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Induction of Heat Shock Proteins in Short-Term Cultured Hepatocytes Derived from Normal and Chronically Griseofulvin-Treated Mice†

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Freshly isolated mouse hepatocytes were tested with respect to the induction of heat shock (stress) proteins by elevated temperature, sodium arsenite and ethanol treatment. With heat, arsenite and ethanol treatments, the synthesis of a protein with a molecular weight of 68 kD (heat shock protein 68) was predominantly elevated; arsenite and ethanol exerted their effects on heat shock protein synthesis in a dose-dependent manner. Hepatocytes derived from livers of chronically griseofulvin-pretreated mice differed in their response from normal hepatocytes in that ethanol was ineffective in these cells. These results indicate that different modes and pathways of the stress response exist, depending on the nature of the inducing agent but also on pretreatment conditions. *In vivo*, pathologic alterations of cells and organs (e.g., in the course of chronic diseases) can, therefore, be expected to modulate the stress response.

The heat shock (stress) response is a reaction of cells to diverse metabolic, chemical and physical stress conditions, including hyperthermia, hypoxia, lytic viral infections, pH changes, treatment with various chemicals and mechanical trauma, which is highly conserved during evolution [for review, see Refs. (1–3)]. It is regulated at both transcriptional and translational levels and leads to the appearance of a set of proteins, called heat shock or stress proteins (HSPs), often, but not always, followed by reduction of the synthesis of normal proteins (4–6). HSPs repress their own synthesis at both transcriptional and posttranscriptional levels (4, 7). The heat shock response depends on a variety of factors, particularly nature, intensity and duration of stress. This response is extremely rapid and neither new protein nor RNA synthesis is required. HSPs are not necessarily novel proteins but may be produced at basal rates in cells; synthesis is then increased in response to stress conditions (8). However, some tissues (e.g., in the embryo) do not respond (9). The biological role of the heat shock response is still unclear; the ubiquity of the response and its considerable uniformity after diverse stress conditions suggest a multipurpose system with functions involved in the recovery from noxious insults (10). Moreover, it bestows protection on the cells to subsequent environmental stresses, including thermotolerance (8, 11, 12). Alterations of cell shape occur under stressful growth conditions and can, at least partly, be attributed to changes of the cytoskeleton (13). Indeed, an affinity of certain HSPs to cytoskeletal components (e.g., intermediate filaments and microtubules) and similarities between HSPs and cytoskeleton-associated proteins have been reported (13–15).

Studies in our laboratory are mainly concerned with the elucidation of structural and metabolic alterations associated with chronic liver disease and their role in the response of the chronically diseased organ to additional insults. Since the liver is one of the major targets of environmental stresses, including chronic intoxications and viral infections, the HSP response of normal and diseased hepatocytes was investigated. Chronic griseofulvin (GF) intoxication of mice leads to profound alterations of the intermediate filament cytoskeleton of the hepatocytes in addition to induction of porphyria and closely resembles severe alcoholic liver disease (i.e., alcoholic hepatitis). This experimental model, therefore, seemed to be suitable for studies of the influence of chronic stress conditions on the liver. Normal mouse liver cells and cells isolated from mice fed a GF-containing diet for 3 months were exposed to elevated temperature, sodium arsenite and ethanol. It will be shown that both normal and GF pretreated mouse liver cells develop HSPs after heat or sodium arsenite administration in an almost identical manner. However, a significant difference in the response to ethanol between normal and GF-pretreated cells was found in that hepatocytes isolated from GF-intoxicated mice did not respond to ethanol with increased amounts of HSPs in contrast to normal hepatocytes.

MATERIALS AND METHODS

Animals. Male Swiss albino mice (strain Him OF1 SPF, Institute of Laboratory Animal Research, University of Vienna School of Medicine, Himberg, Austria; 35 gm body weight) were fed a standard diet (Altromin, Lage/Lippe, Federal Republic of Germany). To study the effect of chronic GF intoxication, mice received a GF containing (2.5% w/w) powdered standard diet for 3 months (16).

