Regulation of Major Acute-phase Plasma Proteins by Hepatocyte-stimulating Factors of Human Squamous Carcinoma Cells

Heinz Baumann,* Robert E. Hill,‡ Daniel N. Sauder,§ and Gerald P. Jahreis*

*Department of Cell and Tumor Biology, Roswell Park Memorial Institute, Buffalo, New York 14263; ‡Molecular Genetics Sections, MRC Clinical Population Cytogenetics Unit, Western General Hospital, Edinburgh, England; and §Department of Medicine, McMaster University, Hamilton, Canada

Abstract. Human squamous carcinoma (COLO-16) cells release factors which specifically stimulate the synthesis of major acute-phase plasma proteins in human and rodent hepatic cells. Anion exchange, hydroxyapatite, lectin, and gel chromatography of conditioned medium of COLO-16 cells result in separation into three distinct forms of hepatocyte-stimulating factors (designated HSF-I, HSF-II, and HSF-III) with apparent molecular weights of 30,000, 50,000 and 70,000, respectively. None of the preparations contains detectable amounts of thymocyte-stimulating activity. Each of the three HSF forms stimulates the accumulation of mRNA for α_1-antichymotrypsin in the human hepatoma cell line, HepG2. When the same factors were added to primary cultures of adult rat hepatocytes, the expression of the same set of plasma proteins was modulated as by nonfractionated medium. The hormonally induced accumulation of mRNA for acute phase proteins is qualitatively comparable to that occurring in the liver of inflamed rats. Unlike in human cells, in rat liver cells dexamethasone acts additively and synergistically with HSFs. The only functional difference between the three HSF forms lies in the level of maximal stimulation. HSF-I represents the predominant form produced by normal human keratinocytes and closely resembles in molecular size and isoelectric point the activity produced by activated peripheral blood monocytes while the larger molecular weight forms are more prevalent in human as well as mouse squamous carcinoma cells. The observation that HSFs from different sources elicit essentially the same pleiotropic response in hepatic cells led to the hypothesis that the species-specific reaction of adult liver cells to inflammatory stimuli is pre-programmed and that the function of any HSF is to trigger and tune the execution of this fixed cellular process.

THE regulation of plasma protein synthesis in the mammalian liver during an acute systemic injury is most remarkable in that the expression of a subset of proteins, the acute-phase reactants, is drastically increased while that of others is reduced (21, 24). Surprisingly, little is known about the signals mediating this hepatic response and the mechanism leading to the coordinate modulation of structurally nonrelated and genetically nonlinked proteins (7, 9). An essential step towards understanding of this process is defining the source and the structural identity of the modulating activities (18, 33, 37, 42). The functional properties of the proposed factors have to entail the stimulation of the entire species-specific set of acute-phase plasma proteins with kinetics and magnitude of change as produced in vivo. It is therefore essential to establish whether the pleiotropic hepatic response is controlled by one factor or by a set of specific factors.

Analysis of cells involved in or affected by an acute-phase reaction have indicated that activated blood monocytes (31), tissue macrophages (34), and growing keratinocytes (38) release factors with some of the liver-regulating characteristics. In some instances, in vivo and in vitro tests have suggested a structural correlation of the liver-regulating factor with interleukin-1 (IL-1)[1] (11, 39). However, detailed analyses of structural properties of the activity (4, 22, 32, 43) and response of cultured liver cells (4, 26) have cast doubts on the absolute identity of IL-1 and the liver-regulating activity (for the purposes of this paper and in accordance with Ritchie and Fuller [32], designated hepatocyte-stimulating factor [HSF]).

In addition to producing the IL-1-like, epidermal cell-derived thymocyte-activating factor (ETAF), human squamous carcinoma (COLO-16) cells release constitutively a wide spectrum of structurally distinct HSFs (4). This is in contrast

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[1] *Abbreviations used in this paper:* ETAF, epidermal cell-derived thymocyte-activating factor; HSF, hepatocyte-stimulating factor; IL-1, interleukin 1.

to activated peripheral blood monocytes which produce predominantly one form of HSF (32). The existence of structurally different factors provides the opportunity to address the questions concerning (a) the complexity of the factors needed to achieve a full hepatic acute-phase response, and (b) the correlation of HSFs with IL-1/ETAF. To this end, the HSFs were fractionated from conditioned media of the producer cells and tested for their ability to modulate the synthesis of major acute-phase plasma proteins in human hepatoma cells and cultured rat hepatocytes. Although differing in physicochemical properties, each of the partially purified factors, which were devoid of IL-1/ETAF activity, was able to stimulate qualitatively and quantitatively the synthesis of the same set of plasma proteins. This uniform response of liver cells and common specificity of various HSF forms may lend new ideas as to the possible mechanism of regulation of acute-phase proteins.

Materials and Methods

Source of HSF

Conditioned medium from primary cultures of human epidermal cells, COLO-16 cells, lipopolysaccharide-stimulated human peripheral blood monocytes, and mouse squamous carcinoma (MCA-3) cells (generously provided by Dr. M. Kulez-Martin, Department of Experimental Therapeutics, Roswell Park Memorial Institute) served as the source of HSFs (4). The media, collected after conditioning for 24 h (monocytes) or 96 h (epidermal cells), were cleared from suspended cells by centrifugation and were stored at −70°C until further use.

Purification of HSF

Separation of HSF-I, -II, and -III, present in conditioned medium of COLO-16

cells, has been achieved by the following sequence of chromatographic steps: (a) DEAE-Sephadex (A-50, Pharmacia Fine Chemicals, Piscataway, NJ), (b) hydroxyapatite (BDH Chemicals, Poole, England), (c) concanavalin A-Sepharose (Pharmacia Fine Chemicals), and (d) gel filtration on Sephadex G-100. (Details are described in the legends to Figs. 2–4.) Isoelectric focusing of HSFs was carried out for 60–70 KV/h at 4°C in a 110-ml sucrose gradient column containing 1% ampholines, pH 3.5–10 (LKB Instruments, Inc., Gaithersburg, MD).

HSF activity was monitored during purification by two separate assays: monolayers (2 cm^2) of HepG2 cells were treated for 24 h and primary rat hepatocytes for 48 h with dialyzed aliquots of fractions added to the culture medium which contained also 1 μM dexamethasone (4). The specific increase in production of human α_1-antichymotrypsin or rat α_2-macroglobulin over basal level was quantitated by rocket immunoelectrophoresis of aliquots of culture medium and expressed in nanograms secreted per hour and 10^6 cells (4). A standard of conditioned medium of COLO-16 cells, producing maximal response, was included in all HSF activity assays. One unit of HSF activity is defined as the concentration of HSF needed to stimulate the synthesis of α_1-antichymotrypsin in HepG2 cells to a one-third level of that maximally induced by the standard. All quantitations of human HSF activity were carried out on HepG2 cells because of the cells' uniform and highly reproducible responses. However, the unit values obtained for identical HSF preparations, when tested on both HepG2 cells and rat hepatocytes (stimulation of α_2-macroglobulin to one-third maximal level), were within a range of a factor of 3 (compare Figs. 6 and 9). Activity of mouse-derived HSFs were measured exclusively on rat hepatocytes. IL-1 activity was determined on mouse thymocytes using the standard co-stimulator assay previously described (35).

Analysis of Cell Response to HSF

HepG2 cells (20) and primary cultures of hepatocytes, prepared from 10–14-wk-old male Buffalo rats (36) and maintained as monolayer in collagen-coated dishes (3) served as target cells. The effect of HSF treatment on the synthesis of plasma proteins was determined by metabolic labeling of the cells with [^{35}S]methionine (1,000 Ci/mmol, New England Nuclear, Boston, MA) for 6 h,
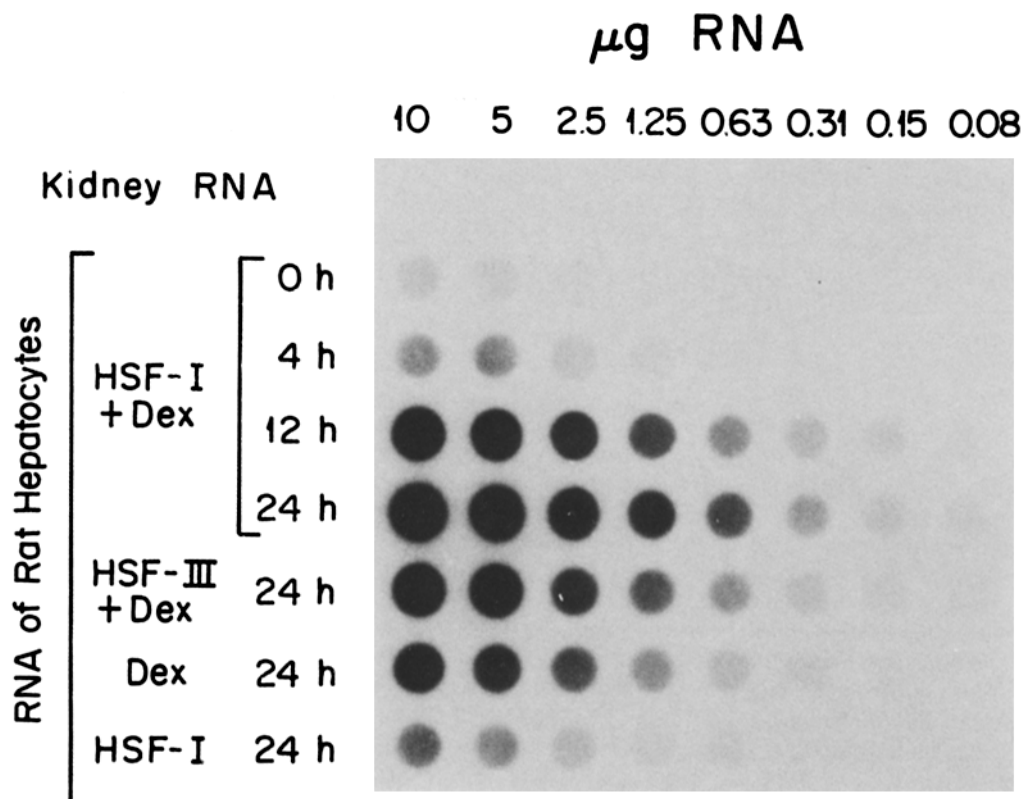

Figure 1. Quantitation of α_1-acid glycoprotein mRNA from established rat hepatocyte cultures. Monolayers of rat hepatocytes were established by culturing in serum-containing medium for 20 h; then the cells were treated with medium supplemented as indicated with 100 U/ml HSF-I and 1 μM dexamethasone, or 80 U/ml HSF-III. After the times indicated, total cell RNAs were extracted. Serial dilutions of the RNAs were blotted into nitrocellulose and hybridized with nick-translated plasmid DNA (pIRL-21) containing cDNA to α_1-acid glycoprotein mRNA. To determine nonspecific hybridization, the same amounts of kidney RNA were applied. The fluorographic image of the dot blots after a 24-h exposure is reproduced.

followed by separation of the secreted proteins by two-dimensional PAGE (3, 4). Plasma proteins which do not separate well on two-dimensional polyacrylamide gels, including α_1-cysteine proteinase inhibitor, fibrinogen, and α_1-acid glycoprotein, were isolated by crossed immunoelectrophoresis using monospecific, polyclonal antibodies, and nonlabeled carrier proteins (3). The relative incorporation of isotype into the separated proteins was used as a measure for change in synthesis.

