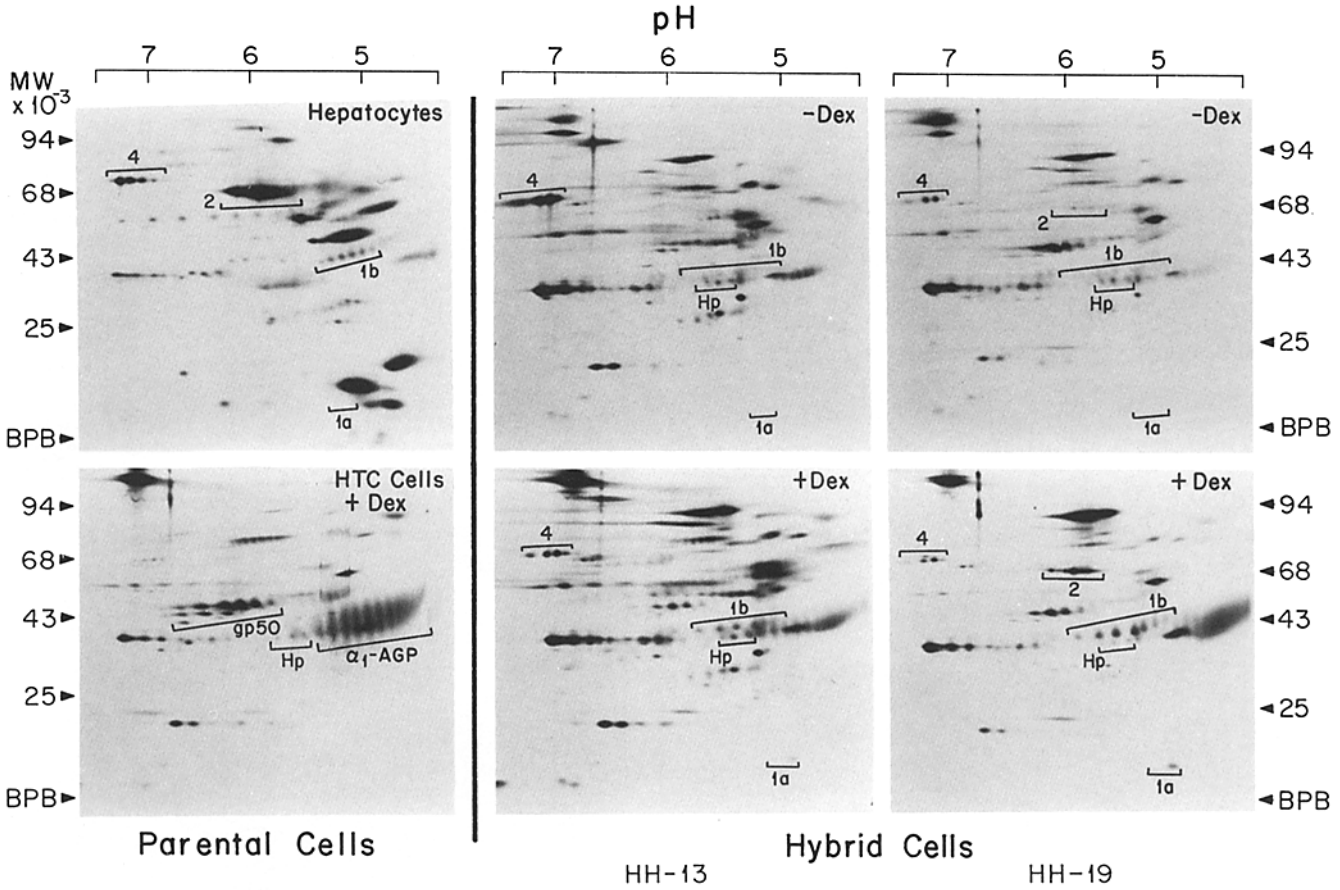

FIGURE 5 Response of hepatocyte-hepatoma hybrid cells to dexamethasone. We have illustrated in this figure, the results of two cloned lines of hybrid cells between primary mouse hepatocytes and rat hepatoma tissue culture cells, HH-13 and HH-19. Both hybrid clones contain all of the following isozyme markers of rat and mouse origin (in parenthesis the chromosome is noted on which the mouse enzymes are located): glucose phosphate isomerase (7); lactate dehydrogenase A (7); mannose phosphate isomerase (9); amlic enzyme (9); peptidase B (10); peptidase C (1); peptidase S (5); 6-phosphogluconate dehydrogenase (4); and superoxide dismutase-1 (16). The total chromosome numbers (mean and standard deviation of 16–20 metaphases) are HTC cells, 60 ± 2; HH-13, 90 ± 8; and HH-19, 96 ± 4.

Confluent cell monolayers were treated for 24 h with or without 1 μM dexamethasone *(Dex)*. After labeling the cells with [^{35}S]methionine (150 μCi/ml) for 6 h, 50-μl aliquots of labeled culture medium were separated by two-dimensional gel electrophoresis. The secretion patterns are compared with those of the parental cells: hepatocytes were labeled immediately after preparation and HTC cells after treatment with 1 μM dexamethasone for 24 h. Spots indicated by numbers represent the following mouse plasma proteins: *1a*, α-haptoglobin; *1b*, β-haptoglobin; *2*, albumin; and *4*, transferrin. The major dexamethasone-regulated glycoproteins in HTC cells are marked with *gp 50* and *α$_1$AGP* (α$_1$-acid glycoprotein) (3). The β-haptoglobin of HTC cells is marked with *Hp*. *BPB*, bromphenol blue.

to be influenced in the liver cells during acute phase reaction by factors other than corticosteroids[1] (2).