Isolation of Mouse Hepatocytes. The animals were anesthetized with Nembutal (60 to 90 μg per gm i.p.). The abdo-

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men was opened by a middle incision and the chest by a lateral incision. Superficial incisions were made through the liver capsule with a razor blade. The left heart ventricle was cannulated and connected to a peristaltic perfusion pump. The cannula was fixed to the ventricle with a mosquito clamp (17). Through the ventricle, the liver was then perfused for 10 min at a flow rate of 2.5 ml per min with a Krebs-Ringer bicarbonate buffer solution containing HEPES (20 mmoles per liter), 0.07 % collagenase Type IV, penicillin (5,000 IU per ml) and streptomycin (5,000 μg per ml). The perfusion medium was continuously gassed with a oxygen-carbon dioxide mixture (95%:5%), and the pH was kept between 7.3 and 7.5. After *in situ* perfusion, the liver was removed, minced and placed into a "digestion chamber." This chamber allowed further enzymatic treatment in a continuously gassed medium without mechanical alteration of the liver cells by rising gas bubbles. The chamber was filled with McCoy cell culture medium (pH 7.4) containing 0.1% collagenase (No. C-2139, Sigma Chemical Corp., St. Louis, Mo.), dispase Grade II (4.8 units per ml), HEPES (20 mmoles per liter), streptomycin (5,000 μg per ml) and penicillin (5,000 IU per ml) (18). During the incubation period (30 min at 37°C), the chamber was placed into a shaking water bath (90 strokes per min). Thereafter, the cell suspension was filtered through a 80 μm mesh nylon net and washed twice with minimal essential medium (MEM) with Earle's salts containing methionine (for heat treatment) or without methionine (for ethanol and sodium arsenite treatments), respectively, by suspension and centrifugation at 50 × *g* for 3 min. Cell yield and viability as checked with a hemocytometer and by trypan blue exclusion were 3 × 10^6 cells per gm liver wet weight and 85 to 90%, respectively.

Heat Shock Treatment. A suspension of freshly isolated liver cells was divided in two equal portions. One portion was incubated at 43°C in a water bath for 20 min. The second portion was kept at 37°C as control. After two washings with methionine-free MEM, the heat-shocked and control cells were labeled by incubation with 2 ml methionine-free medium containing 25 μCi per ml [^{35}S]methionine (specific activity: 1,000 Ci per mmole) for 5 hr at 37°C. At the end of the labeling period, the cells were washed 3 times with 0.9% NaCl, dispersed in 3 ml 1% sodium dodecyl sulfate (SDS) and boiled for 3 min. The proteins were precipitated with 4 volumes absolute ethanol at −20°C overnight and then centrifuged at 1,000 × *g* for 10 min. The pellet was dried under vacuum (12).

Sodium Arsenite Treatment. Freshly isolated cells in suspension were divided in 4 equal portions and washed 2 times with methionine-free MEM. Cells were then incubated with 5 ml methionine-free MEM containing 0, 10, 15 and 20 μ*M* sodium *meta*-arsenite, respectively, for 4 hr at 37°C. For protein labeling, control cells and sodium arsenite-treated cells were incubated (after two washing steps) for 45 min at 37°C with 2 ml methionine-free MEM containing 25 μCi per ml [^{35}S]methionine (19). Protein extraction and precipitation were performed as described for the heat-shocked cells.

Ethanol Treatment. A suspension of freshly isolated cells was divided in 4 equal portions and washed 2 times with MEM containing methionine. Cells were then incubated for 30 min at 37°C with 5 ml MEM (with methionine) containing 0, 4, 6 and 8% ethanol, respectively. After two washing steps with methionine-free MEM, cells were labeled for 4 hr at 37°C with 25 μCi per ml [^{35}S]methionine in 2 ml methionine-free MEM. Protein extraction and precipitation were performed as previously described.

Gel Electrophoresis. The dried pellets were solubilized in 10 m*M* sodium phosphate buffer (pH 7.4) containing 10% β-mercaptoethanol and 5% SDS, and one or two-dimensional polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (20) and O'Farrell (21).

Autoradiography. The SDS polyacrylamide gels were incubated with an autoradiography enhancer (Enlightning®) for 30 min, dried and exposed to a Trimax XD X-ray film at −80°C for various periods of time. The autoradiographies were scanned with a video densitometer 620 (Bio-Rad Laboratories, Richmond, Calif).