To assess change in specific mRNA, total RNA was extracted from the cell monolayers by lysis in guanidine thiocyanate and collected by centrifugation through CsCl (6).

Hybridization Analysis of RNA

To expand our repertoire of specific probes to rat acute-phase plasma protein mRNA, we have established a cDNA library to liver mRNA from an experimentally inflamed male rat (two successive injections of 0.5 ml turpentine, 24 h apart). The preparation of the library was carried out by following exactly the procedure worked out and perfected by Dr. D. Dickinson (Department of Molecular Biology, Roswell Park Memorial Institute). In short, first strand cDNA was synthesized in the presence of actinomycin D (5) followed by second strand synthesis by nick translation reaction (modified from 29). The cDNA was homopolymeric tailed (25), annealed to tailed pBR322, and transfected into JW-355. The library (8,000 colonies) was screened for inflammation-induced sequences (14). The nature of inserts in cloned plasmid DNA was determined by mRNA selection and cell-free translation (30). Products were identified on a two-dimensional polyacrylamide gel by co-migration with immunoprecipitated, cell-free precursor forms (4, 8). The plasmids encoding the following proteins were used (in parenthesis insert size in base pairs): pIRL3, α_1-cysteine proteinase inhibitor, identical to α_1-acute-phase globulin (1,600); pIRL9, haptoglobin (1,300); pIRL14, α-fibrinogen (975); pIRL20, γ-fibrinogen (1,650); and pIRL21, α_1-acid glycoprotein (850). In addition, the following probes were included: p1796 (mouse α_1-antitrypsin [15]), p1706 (mouse albumin [1]) and pα176 (α_{2u}-globulin [23]). The mRNAs for human α_1-antichymotrypsin and apolipoprotein A-1 were probed with cDNA, pHACI (15), and a 2.2-kb human apoA-1 gene fragment inserted in pSV2 (19) (generously provided by Dr. J. L. Breslow, The Rockefeller University, New York), respectively.

To determine the relative concentration of specific mRNAs, serial dilutions of cellular RNA were applied onto nitrocellulose and probed with nick-translated plasmid DNA (17). Fig. 1 shows a representative result of an actual experiment. Hybridization was quantitated by densitometry of the fluorographs, corrected for background reactions (hybridization to equal amounts of kidney RNA), and related to the amount of input RNA.

Measurements of Relative Rates of mRNA Synthesis

To label RNA, the culture medium was removed from rat hepatocytes (8 × 10^6 cells in 75-cm^2 collagen-coated flasks) and replaced by 1 ml of the same medium but containing 1 mCi [^{3}H]uridine (Amersham Corp., Arlington Heights, IL, 60 Ci/mmol). The temperature of the culture was maintained at 37°C. After 15 min, the cells were washed twice with ice-cold phosphate-buffered saline and the total cellular RNA was isolated (6). Filter hybridization of ^{3}H-RNA to immobilized DNA was carried out as described by McKnight and Palmiter (28) and Ucker et al. (40). 10 μg plasmid DNA bound to 5-mm diameter nitrocellulose filter disks ensured DNA excess in all hybridizations. Filters carrying cDNA for different mRNAs were combined and hybridized with the individual RNA preparations for 72 h. The bound radioactivity resistant to RNase was counted twice for 50 min in a Beckman LS7000 liquid scintillation counter. Since a relatively low amount of tritium radioactivity was recovered, a simultaneous measurement of hybridization efficiency by using ^{32}P-cDNA was not done. However, hybridization of ^{32}P-cRNA in separate reactions, but under identical conditions, yielded efficiencies ranging between 8 and 20% which are in conformity with earlier reports (40).

Results

Separation of HSFs of COLO-16 Cells

As reported previously (4), conditioned medium of COLO-16 cells contains HSF activity which migrates on Sephadex G-100 with apparent molecular weights ranging between 15,000 and 100,000 and with predominant peaks at 30,000 and 50,000 (see also Fig. 4). Chromatography of such conditioned medium on DEAE–Sephadex resulted in separation into two HSF forms (Fig. 2). There was no significant differ-
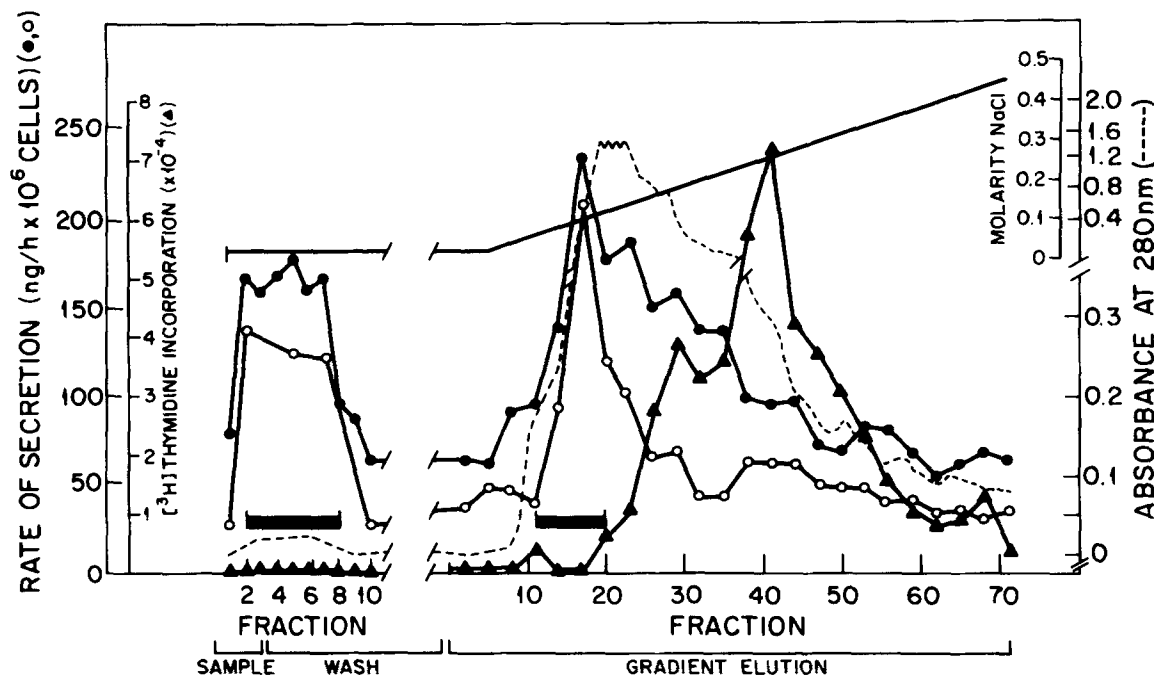

Figure 2. DEAE–Sephadex chromatography of conditioned medium of COLO-16 cells. 400 ml of 4-d–conditioned medium of COLO-16 cells was dialyzed against 10 mM Tris HCl, 25 mM NaCl, pH 8.5, and then applied to a DEAE–Sephadex column (2.6 × 35 cm) in the same buffer. Breakthrough was collected in 50-ml fractions. After washing the column with 2 liters of 10 mM Tris HCl, 25 mM NaCl, the bound material was eluted with a NaCl gradient and collected in 15-ml fractions. Aliquots (0.5 ml) of the fractions after dialysis against phosphate-buffered saline were tested for HSF activity on HepG2 cells (●, α_1-antichymotrypsin) and on rat hepatocytes (○, α_1-macroglobulin) and for stimulation of thymocyte proliferation (△). The basal level of [^{3}H]thymidine incorporation by mitogen-stimulated thymocytes was 5,000–7,000 cpm and was subtracted from all measurements. The value for maximal thymocyte stimulation was 60,000–65,000 cpm. The gradient fraction 11–20, concentrated by ultrafiltration to 20 ml, was used for gel filtration (see Fig. 4). The breakthrough fractions 2–8 were pooled, adjusted to pH 6.8, and used for hydroxyapatite chromatography (Fig. 3).

ence in the profile of HSF activity when tested for stimulation of α_1-antichymotrypsin in HepG2 cells or α_2-macroglobulin in rat hepatocytes. The breakthrough fractions contained a substantial amount of HSF but no detectable IL-1 activity. The presence of an IL-1 inhibitor activity was ruled out by mixing experiments (data not shown). The major bound HSF activity eluted slightly ahead of the bulk proteins and separated clearly from the IL-1 activity. The fraction of bound HSF activity, but essentially free of IL-1 activity (indicated by bar in Fig. 2), has been denoted HSF-I and was used for subsequent experiments.

The nonbound material was further chromatographed on hydroxyapatite (Fig. 3). Again, a separation into two fractions was obtained. The bound HSF activity appeared immediately after applying the phosphate gradient but showed extensive trailing during subsequent elution. Only the fractions with the highest specific HSF activity, denoted HSF-II, were used for further analysis.

