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Changes in Expression of mRNA Coding for Glutathione S-Transferase Subunits 1-2 and 7 in Cultured Rat Hepatocytes

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SUMMARY

mRNA hybridizing to probes for glutathione S-transferase (GST) subunits 1 and 2 (probe pGSTr 155) and subunit 7 (probe pGSTr 7) has been measured by Northern blot analysis in adult rat hepatocytes both in conventional monoculture and in co-culture with epithelial cells. In addition, several media conditions were used, namely with and without fetal calf serum (FCS) and with and without nicotinamide or dimethyl sulfoxide (DMSO). In monoculture, mRNA coding for subunits 1 and 2 was extensively reduced in the presence of FCS. In the absence of FCS, after an initial decrease, an increase of subunits 1 and 2 mRNA was noticed on day 6. When nicotinamide or DMSO was added to the medium, the GST subunits 1 and 2 mRNA level increased during the culture period. In co-culture, an initial reduction in levels of mRNA encoding subunits 1 and 2 was less marked and the values measured increased with co-culture time. Nicotinamide tended to reduce these mRNA levels, whereas DMSO increased them. In contrast, in conventional culture, mRNA encoding subunit 7 was expressed *de novo* and this induction was prevented by DMSO but not by nicotinamide. Similar results were obtained with co-culture.

GSTs (EC 2.5.1.18.) are a family of dimeric multifunctional proteins that catalyze the conjugation of electrophilic metabolites of xenobiotics, the reduction of lipid and DNA hydroperoxides, and the key steps in the biosynthesis of prostaglandins and leukotriene C_4. They also bind a number of lipophilic nonsubstrate ligands (1–3). The subunits of the rat enzymes have been named numerically, in chronologic order of their characterization (4). Eleven subunits have been designated and, on the basis of primary structure, they belong to at least three gene families, namely the α family (subunits 1, 2, 8, and 10), the μ family (subunits 3, 4, 6, 9, and 11), and the π family (subunit 7) (2, 3). There are several reports of heterogeneity within subunit 1 (5–7).

Each rat tissue, of which the liver has been most frequently studied, has its own characteristic isoenzyme composition. In addition, the isoenzyme profile of the liver has been shown to change with development and also to be modulated by endogenous hormones and compounds such as enzyme inducers and carcinogens (2, 3). The major subunits represented in the adult liver are 1, 2, 3, and 4. Subunit 7 is normally absent in hepatocytes *in vivo* but appears at an early stage in chemically induced carcinogenesis. It is associated with preneoplastic foci and hyperplastic nodules and is a major component of the eventual hepatoma (8–10). It is also expressed in rat hepatocytes after 48 hr *in vitro* (11).

Cultured rat hepatocytes have previously been used to study the expression of GST isoenzymes *in vitro* and it has been demonstrated that GST protein levels and enzyme activity *in vitro* vary with the culture conditions used (12). In some cases, especially in co-culture of hepatocytes with hepatic epithelial cells, GST activity was maintained over 16 days at a level comparable to or even exceeding *in vivo* values. It has also been shown by HPLC analysis, using the method of Ostlund-Farrants *et al.* (13), that changes in enzyme activity are associated with changes in the isoenzyme profile, including a loss of subunits 1 and 2 and the *de novo* expression of subunit 7 (14, 15).

In the present work, the expression of GST mRNA levels coding for subunits 1 and 2 and subunit 7 in long term primary cultures of rat hepatocytes has been studied. The subunit 1/2 cDNA probe hybridizes strongly to a heterogeneous mRNA population (980 ± 75 nucleotides) that is mRNA coding for GST subunit 1 and less strongly to another mRNA species (1150 ± 100 nucleotides) that is subunit 2 mRNA (16, 17). The subunit 7 DNA insert hybridizes to mRNA of approximately

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ABBREVIATIONS: GST, glutathione-S-transferase; FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; HPLC, high pressure liquid chromatography; DMSO, dimethyl sulfoxide.