Reagents and Chemicals. The following products were used: GF (Glaxo, Greenford, England); Nembutal (La Société Sanofi Santé Animale, Paris, France); HEPES (Flow Laboratories, Meckenheim, Federal Republic of Germany); collagenase Type IV and collagenase No. C-2139 (Sigma); penicillin (GIBCO, Paisley, Scotland); streptomycin (GIBCO); McCoy cell culture medium (Flow Laboratories); dispase Grade II (Boehringer Mannheim, Federal Republic of Germany); MEM, with Earle's salts/without L-glutamine (GIBCO); [^{35}S]methionine (New England Nuclear, Boston, Mass.); SDS (Merck, Darmstadt, Federal Republic of Germany); sodium *meta*-arsenite (Merck); β-mercaptoethanol (Fluka, Buchs, Switzerland); Enlightning® (New England Nuclear), and Trimax XD-100 NIF X-ray film (3M Company). All other chemicals were of the purest grade available and obtained from Merck.

RESULTS

Induction of HSPs by Heat Treatment. Autoradiography of the electrophoretically separated proteins of normal mouse liver cells revealed, after exposure to 43°C for 20 min, a pronounced induction of 68 kD protein (HSP 68) and a less intense induction of a 87 kD protein (HSP 87) concomitant with a reduction of the synthesis of other proteins (Figure 1, A and B). The response of liver cells from GF-pretreated mice was similar, but weak induction of an additional protein corresponding to a molecular weight of 70 kD (HSP 70) was found (Figure 1, C and D).

Induction of HSPs by Sodium Arsenite Treatment. Sodium arsenite treatment of normal mouse liver cells in concentrations of 10, 15 and 20 μ*M* led to a considerable and dose-dependent induction of HSP 68. HSP 87 synthesis was slightly elevated, but not in a dose-dependent manner. Label incorporation into other proteins except proteins with molecular weights of 32 and 34 kD (P32/34; *vide infra*) was reduced (Table 1, Figure 2). Liver cells from GF-treated mice showed a dose-dependent (although in comparison to normal mouse liver cells somewhat less pronounced) induction of HSP 68 and HSP 87 (Table 1, Figure 3). The synthesis of HSP 70 was slightly elevated (Figure 3), that of actin reduced (Table 1, Figure 3). HSP 74 synthesis was increased only by treatment with 20 μ*M* sodium arsenite (Table 1, Figure 3). Upon exposure of normal and GF-pretreated hepatocytes to 10 μ*M* sodium arsenite, the synthesis of two additional proteins with molecular weights of 32 and 34 kD (P32/34) was increased, but reduced with higher sodium arsenite concentrations (Table 1, Figures 2 and 3).

Induction of HSPs by Ethanol Treatment. Exposure of normal mouse liver cells to 4, 6 and 8% ethanol resulted in a dose-dependent induction of HSP 68. HSP 87 synthesis was slightly elevated in cells treated with 4 and 6% ethanol, whereas 8% ethanol was ineffective. Synthesis of other proteins (e.g., P32/34; actin, HSP74) was reduced (Table 2, Figure 4) concomitantly. The isoelectric point values of HSPs induced by ethanol treatment as estimated by two-dimensional gel electro-