Nonetheless, the nonbound fraction still showed significant HSF activity, and this was denoted HSF-III. This HSF form could be quantitatively recovered from the large breakthrough volume by chromatography on concanavalin A–Sepharose.

Chromatography on Sephadex G-100 of concentrated fractions containing HSF-I, -II, and -III revealed that three distinct molecular weight forms of HSF had been recovered from the original conditioned medium (Fig. 4). A significant co-migration of IL-1 activity was not observed. These HSF forms, obtained from Sephadex G-100 columns (as indicated in Fig. 4), were then used for studying the hepatic response. When the same purification scheme was applied to culture medium that had not been conditioned by COLO-16 cells, no HSF activity was recovered. This ruled out an isolation of a not previously recognized factor present in fetal calf serum.
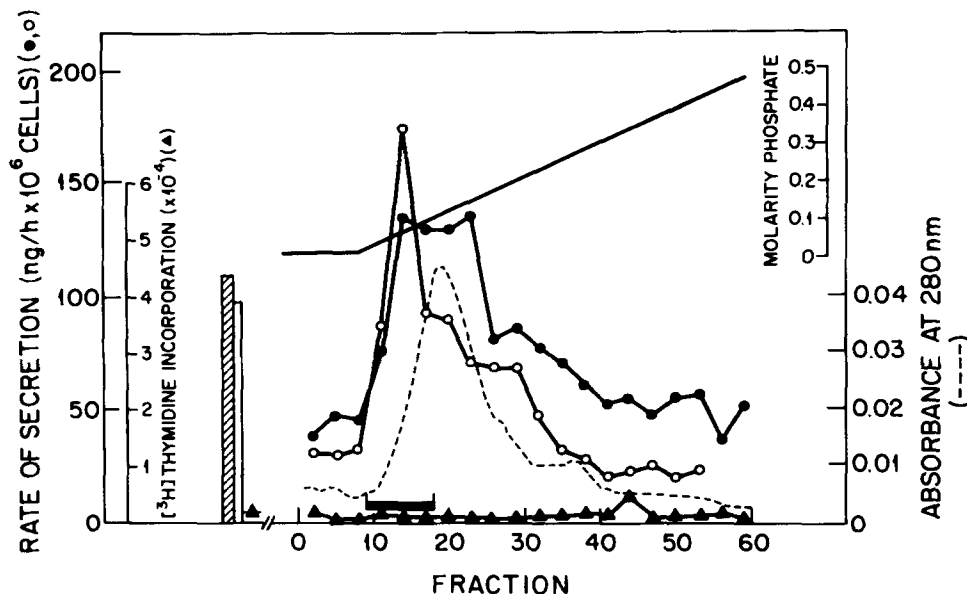
Five independent purifications of HSFs from conditioned medium of COLO-16 cells yielded the same qualitative separation as illustrated in the example reproduced in Figs. 2–4. In each case, we found that the different HSF forms were present essentially in the same relative proportion and that the purification had recovery values comparable to those

shown in Table I. However, the absolute amounts of HSF activity actually obtained varied considerably from one preparation to the other. This variability could be mainly attributed to the quite different concentrations of HSF activity in the conditioned media, ranging from 50 to 900 U/ml. Since all the media were collected during the same culture period of COLO-16 cells (that is, during growth phase before reaching confluency), it appears that production of HSFs is dependent not only on growth but also on other, not yet known, cellular processes and/or culture conditions. The quantitation of the purification (Table I) indicated a major reduction of activity during the last chromatographic step. We found that ultrafiltration of the low protein–containing samples, either before or after gel filtration was a major cause for loss of both protein and activity. Therefore, to improve recovery of HSF activity intended for cell regulation studies, we added 0.1% fetal calf serum to the HSF fraction before the last concentrating step.

Chromatographic Comparison of HSFs from COLO-16 Cells with Those from Other Cell Types

Having observed that COLO-16 cells release several forms of HSFs, the questions arose as to (a) whether nontransformed epidermal cells release a similar spectrum of HSFs; (b) whether one of the forms shares structural similarities with HSF of peripheral blood monocytes, and (c) whether mouse squamous carcinoma cells also are producers of HSFs equivalent to the human factors.

Size fractionation of conditioned medium from normal human keratinocytes revealed the presence of HSF activity with an apparent molecular weight of 30,000 (Fig. 5A). When conditioned medium was fractionated as outlined in Figs. 2–4, essentially all activity showed a chromatographic behavior identical to HSF-I of COLO-16 cells (data not shown). Since previous analysis of blood monocytes also had indicated a predominant form of HSF with a molecular weight of 30,000 (4, 22, 32, 43), we applied the same purification scheme to conditioned medium of these cells. The majority of the activity could again be recovered in the same fractions as HSF-I from COLO-16 cells (Fig. 5B). Isoelectric focusing of this

Figure 3. Hydroxyapatite chromatography of HSF-II. Nonbound material of the DEAE–Sephadex chromatography (Fig. 2) was applied to a hydroxyapatite column (1.6 × 16 cm) in 10 mM sodium phosphate, pH 6.8. The breakthrough material (containing HSF-III) was collected as one fraction (600 ml). The HSF activity is indicated by vertical bars. Open bars, stimulation of α_1-antichymotrypsin in HepG2 cells. Hatched bars, α_2-macroglobulin in rat hepatocytes. The activity was concentrated by chromatography on a 2-ml column of concanavalin A–Sepharose and eluted in 10 ml 0.5 M α-methylmannoside in phosphate-buffered saline. The hydroxyapatite column was washed with 1 liter of 10 mM phosphate

buffer and then eluted with a phosphate gradient in fractions of 5 ml. The eluate was assayed as in Fig. 2. The fractions, 9–18, containing HSF-II, were pooled and concentrated to 10 ml by ultrafiltration.
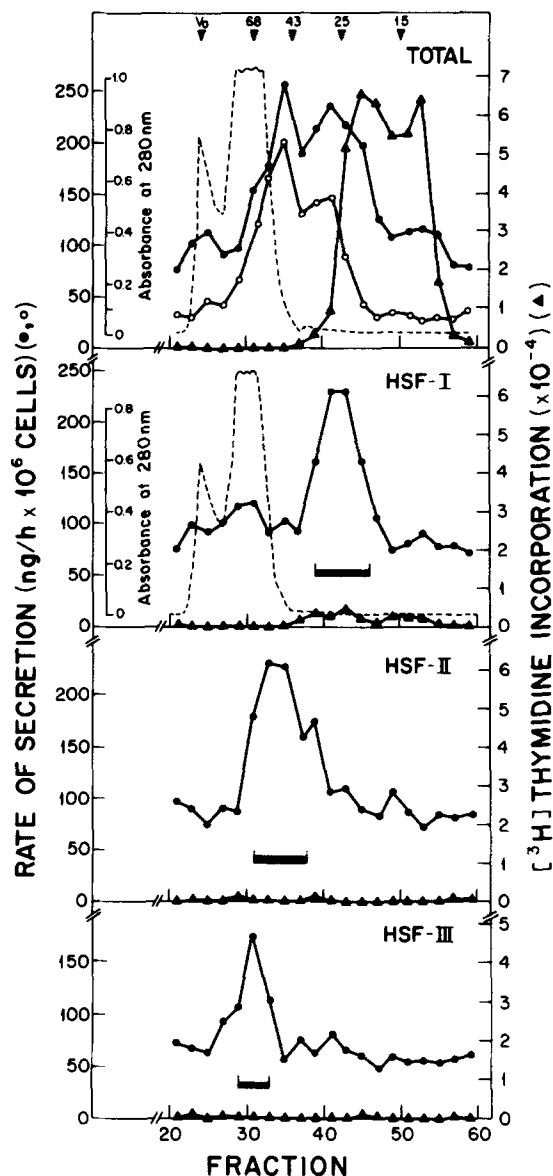
Figure 4. Gel chromatography of COLO-16–derived HSFs. 2 ml of 10-fold–concentrated conditioned medium of COLO-16 cells (*TOTAL*) or 2 ml of the concentrated fractions containing *HSF-I* (DEAE-bound, Fig. 2), *HSF-II*, (hydroxyapatite bound, Fig. 3), and *HSF-III* (hydroxapatite breakthrough and concanavalin A bound, Fig. 3) were chromatographed against the gravity on a column of Sephadex G-100 (1.6 × 100 cm) in phosphate-buffered saline. The eluate was collected in 3-ml fractions and assayed as in Fig. 2. Bars indicate the pooled fraction used for further studies. No absorbance at 280 nm could be detected in the eluate from the chromatography of HSF-II and -III. In the top panel, the elution position of molecular weight marker proteins are indicated (values in thousands).

peak yields a single band of activity with an apparent isoelectric point (pI) of 5.5. A minor portion of the total HSF activity produced by human monocytes chromatographed like HSF-II of COLO-16 cells. Due to the low amount of activity available, no determination of pI values could be done yet.

The mouse squamous carcinoma cell line MCA-3 also was found to produce HSF activity. However, the relative amount of activity released by these cells was much less than by COLO-16 cells. To obtain a maximal response of rat hepatocytes (HepG2 cells did not respond to the mouse factor; see

Table I. Purification of HSFs

Fraction	Protein	HSF activity	Specific activity
	mg	*U*	*U/mg protein*
Conditioned medium of COLO-16	2,800	320,000	114
DEAE-Sephadex bound	88	50,000	568
DEAE-Sephadex breakthrough	2.8	48,000	17,100
Hydroxyapatite bound	0.3	12,400	41,300
Hydroxyapatite breakthrough	0.2	14,000	70,000
Sephadex G-100			
HSF-I	0.076	12,000	156,000
HSF-II	0.011	1,200	109,000
HSF-III	0.005	1,800	360,000

Conditioned medium of COLO-16 cells (400 ml of the same preparation as in Fig. 2) was fractionated as illustrated in Figs. 2–4, with the modifications that the fractions eluted from DEAE-Sephadex, hydroxyapatite, and concanavalin A (HSF-I, -II, and -III, respectively) were concentrated to 3 ml by ultrafiltration and that the entire samples were chromatographed on Sephadex G-100. Total protein amount and HSF activity were determined in the pooled fraction obtained from the indicated chromatographic step.

also reference 4), conditioned medium of 10–20 times greater concentration than COLO-16 medium had to be used. Fractionation of MCA-3 medium on Sephadex G-100 resulted in separation into two major peaks with apparent molecular weights of 50,000 and 70,000 (Fig. 5C). The 50,000-mol-wt component could be purified like HSF-II from COLO-16 cells (data not shown). This HSF form was at least as effective as the human HSF-II in stimulating the synthesis of major acute-phase proteins in rat hepatocytes (see Table III below). DEAE chromatography of MCA-3 medium also indicated the presence of activity that binds to the anion exchanger. Whether this portion represents an HSF form comparable in charge and size with HSF-I remains to be shown. At present, we do not have any indication as to the similarity of the higher molecular weight form (>50,000) with HSF-III.