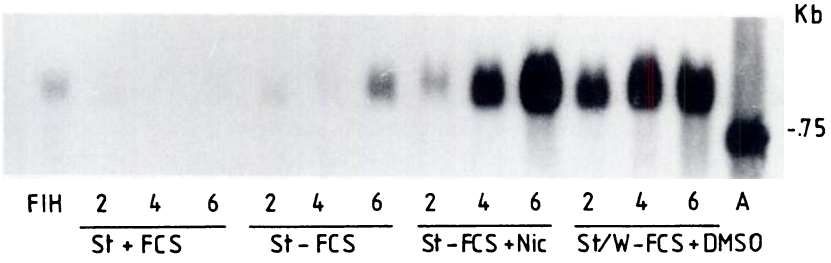

Fig. 1. Northern blot analysis of mRNA encoding GST subunits 1 and 2 in monocultured rat hepatocytes. *Numbers* refer to the days of culture. RNA was isolated from freshly isolated hepatocytes (*FIH*) and monocultured hepatocytes and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr 155, and autoradiography; 20 μg of RNA were loaded in each lane. *A*, pBR DNA size markers. *St*, standard medium; *Nic*, nicotinamide; *W*, Williams' E medium.

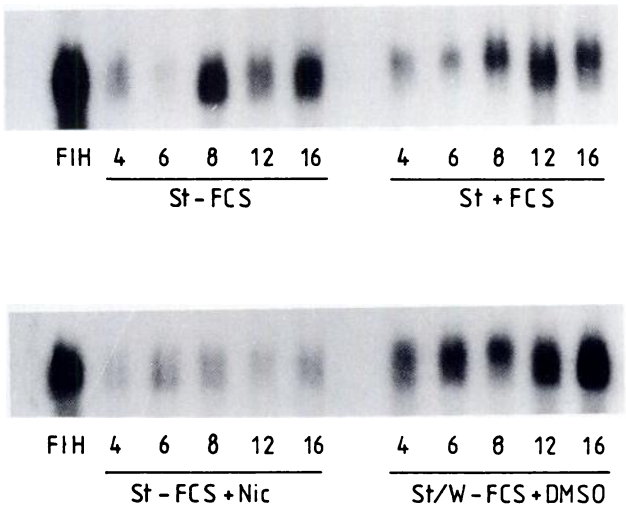

Fig. 2. Northern blot analysis of mRNA encoding GST subunits 1 and 2 in co-cultured rat hepatocytes. *Numbers* refer to the days of culture. RNA was isolated from freshly isolated hepatocytes (*FIH*) and co-cultured hepatocytes and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr 155, and autoradiography; 20 μg of RNA were loaded in each lane. *St*, standard medium; *Nic*, nicotinamide; *W*, Williams' E medium.

940 nucleotides (8). In order to establish whether previously observed changes in enzyme activity and enzyme protein *in vitro* were correlated with variations in the corresponding mRNA levels of GST subunits, the mRNA levels of conventionally and co-cultured rat hepatocytes have been measured, using different culture media conditions, i.e., media with or without FCS and media with added nicotinamide or DMSO.

Experimental Procedures

Materials. Culture media and FCS were obtained from GIBCO (Scotland). Collagenase, bovine albumin, and bovine insulin were products from Sigma Chemical Co. Hydrocortisone hemisuccinate was a product of Roussel (Paris). The nick translation kit, nitrocellulose Hybond C, and [α^{32}P]dCTP were obtained from Amersham International (UK). All other compounds were readily available commercial products and were used without further purification.

Isolation of hepatocytes and culture procedures. Hepatocytes from adult male Sprague-Dawley rats that weighed 180–200 g were isolated by the two-step collagenase perfusion method, as previously described (18). The liver was first washed for 10 min with HEPES buffer, pH 7.6, and then for 20 min with a 0.025% collagenase solution buffered with HEPES. Cells were collected in Leibovitz-15 medium and seeded at a density of 10×10^6 cells/175-cm^2 flasks in 25 ml of medium containing 10% FCS. The standard medium consisted of 75% minimal essential medium and 25% medium 199, containing 200 μg/ml bovine albumin, 10 μg/ml bovine insulin, and 10% FCS. The medium was first changed 4 hr after cell seeding and every day thereafter. Co-cultures were set up by adding 1.4×10^7 rat liver epithelial cells/flask in order to reach confluency within 24 hr.