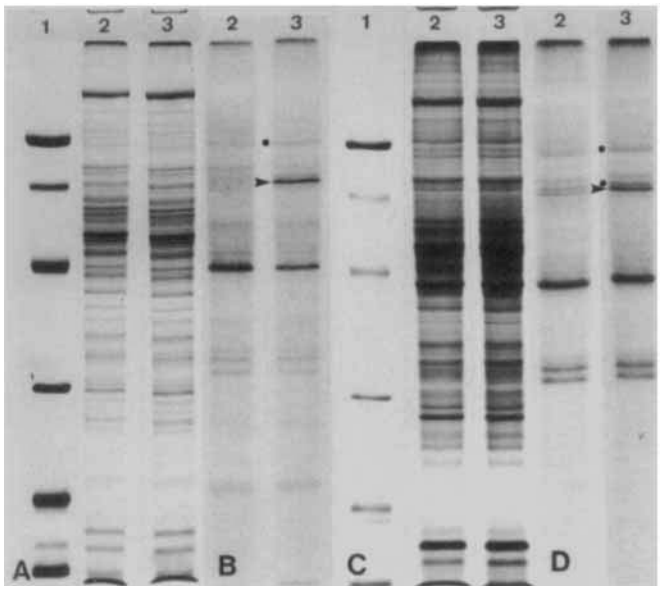

FIG. 1. Induction of HSPs in freshly isolated normal mouse liver cells [(A) and (B)] and cells derived from GF-pretreated mouse liver [(C) and (D)] by exposure to 43°C for 20 min (= heat shock) followed by incubation with [^{35}S]methionine (25 μCi per ml) for 5 hr at 37°C. (A) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards (from *top* to *bottom*: 92.5, 66.2, 45, 30 and 21.5 kD); *Lane 2* = normal mouse liver cells incubated at 37°C (control cells); *Lane 3* = normal mouse liver cells exposed to 43°C (heat-shocked cells). (B) Autoradiography of *Lanes 2* and *3* shown in (A). Note predominant label incorporation into a protein with molecular weight of 68 kD (HSP 68; *arrowhead* in *Lane 3*) and a less intense induction of the synthesis of an 87 kD protein (*dot* in *Lane 3*) in heat-treated cells. The synthesis of other proteins is reduced (compare *Lanes 2* and *3*). (C) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards [see (A), *Lane 1*]; *Lane 2* = GF-mouse liver cells incubated at 37°C (control cells); *Lane 3* = GF-mouse liver cells exposed to 43°C (heat-shocked cells). (D) Autoradiography of *Lanes 2* and *3* shown in (C). Note increased synthesis of HSP 68 (*arrowhead* in *Lane 3*) and weak induction of 70 and 87 kD proteins (*dots* in *Lane 3*).

TABLE 1. Effect of sodium arsenite (AS) treatment of normal [mouse liver cell (mlc)] and GF-pretreated mouse hepatocytes (GF-mlc) on HSP synthesis and the synthesis of other proteins (P32/34, actin)

Treatments	Protein synthesis[a]				
	P32/34	Actin	HSP 68	HSP 74	HSP 87
mlc AS 10 μM	285	65	632	79	126
mlc AS 15 μM	227	53	846	67	118
mlc AS 20 μM	114	41	941	67	134
GF-mlc AS 10 μM	142	83	266	91	120
GF-mlc AS 15 μM	115	77	400	91	135
GF-mlc AS 20 μM	109	70	461	132	152

[a] Protein synthesis was evaluated by analysis of autoradiograms shown in Figures 2 and 3 with a computer video densitometer reflecting [^{35}S]methionine incorporation. Values are per cent of total label incorporation into proteins of cells after stress treatments in relation to incorporation into unstressed control cells (incorporation into proteins of control cells = 100%).

phoresis (gel not shown) were between 5.55 and 5.95 pH (isoelectric variants) for HSP 68, and 4.9 for HSP 87.

Ethanol administration to GF-pretreated mouse liver cells did not lead to increased synthesis of HSPs (Table 2, Figure 5).

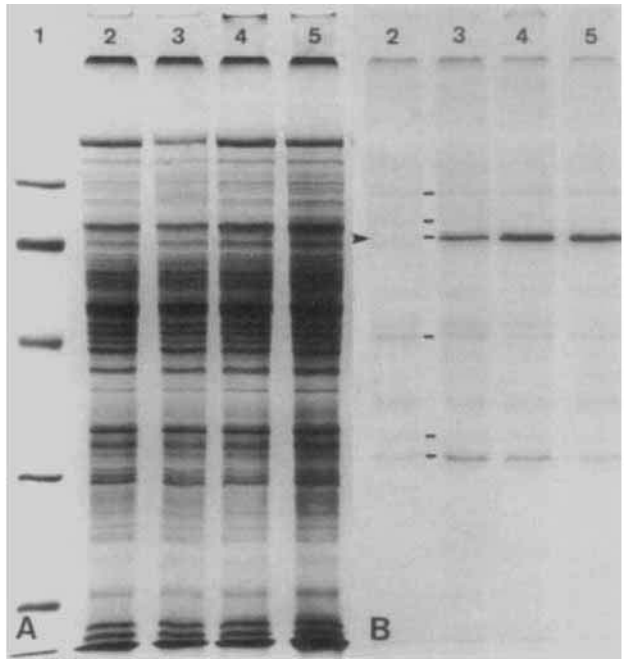

FIG. 2. Induction of HSPs in freshly isolated normal mouse liver cells after exposure to different concentrations of sodium arsenite for 4 hr at 37°C followed by incubation with [^{35}S]methionine (25 μCi per ml) for 45 min. (A) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards (see Fig. 1); *Lane 2* = control normal mouse liver cells without addition of sodium arsenite; *Lane 3* = normal mouse liver cells exposed to sodium arsenite in a concentration of 10 μM; *Lane 4* = 15 μM sodium arsenite; *Lane 5* = 20 μM sodium arsenite. (B) Autoradiography of SDS-PAGE (approximately equal amounts of radioactivity were loaded) shown in Fig. 2A, *Lanes 2* to *5*. *Bars* between *Lanes 2* and *3* denote (from *top* to *bottom*): HSP 87; HSP 74; HSP 68; actin; P34, and P32. A dose-dependent increase in label incorporation predominantly into a 68 kD protein is seen (*arrowhead; Lanes 2* to *5*).