Properties of the HSFs Purified from COLO-16 Cells

The three HSF forms separated from conditioned medium of COLO-16 cells do not only differ in size when analyzed to gel filtration but also in isoelectric points (Table II). HSF-III, unlike HSF-I and -II, failed to focus into discrete bands and appears quite uniformly spread over a pH range from 5 to 7.5. Determination of the pI of active HSF was additionally complicated by the fact that the activity of all forms was acid labile. Treatment of the HSF preparations with buffers of different pH indicated a gradual loss of activity when the pH dropped below 6. Exposure of HSFs to pH 5.0 for 12 h at 4°C resulted in a 90% reduction of the activity (data not shown).

A major structural distinction between the HSFs is that HSF-II and -III bind to concanavalin A while HSF-I does not show any detectable affinity. Although the lectin binding suggested the presence of carbohydrate residues, their actual existence still has to be verified by biochemical analysis. More detailed presentation of the biochemical properties and the structural relatednesss of the different HSF forms will be the subject of a future study.

The separation of HSF activities into three structurally distinct forms and simultaneous removal of thymocyte-stimulating activity is, however, sufficient to address the question concerning the qualitative and quantitative effect of distinct
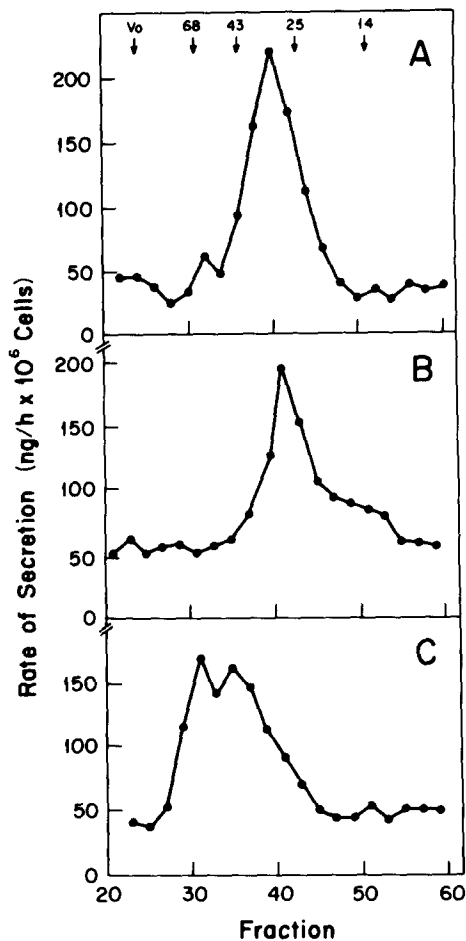
Figure 5. Gel filtration of HSFs from different cell sources. 3-d-conditioned medium of normal human keratinocytes (A) and mouse squamous carcinoma (MCA-3) cells (C) were concentrated 10-fold by ultrafiltration. Aliquots of 2 ml were separated on a Sephadex G-100 column as in Fig. 4. In B, 30 ml of conditioned medium of human blood monocytes activated by S. aureus toxin A (4, 35) was chromatographed first on a DEAE–Sephadex column (similar to Fig. 2) and the bound fraction, after concentrating to 2 ml, was separated on Sephadex G-100. The HSF activity in the eluate of each column was determined only on rat hepatocytes, since HSF of MCA-3 cells failed to significantly stimulate HepG2 cells.

Table II. Properties of HSFs from COLO-16 Cells

| Form | Molecular weight | Isoelectric point | Con A binding | Maximal stimulation | |
				HepG2 cells	Rat hepato-cytes
				%	%
HSF-I	30,000	5.5	–	100	100
HSF-II	50,000	7.2 and 8.5–9.0	+	50–70	100
HSF-III	70,000	?	+	50	50

HSF preparation on the liver response. Specifically, we asked whether all three HSF forms have the same biological activity, meaning the ability to induce all the changes in plasma protein expression associated with acute-phase reaction, or whether each form influences only a part of the hepatic response. Therefore, we first defined basic properties of the regulatory activities by analyzing the effects of HSFs on mRNA concen-

tration and cellular production of α_1-antichymotrypsin in the phenotypically stable HepG2 cells. Since the acute-phase response of nontransformed liver cells encompasses a wider spectrum of modulated plasma proteins than exists in HepG2 cells, we extended our characterization of HSF activities to the regulation of all major acute-phase plasma proteins in adult rat hepatocytes. Analyzing the effect of human factors on rat cells also allowed us to determne to what degree the species-specific liver response is dependent upon the inducing agent.

Response of Human Hepatoma Cells to HSFs

When serial dilutions of HSF-I, -II, and -III were tested for their effect on α_1-antichymotrypsin synthesis in HepG2 cells, we observed that, at submaximal HSF concentrations, a similar relationship existed between the cell response and HSF concentration (Fig. 6). The factors differed, however, in their maximal level of stimulation. The results from five preparations consistently showed that (a) HSF-I was most effective and could modulate the synthesis at least to the level of nonfractionated conditioned medium; (b) the maximal stimulation by HSF-II ranged between 50 and 70%; and (c) the activity of HSF-III was always limited to 50% maximal stimulation. Combination of maximal amounts of HSF-III and HSF-II did not result in a response that surpassed that of HSF-II alone. Likewise, addition of a maximal concentration of HSF-III to a submaximal concentration of HSF-I did not
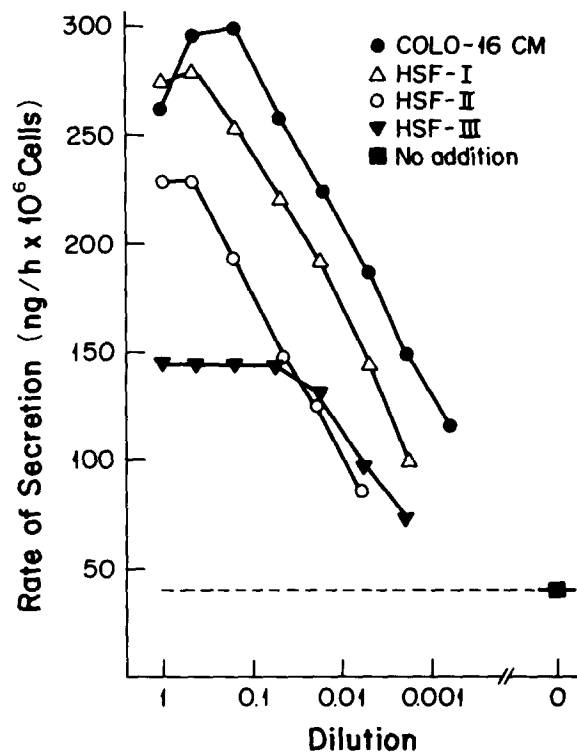

Figure 6. Activity of HSFs from COLO-16 cells. Fractions from G-100 columns containing HSF-I, -II, and -III (Fig. 4) were concentrated by ultrafiltration to 2 ml and added to 4 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum (equal dilution 1). Serial dilutions of these preparations were tested for their ability to stimulate the synthesis of α_1-antichymotrypsin in HepG2 cells. The means of duplicate cultures are shown. The maximal level reached by nonfractionated conditioned medium of COLO-16 cells (COLO-16 CM) was defined as 100% (Table II).

lead to an enhancement of the HSF-I response (data not shown).

The difference in the ability of the various HSF forms, in particular HSF-III, to induce the α_1-antichymotrypsin necessitated a modification of the definition of HSF activity units introduced by Ritchie and Fuller (31) which is based on titration of HSF preparation to the level of half-maximal stimulation of acute-phase protein synthesis. To have an estimate for the activity of all HSF forms but without introducing separate unit definitions for each HSF form, we chose as a unit of HSF activity the concentration of HSF needed to stimulate the synthesis of α_1-antichymotrypsin in HepG2 cells to 33% maximal level. The 100% level was defined as the level reached maximally by nonfractionated conditioned medium of COLO-16 cells. By measuring the effect of HSFs from human monocytes and COLO-16 cells on α_1-antichymotrypsin synthesis in HepG2 cells and on fibrinogen synthesis in rat hepatocytes (using the same assay system as Ritchie and Fuller [31]), we could calculate that about four of our HSF units equal one HSF unit of Ritchie and Fuller.

The stimulation of α_1-antichymotrypsin production is a consequence of an increase in functional mRNA (4). As depicted in Fig. 7, the HSF-mediated accumulation of mRNA is rapid and attains its maximal level within 12 h. There is no significant difference in the time course of the response to the three HSF forms. In each case, the relative change of mRNA concentration achieved within 24 h is comparable to that of cellular synthesis of α_1-antichymotrypsin (Figs. 6 and 7). The

response is gene specific and is not a result of a general increase of mRNA for secretory proteins as illustrated by the nonaffected or even slightly reduced level of mRNA for apolipoprotein A-1. The apparent absence of a lag period in induction of α_1-antichymotrypsin mRNA suggested that the cellular reaction triggered by HSF might influence directly the α_1-antichymotrypsin gene expression and not be dependent on generating an intermediary regulatory protein (2, 41). This notion is supported by the observation that the HSF-stimulated mRNA accumulation is not at all affected in HepG2 cells whose protein de novo synthesis has been completely inhibited by cycloheximide during the HSF treatment (Fig. 8 shows the results with HSF-I; the same results were obtained when HSF-II and -III were used). Furthermore, HSF had no detectable influence on the relative amounts or basal level of α_1-antichymotrypsin mRNA in actinomycin D-treated cells, suggesting that HSF does not exercise a significant change in posttranscriptional stability of the cellular mRNA for this protein.