Four hours after seeding, 7×10^{-5} M hydrocortisone hemisuccinate was added to conventional cultures and 3.5×10^{-6} M to co-cultures. These concentrations were maintained during the culture period. Four different media conditions were tested, namely: standard medium plus FCS; standard medium minus FCS; standard medium minus FCS containing 25 mM nicotinamide; and 50% standard medium and 50% Williams' E medium minus FCS with 2% DMSO.

Cell harvesting. Cultures were washed with HEPES buffer (pH 7.6; 4°). Cells from 175-cm^2 flasks (six flasks or approximately 60×10^6 hepatocytes/assay) were removed by scraping, immediately frozen in liquid nitrogen, and stored at −80°.

Isolation of RNA. Total RNA from hepatocytes was prepared by a guanidine-thiocyanate-CsCl procedure. Cell pellets were lysed by suspension in 5 M guanidium thiocyanate 2% lauryl sarcosine, and 0.6 M β-mercaptoethanol. Homogenates were centrifuged at 12,000 × g for 15 min. Supernatants were then centrifuged on a 5.7 M cesium chloride cushion for 22 hr at 85,000 × g. RNA pellets were dissolved in 1 M Tris·HCl, pH 7.4, 1 mM EDTA, pH 7.4, and 1% lauryl sarcosine and precipitated by ethanol/sodium acetate. The RNAs were washed, dried, dissolved in sterile water, and stored at −80°.

Northern blots. RNA (20 μg) from cultured hepatocytes was subjected to electrophoresis in a denaturing formaldehyde/1.5% agarose gel and transferred onto nitrocellulose filters. These filters were prehybridized and hybridized with nick-translated probes (400–600 10^6 cpm/μg of DNA). pGSTr 155 (16) is complementary to mRNA coding for GST subunit 1 and may cross-hybridize with subunit 2; pGSTr7 (8) is complementary to GST 7 mRNA. After hybridization, filters were washed, dried, and autoradiographed at −80°. In control experiments, filters were stripped of the previous probe and rehybridized with a nick translated albumin or actin probe (19). In addition to the study, RNA of freshly isolated and cultured rat hepatocytes has been hybridized with the pKM$_2$ cDNA probe (20).

Results

Assay of mRNA Encoding GST Subunits 1 and 2 in Cultured Rat Hepatocytes

Conventional culture. Subunits 1 and 2 together represent about 50% of the total GST protein and about 22% of the total

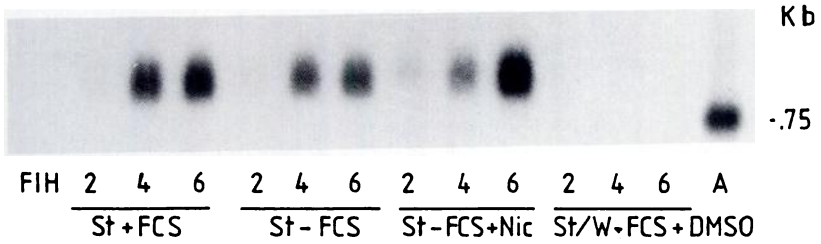

Fig. 3. Northern blot analysis of mRNA encoding GST subunit 7 in monocultured rat hepatocytes. *Numbers* refer to the days of culture. RNA was isolated from freshly isolated hepatocytes (*FIH*) and monocultured hepatocytes and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr 155, and autoradiography; 20 μg of RNA were loaded in each lane. *A*, pBR DNA size markers.

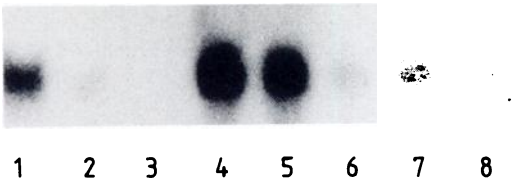

Fig. 4. Analysis of mRNA encoding GST subunit 7 in fetal rat hepatocytes and newborn rat hepatocytes compared with adult rat hepatocytes and cultures of adult rat hepatocytes. *Lane 1*, Northern blot analysis of RNA isolated from fetal rat hepatocytes; *lane 2*, 5-day old newborn rat hepatocytes; *lane 3*, hepatocytes from 2-month-old rats; *lane 4*, conventional cultured rat hepatocytes, 4 days in standard medium plus FCS; *lane 5*, co-cultured rat hepatocytes, 4 days in standard medium plus FCS; *lane 6*, conventional cultured rat hepatocytes, 4 days in 50% standard medium/50% Williams' E medium minus FCS plus DMSO; *lane 7*, co-cultured rat hepatocytes, 4 days in 50% standard medium/50% Williams' E medium minus FCS plus DMSO; *lane 8*, co-cultured rat hepatocytes, 4 days in 50% standard medium/50% Williams' E medium minus FCS plus DMSO but separated from the epithelial cells. Ten micrograms of RNA were loaded in each lane and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr7, and autoradiography.