DISCUSSION

Exposure of freshly isolated mouse hepatocytes *in vitro* to various stress situations, such as elevated temperature, sodium arsenite, and ethanol, led to a rapid induction of a set of proteins, termed "heat shock" or "stress" proteins. Thus, in their stress response, mouse hepatocytes *in vitro* closely resembled other cells of diverse origins, from bacteria to man [see Ref. (1) for further information and references]. The protein patterns induced, however, somewhat differed depending on the nature of the stressor as well as on the pretreatment regimen. Different patterns of HSPs after induction by heat, arsenite, ethanol and hypoxia, and dependence of HSP concentrations and kinetics of synthesis on the extent of external stress have also been found in Chinese hamster fibroblasts by Li and Werb (22). Hepatocytes derived from long-term GF-treated mice differed from normal hepatocytes in their stress response in that, after arsenite and heat treatments, the synthesis of proteins little or not stimulated in stressed normal hepatocytes (e.g., HSP 70) was elevated. Moreover GF-pretreated cells were refractory with respect to stimulation of stress protein synthesis by ethanol. These results not only suggest different modes and pathways of the stress response, depending on the nature of the inducing agent, but also its modulation in pathologically altered (chronically stressed?)

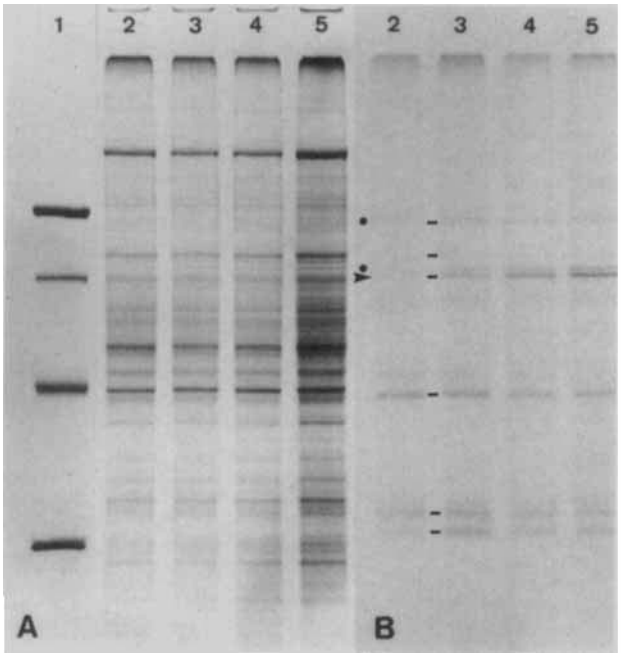

FIG. 3. Induction of HSPs in freshly isolated GF-mouse liver cells after exposure to different concentrations of sodium arsenite for 4 hr at 37°C followed by incubation with [^{35}S]methionine (25 μCi per ml) for 45 min. (A) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards (see Fig. 1); *Lane 2* = GF-mouse liver cells without addition of sodium arsenite; *Lane 3* = GF-mouse liver cells exposed to 10 μM sodium arsenite; *Lane 4* = 15 μM sodium arsenite; *Lane 5* = 20 μM sodium arsenite. (B) Autoradiography of SDS-PAGE shown in Fig. 3A, *Lanes 2* to *5*. *Bars* between *Lanes 2* and *3* denote (from *top* to *bottom*): HSP 87; HSP 74; HSP 68; actin; P34, and P32. Note conspicuous label incorporation into the 68 kD protein (*arrowhead*) and less pronounced incorporation into 70 and 87 kD proteins (*dots*). Label incorporation into proteins with 32 and 34 kD molecular weights is enhanced in cells treated with 10 μM sodium arsenite, but reduced in cells treated with higher arsenite concentrations.