Although glucocorticoids were found to have stimulatory effects on the expression of several acute-phase proteins in rodent liver cells, including the mouse and rat α_1-antichymotrypsin homologs (2, 3, 9, 22), there is no discernible effect of dexamethasone on the HSF action and on the stimulation and stability of cytoplasmic mRNA for α_1-antichymotrypsin in HepG2 cells (Fig. 8). This apparent lack of a requirement of glucocorticoids for HSF activity in HepG2 cells is in sharp contrast to HSF-regulated plasma proteins in rat hepatocytes.
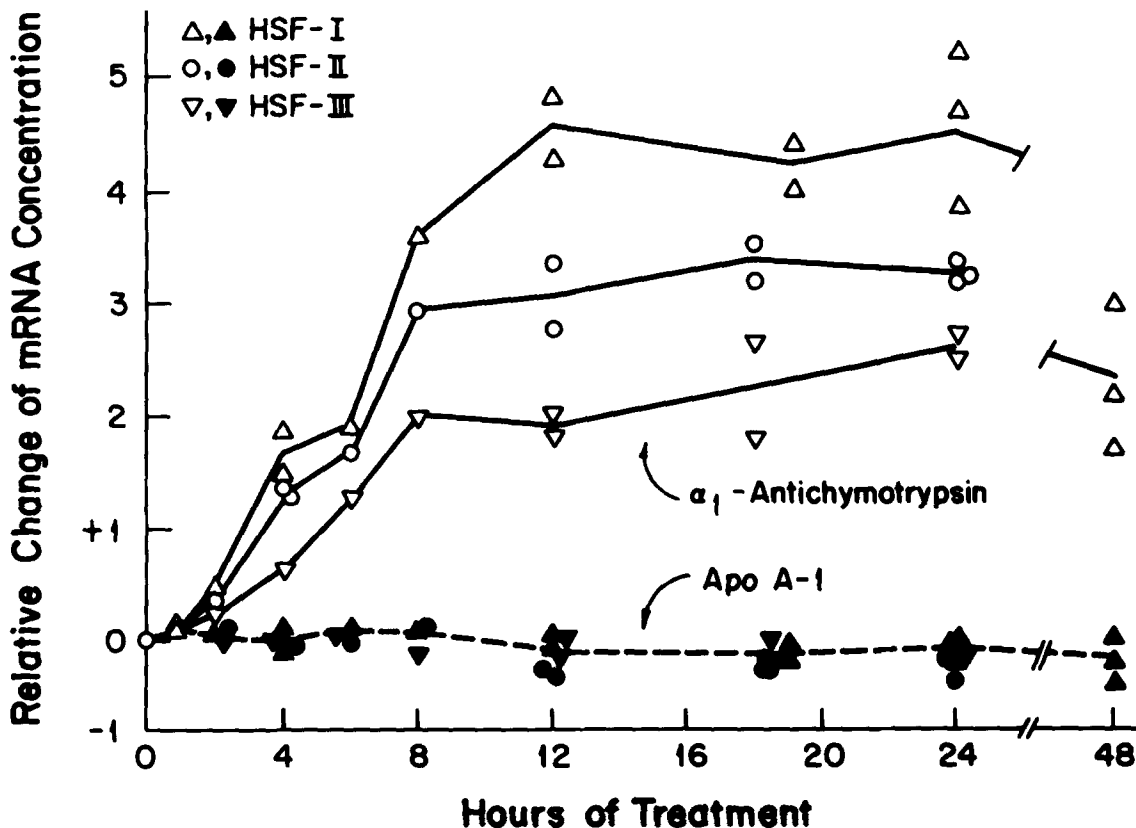

Figure 7. Time course of mRNA induction by HSF in HepG2 cells. Monolayers of HepG2 cells were treated with medium containing HSF-I (100 U/ml), HSF-II (100 U/ml), or HSF-III (10 U/ml). Cellular RNA were extracted at the time indicated. The concentration of mRNAs for α_1-antichymotrypsin (open symbols) and apolipoprotein A-1 (apo A-1, closed symbols) were measured by dot blot hybridization. The values obtained from three separate experiments are expressed as the relative increase above or decrease below the level of the mRNAs in cells not treated with HSFs.
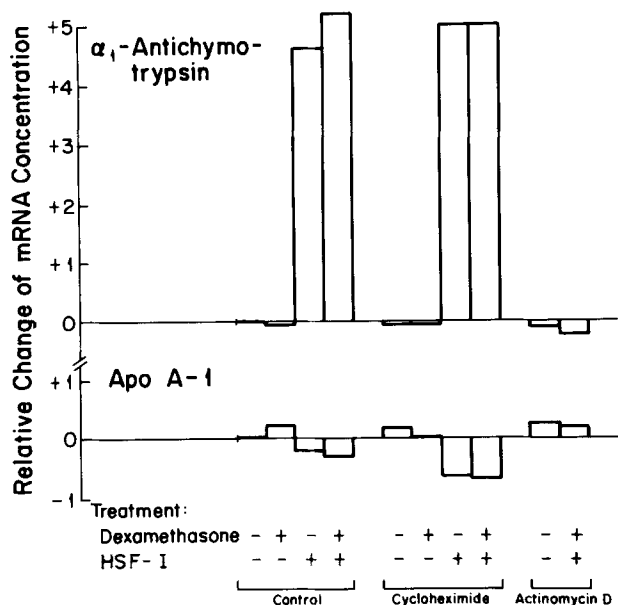
Figure 8. The effect of inhibitors of protein and RNA synthesis on stimulation of α_1-antichymotrypsin mRNA in HepG2 cells. Monolayers of HepG2 cells in 25-cm^2 flasks were cultured for 18 h in medium alone, in medium containing either 1 μM dexamethasone, 100 U/ml HSF-I, or a combination of dexamethasone and HSF-I. One set of cultures were kept free of inhibitor (*Control*), one was pretreated for 2 h and then maintained in the presence of 5 μg/ml *cycloheximide*, and another set was similarly pre-treated and maintained in the presence of 5 μg/ml *actinomycin D*. The concentration of inhibitors was sufficient to reduce protein and RNA synthesis more than 95%. Total cellular RNA were extracted after the treatments. Cycloheximide treatment lowered the yield of RNA per monolayer by 20–25% and actinomycin by 35–45% when compared to cells maintained in the absence of inhibitors. Relative concentration of mRNA for α_1-antichymotrypsin and apolipoprotein A-1 were quantitated by dot blot hybridization. The means of duplicate cultures were related to nontreated control cells.

Response of Rat Hepatocytes to HSFs

If HSFs actually represent the universal inducers of the hepatic acute-phase reaction (32), the definition of their biological activity should include the stimulation of the entire set of acute-phase reactants. Since none of the available hepatoma cell lines show regulated expression of more than a few acute phase plasma proteins, we had to resort to primary culture of adult hepatocytes. Although the differentiated phenotype of cultured rat hepatocytes is not optimally preserved in tissue culture (12, 16), these cells have still proven to be one of the best in vitro systems in that they react to inflammatory stimuli by increased synthesis of almost all major acute-phase plasma proteins (4, 22, 31–34). Rat liver cells are unique in that a full response is dependent on glucocorticoid (4). Therefore, to properly assess the activities of HSF-I, -II or -III, we had to determine separately the contribution of the steroid, the HSFs, and the combination of the two, to the regulated expression of each acute-phase plasma protein. In most cases, however, it was sufficient to quantitate the HSF activity by determining the factors specific effect on the expression of individual plasma proteins in dexamethasone-treated cells (3, 4, 22, 31–34, 43). Nevertheless, one has to keep in mind that dexamethasone treatment alone is able to stimulate the production of several plasma proteins above the level of nontreated control cells (2–4, 12).

When we tested in rat hepatocytes the activity of HSF-I, -II, or -III to stimulate the cellular production of the marker acute-phase reactants α_1-acid glycoprotein, fibrinogen, and α_2-macroglobulin, above the level established by dexamethasone, we obtained essentially identical concentration-dependent responses for each protein (Fig. 9). The relative effectiveness of the HSF forms was very comparable to that determined in HepG2 cells (Fig. 6). The only difference was that in rat hepatocytes, HSF-II was equally effective as HSF-I or nonfractionated conditioned medium.

To assess the specific influence of the HSFs on a wider spectrum of plasma proteins and to determine in each case the contribution of dexamethasone to the overall regulation, we measured the relative change in cellular synthesis of major acute-phase proteins (Table III, representative of three independent experiments). Surprisingly, HSF-I, -II, and -III stim-
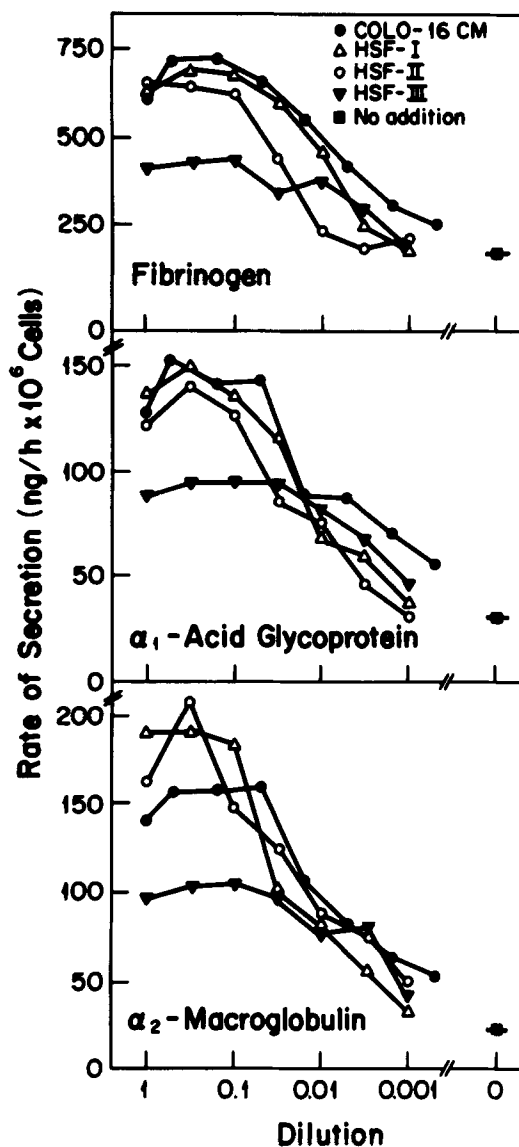

Figure 9. HSF concentration-dependent response of rat hepatocytes. Cultures of rat hepatocytes from one preparation were treated for 48 h with the same media and corresponding dilutions as used on HepG2 cells in Fig. 6. 1 μM dexamethasone was added to each culture. The rate of synthesis of fibrinogen, α_1-acid glycoprotein, and α_2-macroglobulin was determined by rocket immunoelectrophoresis. The means of duplicate cultures are reproduced.