activity toward CDNB present in freshly isolated, differentiated, adult rat hepatocytes (2, 3, 14). Their mRNAs are well represented in Northern blot analysis (Fig. 1, *lane 1*). In subsequent culture, when hepatocytes are maintained in a medium containing FCS, there is a decrease in subunit 1/2 mRNA levels which persists over 6 days (Fig. 1, *lane 1* compared with *lanes 2–4*), whereas in the absence of FCS, subunit 1/2 mRNA levels start to increase again on day 6 (Fig. 1, *lanes 5–7*). These changes in mRNA levels are consistent with changes in total GST CDNB activity (12).

In contrast, when either nicotinamide or DMSO is included in the culture medium, there is no initial decrease in subunit 1/2 mRNA levels compared with freshly isolated hepatocytes, and an 8- to 10-fold elevation of these mRNAs is apparent after 6 days in culture (Fig. 1, *lanes 8–13*). Although these changes in mRNA levels could result in our observed 3-fold increase in total GST CDNB activity (12), previous HPLC analysis of GST subunits from nicotinamide-treated hepatocytes has shown that levels of subunits 1 and 2 are maintained and do not exceed those found in freshly isolated hepatocytes (14). These results show that mRNA and protein levels of subunit 1/2 do not run in parallel. Further investigation is needed to understand this discrepancy.

Albumin and actin cDNA probes were used to assess the fidelity of differentiated gene expression in hepatocytes in culture (results not shown). In hepatocytes maintained in media with or without FCS, albumin mRNA levels, like those of subunits 1 and 2, also decreased during 6 days in culture but, in contrast, remained stable if these cells were cultured in the presence of nicotinamide or DMSO. Actin mRNA levels, which in freshly isolated hepatocytes are very low, increased upon culture in all medium conditions.

Co-culture. The hepatic epithelial cells used to set up the co-cultures do not contain mRNA for subunits 1 and 2. In co-culture (Fig. 2), the different medium conditions have less influence on the subunit 1/2 mRNA levels than in conventional culture. This is consistent with earlier observations that the variations in GST enzymic activity produced by the different culture media were less expressed in co-culture (12).

Better maintenance of the characteristics of differentiated hepatocytes during co-culture, using different medium conditions, is also evident by the expression of mRNA encoding albumin (results not shown).

On this basis, therefore, hepatocytes cultured in the presence of epithelial cells appear to be less affected by factors in the medium than are hepatocytes in monoculture. In addition, the presence of epithelial cells is of more importance for the long term stability of the hepatocytes in culture.

Assay of mRNA for GST Subunit 7 in Cultured Rat Hepatocytes

Conventional culture. mRNA coding for subunit 7 is not detectable in RNA extracted from normal adult liver, although immunohistochemical studies show that it is present in bile duct cells (21). In an earlier study, when GST subunits were separated by HPLC (14), the expression of subunit 7 was observed after culture for 4 days. In the present work it is evident that, immediately after isolation by collagenase perfusion, rat hepatocytes do not express detectable levels of subunit 7 mRNA (Fig. 3, *lane 1*). However, after 2 days of culture, in either the presence or absence of FCS, a small amount of mRNA for subunit 7 is detected, which increases during the 6 day culture period (Fig. 3, *lanes 2–7*). If nicotinamide is added