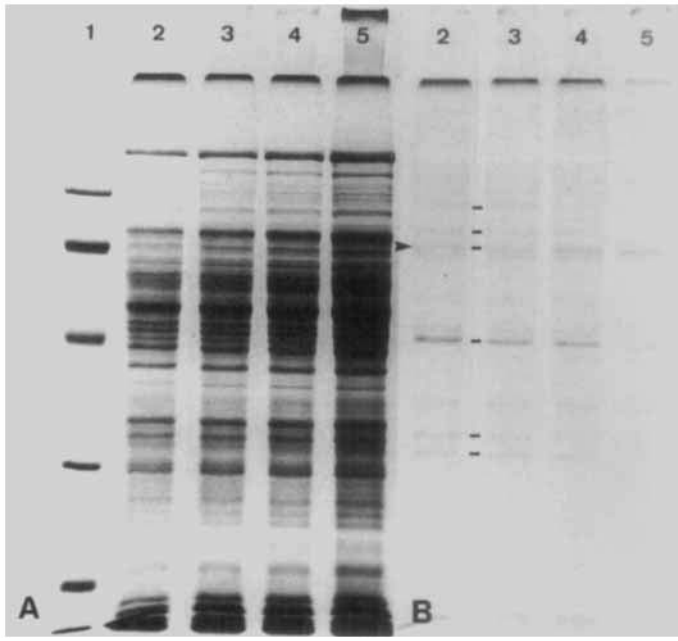

FIG. 4. Induction of HSPs in freshly isolated normal mouse liver cells after exposure to different concentrations of ethanol for 30 min at 37°C followed by incubation with [^{35}S]methionine (25 μCi per ml) for 4 hr at 37°C. (A) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards (see Fig. 1); *Lane 2* = untreated normal mouse liver cells (control cells); *Lane 3*, normal mouse liver cells exposed to 4% ethanol; *Lane 4* = 6% ethanol; *Lane 5* = 8% ethanol. (B) Autoradiography of SDS-PAGE (approximately equal amounts of radioactivity were loaded) shown in Fig. 4A, *Lanes 2* to *5*. *Bars* between *Lanes 2* and *3* denote (from *top* to *bottom*): HSP 87; HSP 74; HSP 68; actin; P34, and P32. Note dose-dependent induction of HSP 68 (*arrowhead*) synthesis. Reduction of label incorporation into other proteins is particularly conspicuous in cells treated with 8% ethanol (*Lane 5*).

TABLE 2. Effect of ethanol (ETH) treatment of normal [mouse liver cell (mlc)] and GF-pretreated mouse hepatocytes (GF-mlc) on HSP synthesis and the synthesis of other proteins (P32/34, actin)

Treatments	Protein synthesis[a]				
	P32/34	Actin	HSP 68	HSP 74	HSP 87
mlc ETH 4%	89	81	131	82	128
mlc ETH 6%	69	85	159	89	121
mlc ETH 8%	62	59	300	64	100
GF-mlc ETH 4%	76	91	109	100	86
GF-mlc ETH 6%	85	125	115	93	86
GF-mlc ETH 8%	85	107	94	109	70

[a] Analysis of autoradiograms shown in Figures 4 and 5. For details of determinations, see Table 1.

cells. With respect to the first point, several lines of evidence suggest functional differences of HSPs induced by different stress conditions. For example, although arsenite induced HSPs in some cell lines, neither thermotolerance nor protection against its own toxicity ensued in contrast to the effect of heat and ethanol treatments (12, 23).

The differences between the effects of heat (and arsenite) and ethanol on GF-pretreated hepatocytes are remarkable, since in most cell systems ethanol closely mimics the action of heat, and both induce tolerance to itself as well as cross-tolerance (as for example, shown with Morris hepatoma MH-7777 cells) (12). The reason for the difference between the effects of elevated temperature and arsenite on one hand and ethanol on the other on GF-pretreated hepatocytes is open to speculation: (i) the lack of ethanol effect on GF-pretreated hepatocytes could resemble the inability of embryonal carcinoma and mouse early embryonic cells to respond to heat treatment with HSP synthesis. This could be due to the fact that, in these cells, several HSPs are spontaneously expressed and, therefore, no further HSP synthesis is induced by heat treatment (9). It is possible, that chronic GF treatment has already induced certain types of HSPs, thus preventing further induction by ethanol. It has indeed been shown that HSPs regulate their own synthesis: accumulation of a certain quantity of HSPs is a signal to release the block of normal protein synthesis and repress HSP production (4, 7). (ii) Moreover, the different behavior of GF-pretreated mouse hepatocytes could be due to the altered metabolic situation resulting from long-term GF feeding or selection of cer-