Table III. Effect of HSFs on the Relative Synthesis of Plasma Proteins in Cultured Rat Hepatocytes

Treatment	Incorporation into total medium protein	Relative incorporation ($\times 10^4$)							
		α_2-macro-globulin	α_1-antichy-motrypsin	α_1-acid gly-coprotein	α_1-cysteine proteinase inhibitor	Haptoglobin (prohaptoglobin) + β-haptoglobin	Fibrinogen	Hemopexin	Albumin
	cpm/μg total cell protein								
0 h control	7,150	<1	64	5	17	162	174	52	911
48 h control	5,070	<1	24	7	4	82	62	102	824
Dex	6,660	42	122	61	22	131	151	78	1,030
CM COLO	5,570	<1	296	34	15	140	206	91	620
CM COLO + Dex	9,200	259	871	209	116	285	601	200	603
HSF-I	4,950	<1	152	42	12	133	183	110	599
HSF-I + Dex	8,400	268	484	186	97	312	544	198	779
HSF-II	5,390	<1	260	39	20	150	220	104	618
HSF-II + Dex	10,130	300	1,065	201	105	304	512	217	744
HSF-III	5,100	<1	57	22	10	95	120	87	660
HSF-III + Dex	7,180	101	408	115	63	224	305	180	693
HSF-II of MCA-3 + Dex	10,870	435	910	227	119	331	670	207	630

Duplicate monolayers of rat hepatocytes in 24-well dishes were treated for 48 h with culture medium alone (*control*); medium containing nonfractionated conditioned medium of COLO-16 cells (150 U/ml (*CM COLO*); same plus 1 μM dexamethasone; HSF-I, -II, or -III (100 U/ml each) plus 1 μM dexamethasone; and HSF-II isolated from conditioned medium of MCA-3 cells (100 U/ml) plus dexamethasone. After labeling of the cells with [^{35}S]methionine (100 μCi/ml) for 6 h, the radioactivity incorporated into total medium protein was measured. 50 μl of labeled culture medium from each well was separated by two-dimensional gel electrophoresis or by crossed immunoelectrophoresis (α_1-acid glycoprotein, α_1-cysteine proteinase inhibitor and fibrinogen). The radioactivity present in the indicated plasma proteins was measured and related to the total amount of radioactivity used for analysis. The values represent means of the duplicate cultures.

ulated the synthesis of the same set of major acute-phase plasma proteins including all rat-specific ones, while albumin was decreased. The qualitative effect was not significantly different from that described for nonfractionated medium (see also reference 4). Although each HSF response had a similar glucocorticoid requirement, the magnitude of overall change was dependent upon the type of HSF used. Hepatocytes exposed to maximal concentrations of HSF-III displayed only one-half stimulation of all major acute-phase proteins as compared to those treated with HSF-I or -II. The relative proportion of the changes elicited by each HSF is, however, similar. The uniformity of the hepatic response was also apparent when the hepatocytes were treated with mouse HSF (e.g., HSF-II of MCA-3 cells, Table III).

By comparing the regulation of individual proteins, remarkable differences in the response to HSF alone, dexamethasone alone, and a combination of both, became apparent. Two extreme examples are found in (*a*) α_2-macroglobulin, whose synthesis was not inducible by HSFs alone, but was primarily stimulated by HSF in the presence of dexamethasone; and (*b*) α_1-acid glycoprotein whose synthesis was similarly stimulated by both hormones. In general, however, the synergistic action of dexamethasone and HSFs prevailed. Furthermore, the presence of dexamethasone was even essential for maintenance of basal level expression of several proteins, e.g., α_1-antichymotrypsin, fibrinogen, and haptoglobin.

The broad effect of each HSF form was not only manifested at the level of protein synthesis but also at the level of mRNA (e.g., Fig. 1). The similarity of action is already detected when the time course of mRNA accumulation was measured (Fig. 10, shown for HSF-I; essentially the same results were obtained with HSF-II and HSF-III). In these experiments, the hepatocytes were cultured for the first 20 h in serum-containing medium but without additional hormones. During this period the cells established their typical cell morphology and membrane organization (36) and recovered their responsiveness to HSFs. The responsiveness to dexamethasone, however,

was never impaired as a consequence of cell preparation (data not shown). After this cellular adaptation period, the hormone treatments were started. By choosing this regimen, we could unambiguously assess the regulatory influence of HSF and dexamethasone on the basal expression of plasma protein mRNAs of receptive cells. As is apparent from Fig. 10, the accumulation of mRNA for different acute-phase proteins followed an identical time course, suggesting that hormonal treatment controls each mRNA species by a similar or even common mechanism. The kinetics differed, however, from that of α_1-antichymotrypsin mRNA in HepG2 cells (Fig. 7) by an initial lag period. This difference in the response seems to be more general to rat hepatocytes since a lag period was observed regardless of whether the cells were treated with HSF or dexamethasone alone (Fig. 10 shows the example of α_1-acid glycoprotein). Although conditions as well as hormonal environments are not comparable, the time course of accumulation for the same mRNAs in vivo during an inflammation is surprisingly similar. A major distinction lies, however, in the magnitude of change induced.

To complete the characterization of the biological activities of HSFs, we determined to what degree the HSF-mediated regulation of mRNA for the major acute-phase plasma proteins compares to that occurring during inflammation in vivo. This analysis was important for assessing not only the activity of the HSFs but also the reproducibility of a hepatic acute-phase response in cultured rat hepatocytes. For this purpose, monolayers of hepatocytes were established and then treated for 24 h with HSF-I or -II, with dexamethasone, and with combinations of the HSFs and dexamethasone (HSF-III was omitted due to its lower induction level). The changes in relative amount of mRNA for several acute-phase plasma proteins and control proteins were quantitated before and after the treatments (Table IV).

When the amount of mRNA in the control cultures was related to that present at 0 h, an interesting phenomenon became apparent. During the first 20 h, a substantial portion
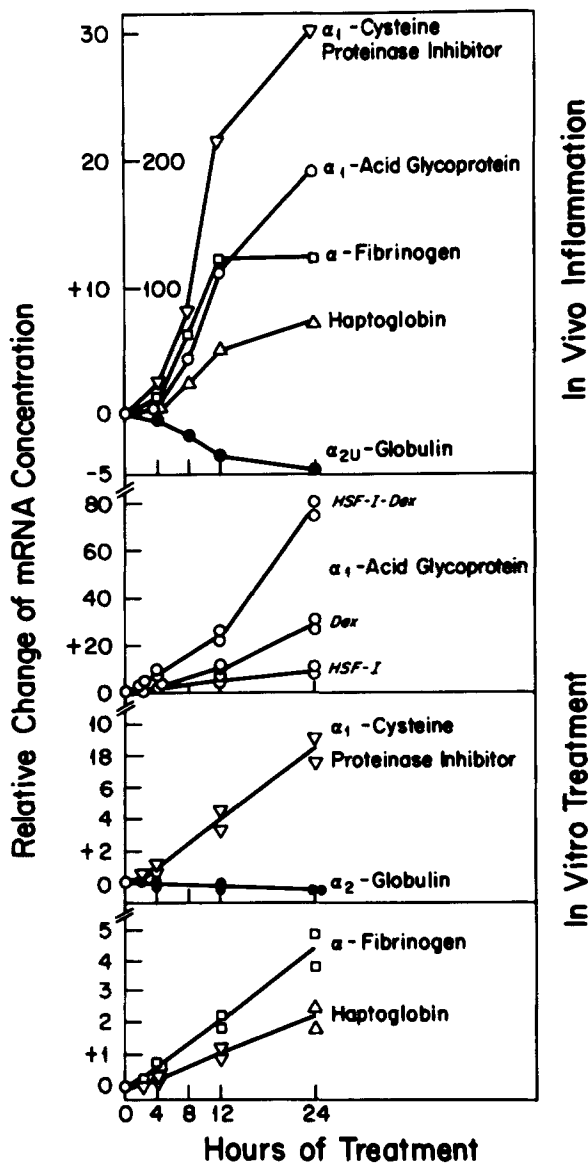
Figure 10. Time course of mRNA induction in rat hepatocytes. Monolayers of rat hepatocytes were established in 75-cm² flasks. After 20 h in culture, treatments of duplicate monolayers were started (0 h). The media contained either HSF-I (100 U/ml), 1 µM dexamethasone, or both. After various times, total cellular RNA was extracted and measured for the relative change of mRNA for the plasma proteins indicated. Only in the case of α_1-acid glycoprotein, the results obtained with all treatments are reproduced; in all other cases, the presentation of data are limited to HSF-I plus dexamethasone. The top panel shows the time course of mRNA accumulation after an in vivo inflammation (0–200 scale applies to α_1-acid glycoprotein).

of the mRNA for several plasma proteins was lost, a finding that has also been reported elsewhere (16). This decline in some, but not all, instances is reflected in a reduced synthesis and secretion of the proteins (Table III and reference 4). Treatment of the hepatocytes with HSFs and dexamethasone-induced changes in the amount of mRNA for major acute-phase plasma proteins which closely correlate with the changes in protein synthesis (Table III). This strongly suggests that the HSF-modulated protein production is mainly regulated at the mRNA level. A remarkable exception is albumin, where a several-fold decrease of mRNA does not manifest in a similar

reduction in protein synthesis (compare Tables III and IV; see also reference 4).