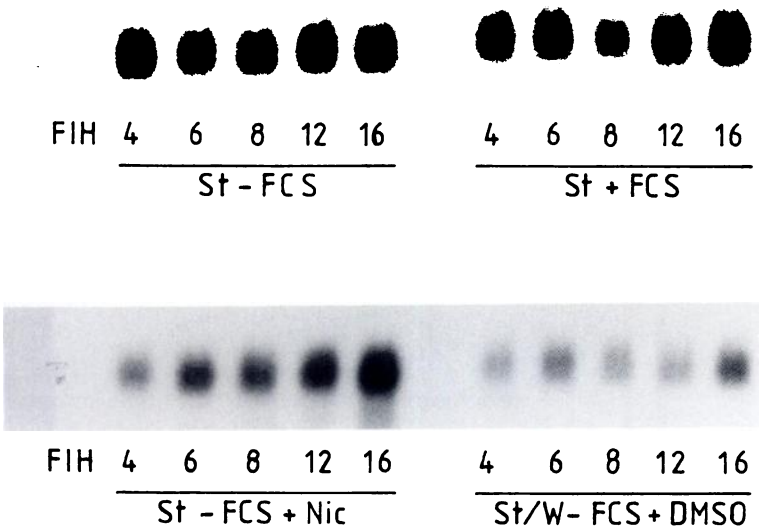

Fig. 5. Northern blot analysis of mRNA encoding GST subunit 7 in co-cultured rat hepatocytes. *Numbers* refer to the days of culture. RNA was isolated from freshly isolated hepatocytes (*FIH*) and co-cultured hepatocytes and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr 155, and autoradiography; 20 μg of RNA were loaded in each lane. *St*, standard medium; *Nic*, nicotinamide; *W*, Williams' E medium.

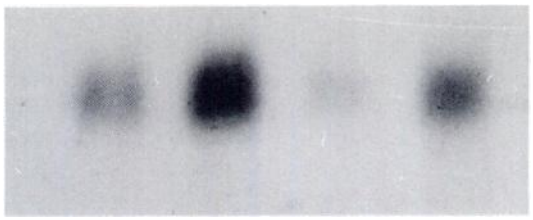

Fig. 6. Analysis of mRNA encoding GST subunit 7 of DMSO-treated co-cultured rat hepatocytes separated from the rat liver epithelial cells. Northern blot analysis of RNA isolated from rat hepatocytes co-cultured in 50% standard medium/50% Williams' E medium minus FCS plus DMSO medium for 4 days and separated from the epithelial cells (*lane 3*) and co-cultured for 16 days in the same culture medium conditions and separated from the epithelial cells (*lane 4*). *Lanes 1* and *2*, respectively, 4 and 16 days of co-culture in 50% standard medium/50% Williams' E medium minus FCS plus DMSO, but hepatocytes not separated from the epithelial cells. Ten micrograms of RNA were loaded in each lane and analyzed after transfer from a 1% agarose gel to a nitrocellulose membrane, hybridization with ^{32}P-labeled pGSTr 7, and autoradiography.

to the culture medium, the content of subunit 7 mRNA is even greater (Fig. 3, *lanes 9* and *10*), whereas the addition of DMSO results in very low levels of subunit 7 mRNA (Fig. 3, *lanes 11–13*).

This dedifferentiation of hepatocytes with respect to expression of subunit 7 has a direct parallel in the expression of the fetal form of pyruvate kinase (pkM_2). A low level of subunit 7 mRNA, evident in freshly isolated fetal rat hepatocytes (Fig. 4, *lane 1*) and almost lost within 5 days post partum (Fig. 4, *lane 2*), is completely lost in hepatocytes isolated from adult rats (Fig. 4, *lane 3*) but *de novo* expressed in monocultured and co-cultured adult rat hepatocytes whatever the medium conditions (Fig. 4, *lanes 4–8*). Similar observations are made with respect to mRNA encoding fetal pyruvate kinase pKM_2, the level of which is also reduced in cultured hepatocytes by the incorporation of DMSO in culture media (results not shown).

Co-culture. Rat hepatocytes and rat liver epithelial cells co-cultured in a medium with or without FCS express subunit 7 mRNA (Fig. 5, *lanes 2–11*). The level reached after 4 days of co-culture is maintained for a further 12 days. Co-cultures treated with nicotinamide and DMSO also express subunit 7 mRNA (Fig. 5, *lanes 12–21*). Because hepatocytes and epithelial cells were not separated at the end of each culture period and because the rat liver epithelial cells used in co-culture contain subunit 7 mRNA, the relative contributions of the two cell types to the hybridization signal in RNA isolated from co-cultures treated with DMSO (Fig. 6) have been estimated. Hepatocytes treated in co-culture with DMSO and separated from the epithelial cells by collagenase treatment (Fig. 6, *lanes 3 and 4*) yield RNA that contains less subunit 7 mRNA than RNA purified from the mixed co-cultures (Fig. 6, *lanes 1 and 2*). The epithelial cells, therefore, contribute the greater proportion of subunit 7 mRNA present in RNA isolated from co-cultures. Hence, on the basis of expression of subunit 7, co-cultured hepatocytes maintain better differentiation than do monocultures.