To demonstrate that haptoglobin synthesis by Hepa-1 cells and by nontransformed liver cells can be regulated by common effectors, we tested the influence of glucagon and 8-bromo-cAMP. As found for adult hepatocytes, both components inhibited haptoglobin production by Hepa-1 cells (Table II). It is, however, not apparent whether this effect is achieved via the same mechanism as in hepatocytes. Since Hepa-1 cells, unlike adult hepatocytes, required initial stimulation by dexamethasone to obtain measurable haptoglobin synthesis, it is conceivable that glucagon or 8-bromo-cAMP interfered with the overall steroid action rather than specifically affecting haptoglobin expression. Whether the reduction of haptoglobin synthesis in Hepa-1 cells upon treatment with glucagon or cAMP analog is a consequence of a change in posttranscriptional stability of mRNA is not known. When cloned probes for haptoglobin sequences become available, we shall be able to test which of the possibilities applies.

The demonstration that haptoglobin production in Hepa-1 cells is regulated by dexamethasone has led to the question whether this property of tumor cells is also manifested in nontransformed liver cells, or whether their steroid response is a unique acquisition of Hepa-1 cells. Recent analysis of adult hepatocytes[1] as well as analysis of fetal hepatocytes (Fig. 4) has failed to show similar behavior when these cells are treated with dexamethasone in tissue culture. The observation that production of other plasma proteins is modulated by steroid treatment excludes the possibility that the hepatocytes, when placed in tissue culture, lose their responsiveness to dexamethasone. If there is indeed a dexamethasone-responsive haptoglobin expression in normal hepatocytes, this regulation might be restricted to a specific stage in the hepatocytes' development. It remains to be seen whether such a hypothetical steroid-sensitive state of hepatocytes exists only during the developmental period of the first four postnatal weeks. In all studies involving isolated hepatocytes, however, an important fact has to be considered, i.e., that hepatocyte

preparations do not consist of a homogeneous cell population from a defined developmental stage. It might well be that this heterogeneity does not allow assessment of the properties of a subpopulation of liver cells.

Because we could not detect glucocorticoid-regulated haptoglobin production in primary hepatocytes, we attempted to determine whether the mouse haptoglobin synthesis becomes sensitive to dexamethasone once hepatocytes are exposed to a transformed environment, or whether the hormonal response of Hepa-1 cells is actually an isolated occurrence. To do so, we employed hybrid cells between mouse hepatocytes and rat hepatoma cells. Several reports have already illustrated that such hybrid cells systems can be very useful in uncovering mechanisms regulating the expression of liver specific genes (31, 33). Our analysis of 10 independent hybrid cell clones (Fig. 5) revealed essentially the same properties of hepatic hybridomas as described by Szpirer et al. (31): hybrid cells express only subsets of the plasma proteins derived from the nontransformed hepatocytes, and the karyotypes indicate loss of chromosomes. Our results differ only in the relative occurrence of mouse plasma proteins in the hybrid clones, most notably the constant presence of mouse transferrin production. Even though the phenotype of the hybrid cells might be influenced by properties of the rat hepatoma cell (8, 22), our observation that all clones express dexamethasone-sensitive mouse heptoglobin synthesis suggests that this regulation might be an intrinsic feature of the mouse gene manifested in the hybrid cell environment. Consequently, the dexamethasone responsiveness of Hepa-1 cells appears not to be a unique acquisition by this tumor cell line. To give a definitive statement, however, we must show how and at what level the synthesis of haptoglobin is modulated in the hybrid cells by the steroid. Results of those determinations will help to delineate the mechanism by which mouse haptoglobin expression becomes regulatable by glucocorticoid. Two possible mechanisms can be envisioned: (*a*) During the transition from fully differentiated hepatocytes to transformed cells, there is a loss of transacting elements which could either directly inhibit glucocorticoid regulation of the haptoglobin gene(s) or override glucocorticoid regulation by maintaining a basal expression equal or above the level achieved by the steroid; or (*b*) in the transformed cell environment the mouse haptoglobin gene(s) are subjected to nonrandom structural alterations, such as methylation (14), that would render the gene(s) sensitive to dexamethasone.

Since haptoglobin represents in many mammalian species an acute phase plasma protein (19), knowledge about its regulation is important in understanding the hepatic acute phase reaction. In this paper, we have presented the existence of two levels of regulation: on one side dexamethasone modulates haptoglobin in hepatoma cells but not in hepatocytes, and on the other side the activity that stimulates the synthesis of this protein in the liver during an acute phase appears not to be glucocorticoid[1] (2). Recent experiments with primary adult hepatocytes showed that activated monocytes produce a factor or factors that provoke an increase in haptoglobin production equivalent to that found in inflamed liver cells. The same factor(s) added to Hepa-1 cells proved to be ineffective (data not presented). To best study this apparent dual regulation of one acute phase reactant, a stable cell system is needed that responds to both effectors. As presented here, a promising approach to obtain such cells is to establish hybridomas between Hepa-1 cells and adult mouse hepatocytes. Cell lines that have retained the hepatomas' glucocorticoid regulation and the hepatocytes' responsiveness to monocyte factor should yield valuable information about modulation of haptoglobin and other acute phase reactants.

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