The response of cultured hepatocytes, although being very specific, is quantitatively less than that observed in liver (Fig. 10 and Table IV). The maximal concentrations of mRNAs attained by in vitro treatments were without exception below those in vivo. The most striking example is mRNA for α_1-cysteine proteinase inhibitor, which in vivo is one of the most prominent acute-phase reactants but whose in vitro regulation is the least reproducible (4, 22). If, however, the initial loss of RNA as well as the gradual deterioration of the hepatic phenotype are taken into consideration, the magnitudes of HSF-mediated mRNA accumulation are quite substantial and, as in the case of α_1-acid glycoprotein, even close to that observed in vivo. Contrary to the rapid loss of α_{2u}-mRNA in vivo, the concentration of this mRNA is maintained constant in tissue culture. It shows even less variation than α_1-antitrypsin mRNA whose expression is known to be not significantly affected by inflammation in vivo (Table IV).

HSFs induce in vitro not only quantitative, but also qualitative changes in mRNA species which are characteristic for acute-phase reaction of the rat liver. As indicated in Table III, rat hepatocytes, like HepG2 cells, synthesize increased amounts of α_1-antichymotrypsin after HSF-treatment. We have noted that in vivo inflammation stimulated the synthesis of α_1-antichymotrypsin by the liver cells and that this change was the consequence of a specific alteration in the mRNA population (Fig. 11*A*). Using as a hybridization probe a cDNA for mouse contrapsin, the structural homolog to human α_1-antichymotrypsin (15), we detected that during the course of inflammation the predominant mRNA form of 2 kb, which is present in normal rat liver, was reduced and that two additional forms of 2.2 and 5.5 kb were increased. This shift in hybridizable mRNA was independent of corticosteroids since liver of adrenalectomized rat showed the same inflammation response. However, the in vivo treatment with dexamethasone was able to mimic a part of that mRNA modulation; that is, to increase the amount of large size forms without affecting the basal level of the 2-kb forms. Sequence analysis of cDNAs corresponding to the different forms of mRNA have indicated that the polymorphism in mRNA is not the result of modification in processing of a single form of transcript; rather, the mRNA actually encodes homologous but nevertheless structurally different proteins (Hill, R. E., manuscript in preparation). Surprisingly, the change in α_1-antichymotrypsin mRNA forms was not manifested in the appearance of structurally distinguishable proteins (4). HSF treatment of hepatocytes in culture was able to induce a similar inflammation-specific alteration in α_1-antichymotrypsin mRNAs. Although the magnitude of change was less prominent in vitro, a proper modulation was still apparent (Fig. 11*B*, representative for three independent HSF-I treatments; HSF-II showed the same result). During the culture period of 48 h in normal medium, the 2-kb mRNA form was generally reduced. The presence of dexamethasone alone prevented the loss of this form and stimulated the appearance of the 2.2-kb form. HSF-I alone was able to elevate slightly the amount of the 2.2-kb form but in combination with dexamethasone, this form became predominant. Under this condition even a low amount of the 5.5-kb form was discernible.

So far we have presented evidence that HSFs regulate protein synthesis (Table III) by increased mRNA accumula-

Table IV. Modulation of mRNA for Acute-phase Plasma Protein in Established Monolayers of Rat Hepatocytes

	Relative change in mRNA							
Treatment	α_1-acid glyco-protein	α_1-cysteine pro-tease inhibitor	Haptoglobin	α-fibrinogen	γ-fibrinogen	Albumin	α_1-antitrypsin	α_{2u}-globulin
20 h	0/+0.5	−0.5/0	−1/−0.5	−2/−3	−1/−1	−2/−3	0/−0.5	0/−0.5
44 h control	0/+3	−0.5/−0.5	−2/−1	−3/−3	−0.5/0	−3/−3	0/−0.5	−0.5/−0.5
Dex	+6/+17	+0.5/+0.5	−0.5/0	0/0	+0.5/+1	−2/−2	0/0	−0.5/0
HSF-I	+1/+7	+1/+1	0/0	0/+0.5	0/0	−3/−4	0/−0.5	−0.5/0
HSF-I + Dex	+115/+56	+19/+8	+3.5/+2	+3/+3	+3/+2.5	−3/−3	0/+0.5	−0.5/0
HSF-II	+4/+3	+1.5/+1	+0.5/0	+0.5/+0.5	+0.5/0	−3/−3.5	0/0	−0.5/−0.5
HSF-II + Dex	+89/+41	+9/+7	+3/+1.5	+2.5/+3	+2.5/+2	−3/−3	0/+0.5	0/0
CM COLO + Dex	+76/+49	+14/+9	+4/+2	+4/+3	+3/+3	−3.5/−3.5	+0.5/+0.5	−0.5/0
24 h inflamed in vivo	+213/+121	+42/+26	+4/+14	+25/+12	+12/+8	−5/−4	+0.5/+0.5	−4/−2

Rat hepatocytes in 75-cm^2 flasks were first cultured for 20 h in serum-containing medium without any further addition of factors. The monolayers were then incubated for an additional 24 h in fresh medium alone (*control*), medium with 1 M dexamethasone (*Dex*), with 100 U/ml HSF-I, 100 U/ml HSF-II, and with a combination of dexmethasone and HSFs. Total cellular RNAs were prepared at 0, 20, and 44 h, and quantitated by dot blot hybridization for the presence of mRNA for the plasma proteins indicated. The values of two independent experiments represent the factor by which the mRNA amount has increased above (+) or decreased below (−) the level of mRNA at 0 h. Changes ranging between −0.5 and +0.5 were considered to be not significant and are indicated by "0". To compare with in vivo conditions, the changes in the mRNA observed in liver 24 h after a turpentine-induced inflammation are included.

tion (Table IV). That this HSF effect is also manifested at the level of mRNA synthesis, we could demonstrate only for α_1-acid glycoprotein mRNA (Table V, experiments limited to HSF-I). Relative rates of mRNA synthesis were determined by metabolic pulse labeling of hepatocytes followed by quantitation of the radioactive mRNA by filter hybridization. To evaluate adequately the hormone action, we also measured synthesis rates in cells immediately after preparation (0 h control) and in cells not treated with hormones (24 h control). To illustrate the validity of the method and specificity of the regulation, we included α_{2u}-globulin mRNA into our analysis because of this RNA's high abundance and stable concentration which are not affected by hormone treatment. Although quite variable from one experiment to another, the results presented in Table V indicate that HSF-I alone had no apparent influence on the relative synthesis rate of α_1-acid glycoprotein mRNA, but could enhance significantly the synthesis in cells treated with dexamethasone. Hence, only the specific increase mediated by HSF in dexamethasone-treated cells appears to be a useful measure for HSF activity on transcription. A surprising finding was that rat hepatocytes, unlike rat hepatoma cells (41), respond to dexamethasone alone by a several-fold increased relative synthesis of α_1-acid glycoprotein mRNA.

The hormone-induced changes in mRNA synthesis correlate qualitatively well with changes in RNA concentrations (Table IV), suggesting that the regulated expression of α_1-acid glycoprotein is at least in part the consequence of increased gene transcription. Unfortunately, measurements of mRNA for haptoglobin and α_1-cysteine proteinase inhibitor in the same cells did not show any significant deviations from basal values (data not shown). These results are not unexpected considering the low magnitude of mRNA stimulation (Table IV).

All of the above presented data confirm that different HSF forms of human cells can each modulate the expression of the same set of major acute-phase plasma proteins in rat hepatocytes. The regulation is manifested at the level of mRNA concentration which resembles qualitatively and to some extent quantitatively the response of liver cells to acute inflammation in vivo. The human HSFs can act on hepatic

Table V. Effect of HSF on Relative Synthesis Rates of α_1-Acid Glycoprotein mRNA

	cpm Hybridized			Relative rate of synthesis (ppm)	
RNA sample	Input	pBR322	AGP	α_{2u}-glob-ulin	AGP	α_{2u}-glob-ulin
	cpm (× 10⁻⁶)					
Experiment I						
0 h control	6.0	6	47	411	7	68
24 h control	1.0	2	8	34	6	32
Dexamethasone	1.3	2	70	41	56	31
HSF-I	0.8	2	10	30	10	35
HSF-I + Dex	1.5	3	326	63	215	40
Experiment II						
0 h control	4.3	4	5	202	<1	46
24 h control	0.9	1	5	29	4	31
Dexamethasone	1.5	2	41	34	26	21
HSF-I	1.7	6	22	63	9	34
HSF-I + Dex	1.1	3	69	30	60	25

In two separate experiments, monolayers of rat hepatocytes were established in 75-cm^2 flasks. After a 20-h culture in medium with 10% fetal calf serum alone, the cells were treated for 4 h with fresh culture medium (*control*), with medium containing either 1 μM dexamethasone, 100 U/ml HSF-I, or 1 μM dexamethasone plus 100 U/ml HSF-I. The cells immediately after adhering to the plates (*0 h*) or after treatment (*24 h*) were labeled with [^{3}H]uridine. The cellular RNA was extracted, hybridized to immobilized pBR322 DNA alone and with cDNA to α_1-acid glycoprotein (*AGP*), and α_{2u}-globulin. The actual amounts of filter-bound radioactivity measured as well as calculated as fraction of the total input radioactivity (in part per million) are reproduced.

cells of different species; the response of the liver cells is, however, strictly species specific.