Discussion

The maintenance of differentiated hepatocytes in culture is desirable to facilitate many studies such as investigations of the mechanisms of detoxification of drugs, carcinogens, and other toxins. To this end, the expression of mRNAs encoding subunits of GST in hepatocytes maintained under several conditions of culture has been characterized and the expression of albumin and actin mRNAs has been used as control for differentiated gene expression.

In conventional monoculture, there is a loss of mRNAs encoding albumin and the α class GSTs of adult rat hepatocytes and these losses can be reversed by addition of nicotinamide or DMSO to the medium. It has been described previously that the addition of 1–2% DMSO to the culture medium induces differentiation in rodent and human tumor cells (22, 23) and maintains differentiation in cultured rat hepatocytes (24). In the latter case, it is confirmed in this study that 2% (v/v) DMSO induces the restoration of albumin synthesis in cultured rat hepatocytes but it is shown that, in the case of the GST α family, normal gene expression is not fully restored, because there is an elevated level of mRNAs relative to the GST subunits. In addition, actin mRNA is also expressed. By all the above criteria, however, except when nicotinamide is added to the culture medium, hepatocytes in co-culture with hepatic epithelial cells exhibit patterns of gene expression more like freshly isolated hepatocytes and are less affected by factors in the media. It is of interest to consider the regulation of the GST α family in the light of these results. The co-regulated expression of an entire gene family by *in vitro* culture conditions is more striking when consideration is taken of the fact that the members of the α family show differential inducibility and tissue-specific regulation *in vivo*. For example, hepatic subunit 1 but not subunit 2 is induced by a number of xenobiotics such as phenobarbital or chemical carcinogens, and subunit 1 mRNA is found only in adult liver and kidney, whereas subunit 2 mRNA is not only present in both fetal and adult liver but is also found in many other tissues (17). It is possible, therefore, that DMSO and co-culture with epithelial cells activate this gene cluster by a common pathway, possibly by a regulator similar to that observed for the globins (25).

The expression of subunit 7 has also been examined, because immunochemical studies show that, in normal liver, bile duct cells consistently stain for subunit 7, whereas staining in normal hepatocytes is very rare, and yet Power *et al.* (11) have reported *de novo* expression of subunit 7 mRNA in 48 hr hepatocyte cultures. In monoculture, it is also observed that subunit 7 mRNA appears after 2 days, the level of which increases over culture time.

Interestingly, the addition of nicotinamide to the medium increases, whereas DMSO decreases, the level of subunit 7 mRNA, a result in contrast to that of the α family expression in that it differentiates between the effect of nicotinamide and DMSO in monoculture. In co-culture, we have observed that the level of *de novo* subunit 7 mRNA in the hepatocyte is less than that in monoculture and that the relative effect of DMSO is also reduced, presumably because the hepatocytes are already more differentiated due to the co-culture conditions.

Because both DMSO (24) and co-culture (26) have been shown to favor maintenance of various specific functions and to inhibit expression of fetal proteins *in vitro*, it may be suggested that the presence of subunit 7 in culture reflects a dedifferentiation process. This conclusion is sustained by the demonstration that subunit 7 mRNA, as well as pKM_2 and α-

fetoprotein mRNA, are synthesized *in vivo* in fetal hepatocytes (27).

The regulation of subunit 7 mRNA is of particular interest for several reasons. Firstly, carcinogens and noncarcinogenic chemical treatment can induce the expression of subunit 7 in normal hepatocytes (28, 29). Secondly, transfection of rat liver cell lines with *ras* oncogenes results in an expression of subunit 7 (30). Because normal adult rat hepatocytes also become able to express *ras* oncogenes when seeded in culture (31), it would be of interest to determine whether any correlation exists between the expression of *ras* oncogenes and GST subunit 7.

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