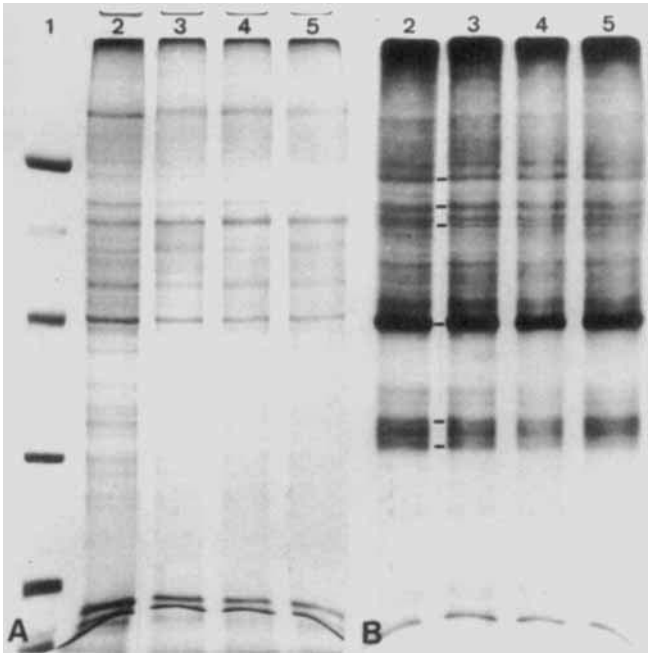

FIG. 5. Induction of HSPs in freshly isolated GF-mouse liver cells after exposure to different concentrations of ethanol for 30 min at 37°C followed by incubation with [^{35}S]methionine (25 μCi per ml) for 4 hr at 37°C. (A) SDS-PAGE (Coomassie blue staining): *Lane 1* = molecular mass standards (see Fig. 1); *Lane 2* = untreated GF-mouse liver cells (control cells); *Lane 3* = GF-mouse liver cells exposed to 4% ethanol; *Lane 4* = 6% ethanol; *Lane 5* = 8% ethanol. (B) Autoradiography of SDS-PAGE shown in Fig. 5A, *Lanes 2* to *5*. *Bars* between *Lanes 2* and *3* denote (from *top* to *bottom*): HSP 87; HSP 74; HSP 68; actin; P34, and P32. Note that ethanol does not induce HSP synthesis in hepatocytes derived from GF-pretreated mice (see also Table 2).

tain cell populations in chronically GF-intoxicated mouse livers.

It also remains to be investigated whether the lack of HSP response to ethanol in chronically GF-poisoned hepatocytes is accompanied by increased or decreased sensitivity of the cells to ethanol and other noxious insults. It is noteworthy in this context that HSPs synthesized in the presence of amino acid analogs (e.g., canavanine or azetidine) in hamster HA-1 cells were nonfunctional with respect to thermotolerance, and the cells became extremely sensitive to heat (24).

Prolonged GF administration to mice is accompanied by neoplastic transformation of hepatocytes and severe derangement of the hepatocellular intermediate filament system of the cytokeratin type, with Mallory body formation resembling chronic alcoholic liver cell damage [for review and further information, see Ref. (25)]. Moreover, heat shock-associated changes in cell shape are correlated with alterations of filamentous cytoskeletal components (microfilaments, microtubules, intermediate filaments), although different cell types respond differently (26). Whether any of these disturbances alone or in combination play a role in the altered stress response to ethanol is an open question, particularly since the mechanisms leading to the stress response of cells and its further metabolic consequences are as yet unclear. Alterations observed in heat-shocked cells (e.g., rat fibroblasts), such as disruption and fragmentation of Golgi complex, mitochondrial changes concomitant with disturbances of energy metabolism, collapse and aggregation of intermediate filaments, nuclear and nucleolar alterations, may be causes but also consequences of heat-induced metabolic derangements (13, 26). Moreover, cell membranes, DNA and several cellular proteins may be targets of hyperthermic insults, as well as of ethanol and other aliphatic alcohols, Ca^{2+} ionophores, viruses and local anesthetics (23).

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