Discussion

The presented purification scheme provided us with preparations of structurally distinct HSFs which, although not being pure, allowed us to address the questions concerning complexity of liver-regulating factors, spectrum of biological activity, and the relationship to thymocyte-stimulating factor, in particular IL-1. Previous studies have already indicated that
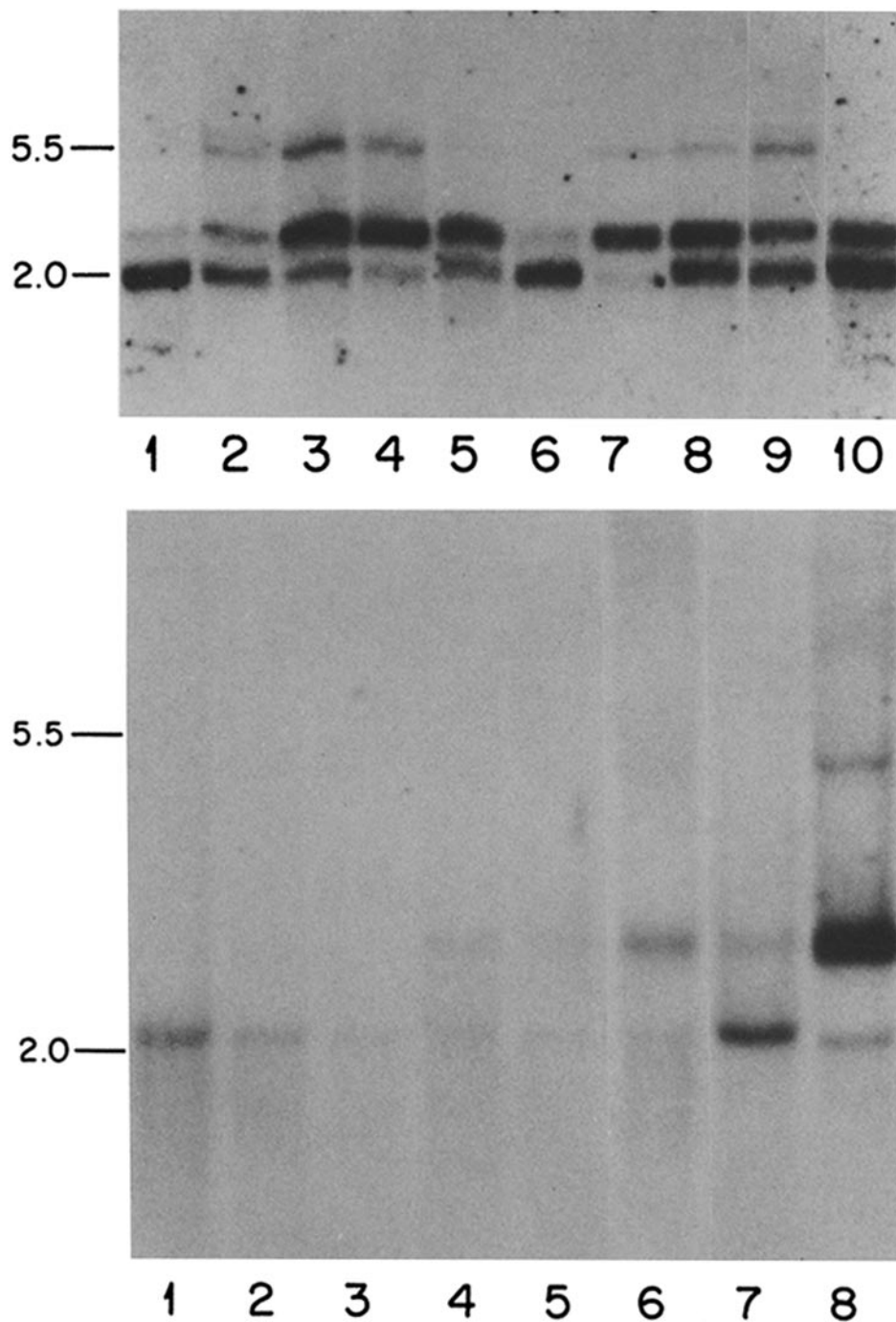
Figure 11. Modulation of mRNAs encoding α_1-antichymotrypsin in rat liver cells. (*Top*) Effect of in vivo treatments. Aliquots of total liver RNA (25 μg) were separated on a 1.5% agarose gel in 2.2% formaldehyde, transferred to nitrocellulose, and probed with nick-translated plasmid DNA (p54) containing cDNA-to-mouse contrapsin, the structural homolog to human α_1-antichymotrypsin (15). The lanes contain liver RNA from a nontreated male rat (*1*), a male rat after 4 h (*2*), 12 h (*3*), 24 h (*4*), or 48 h (*5*) turpentine-induced inflammation, a rat 14 d after adrenalectomy (*6*), a similar adrenalectomized rat but 24 h after turpentine injection (*7*) or 24 h after dexamethasone injection (*8*), and a normal rat treated for 4 h (*9*), or 24 h (*10*) with dexamethasone. (*Bottom*) Effect of in vitro treatments. Hepatocytes were placed in culture, and after 20 h the cells were treated for additional 24 h with medium alone, or medium containing either 1 μM dexamethasone, 100 U/ml HSF-I, or a combination of the two. Total cellular RNA was analyzed as in top panel. The lanes contain RNA extracted from cells 1 h (*1*) and 20 h (*2*) after placing in culture, and from cells after treatment with medium alone (*3*), or medium with dexamethasone (*4*), HSF-I (*5*), and HSF-I plus dexamethasone (*6*). For comparison, RNA from a normal rat (*7*) and 24-h inflamed rat (*8*) were included. The numbers at the left indicate the positions of the ribosomal RNAs and represent the sizes in kilobases.

HSFs exist with apparent molecular weights greater than that of IL-1 (4, 22, 32). We have now achieved the isolation of HSFs from COLO-16 cells that are essentially free of thymocyte-stimulating activity. The thymocyte stimulation assays were carried out with concentrations of HSF preparations up to 10 times higher than that needed to reach maximal stimulation of rat hepatocytes. Whether at even higher concentrations a thymocyte stimulation would become apparent has not been tested and is also not considered to be significant. Although our results indicate that there are indeed HSFs without thymocyte-stimulating activity, we cannot rule out the possibility that IL-1 or IL-1–like factors also contain HSF activity. Functional analysis of homogeneous preparations of

IL-1, which are underway in several laboratories (10, 13), will clarify this point.

The functional definition of HSFs should not be limited to the regulation of a single acute-phase protein (9, 27) but should include the regulation of the entire set of acute-phase reactants (4, 22). Since only primary cultures of adult rodent hepatocytes allow an experimental assessment of the full spectrum of HSF functions, the limitation of culture systems has to be taken into account in the interpretation of data. As indicated in Tables III and IV, HSFs can specifically modulate the expression of acute-phase proteins. However, the quantitative effect on individual sequences differs remarkably from that of liver cells treated in vivo. At present, it is not known

whether this incomplete reproduction of hepatic acute-phase reactions in vitro by HSF is due to (a) the lack of additional, not yet recognized factors; (b) the instability of the cellular phenotype; or (c) a combination of the two. Considering the fact that different human and murine HSF preparations can elicit the same response pattern in rat hepatocytes, we tend to believe that the isolated HSF would be sufficient in properly modulating most, if not all, acute-phase proteins, but that processes such as reorganization of cell morphology, re-establishment of the cell domains, recovery of HSF responsiveness, and metabolic adjustment to the culture environment does influence and modify the execution of the HSF signal.

Despite the drawbacks of the culture systems, the experiments with hepatocytes yielded an interesting feature. The cells' response to different forms of HSFs is remarkably uniform and is strictly rat specific (e.g., stimulation of α_2-macroglobulin and α_1-cysteine proteinase inhibitor). We speculate that the adult hepatocytes are programmed to respond to inflammatory stimuli by a change in the synthesis of a fixed set of plasma proteins. The function of HSFs appears to be to trigger and tune the execution of this program.

To achieve a maximal response of rat hepatocytes to HSFs, the cells have to be cultured in the presence of dexamethasone. Although not yet supported by sufficient experimental evidence, we suspect that the role of glucocorticoids is to maintain the cells' sensitivity to HSF either by stimulating the synthesis, or by preventing the loss of cell elements which are involved in mediating the HSF signal. An additional role of dexamethasone is, of course, the direct influence on the expression of specific acute-phase protein genes, such as α_2-macroglobulin and α_1-acid glycoprotein (Tables IV and V). HepG2 cells seem to differ from rat hepatocytes in that these cells normally contain the postulated elements in amounts not limiting maximal HSF response. This would explain the glucocorticoid-independent HSF action (Fig. 8) and instant inducibility of α_1-antichymotrypsin mRNA accumulation (Fig. 7) in these cells.

HSF-I, -II, and -III show a remarkable difference in their effectiveness (Figs. 6 and 9). Our current and limited knowledge does not provide an explanation for this distinction. Two alternative possibilities could be envisioned. (a) The three HSF forms interact with the same receptors on the surface of the liver cells but possess different stimulating activities, or (b) each HSF has a specific receptor, whereby the number and/or activity of this receptor determined the maximal cell response.

The modulation of acute-phase plasma proteins by HSFs is correlated with a change in mRNA concentration. At the present, we do not know the exact molecular basis for stimulated mRNA expression. The only indication that HSF influences transcriptional activity stems from the results with α_1-acid glycoprotein mRNA in rat hepatocytes (Table V). Moreover, the phenotypic instability of cultured rat hepatocytes and present experimental limitations did not yet allow an accurate assessment as to what extent a modulation of mRNA processing and turnover properties contribute to the acute-phase protein expression. That the latter processes can play an important role in regulation of acute-phase protein mRNA has been recently demonstrated by Vannice et al. (41) for dexamethasone induction of α_1-acid glycoprotein in rat hepatoma cells.

The results of this work have indicated two future directions

of these studies. One direction is toward the characterization of the HSFs. A purification scheme has to be developed, using either biochemical or immunological means, which will allow the isolation of HSFs in pure form and in quantity sufficient to perform structural analyses. It will be of importance to establish whether HSF-I, -II, and -III are structurally related. Once purified HSF is available, identification of the hepatic receptors also will become feasible.

The second direction of our future studies will address the cellular mechanism by which HSF achieves the pleiotropic response of liver cells. It will be essential to elucidate the extent to which changes in transcription, RNA processing, translation, cellular processing, and secretion contribute to the regulated expression of diverse plasma proteins, all of which constitute the hepatic acute-phase reaction.

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