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INFLUENCE OF ETHACRYNIC ACID ON GLUTATHIONE *S*-TRANSFERASE π TRANSCRIPT AND PROTEIN HALF-LIVES IN HUMAN COLON CANCER CELLS

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Abstract—Ethacrynic acid (EA) is a plant phenolic acid that is both an inhibitor and an inducer of glutathione *S*-transferase (GST) activity. To determine contributory factors in the increased GST activity caused by EA treatment, human colon carcinoma HT29 cells were compared with a cloned EA-resistant population (HT6-8) maintained in medium containing 72 μM EA. Several factors are involved in the increased expression of GSTπ in HT6-8. For example, nuclear run-on experiments showed an approximately 2-fold increase in the rate of transcription of GSTπ. In addition, the half-life of GSTπ transcript was increased from 4.1 (wild type, HT29, HT4-1) to 8.4 hr. The half-life of GSTπ protein was 1–2 hr in HT4-1 cells versus 8–9 hr in HT6-8 cells. When either human ovarian carcinoma cells (SKOV3) or human prostatic carcinoma cells (DU145) were treated with EA, the half-life of the GSTπ transcript was also increased. The transcript half-lives of another thiol-metabolism enzyme, γ-glutamylcysteine synthetase (γ-GCS), and a phase II detoxification enzyme, dihydrodiol dehydrogenase (DDH), were also increased in HT6-8, SKOV3 and DU145 cells treated with EA. However, the half-lives of transcripts from "housekeeping genes," such as glyceraldehyde 3-phosphate dehydrogenase (G3PDH), β-actin and β-tubulin, were not changed in these cell lines following EA. Apparently, a number of coordinated factors are involved in EA-enhanced expression of GSTπ and other detoxification enzymes.

Key words: glutathione transferase; half-life; ethacrynic acid; drug-resistance; human colon cancer cell; detoxification

That GST‡ (EC 2.5.1.18) isozymes catalyze the metabolism of a number of structurally different substrates is of particular significance to detoxification of anticancer drugs [1, 2]. Chlorambucil [3] and melphalan [4] have been identified as GST substrates. An increase in GST activity was reported in a rat mammary carcinoma cell line resistant to chlorambucil, which exhibited collateral resistance to other nitrogen mustards [5]. A number of studies have described overexpression of GST in drug-resistant tumor cells. Overexpression of GSTπ has been reported in a doxorubicin-resistant human breast cancer cell line, MCF-7 [6], in cisplatin-resistant cell lines [7], and in several tumors such as colon [8–10], stomach [11], breast [12], kidney [13] and melanoma [14]. In some cases, there is a simultaneous increase or decrease of other forms of GST isozymes. Several regulatory DNA elements have been described for a number of GST isozymes, including GSTπ, and these respond to a wide variety of chemicals [2].

The diuretic drug EA, an α,β-unsaturated ketone, reacts with GSH spontaneously or via GST catalysis in a Michael addition reaction to produce an EA–GS conjugate [15]. It reversibly inhibits GST isozymes by covalent binding [16] and has been used as a modulator of GST in cell lines [17]. It has been suggested that EA inhibits enzymes involved in the metabolic disposition of the cytostatic agent thiotepa in a phase I clinical trial [18]. Moreover, recent clinical observations showed that EA partially reverses chlorambucil resistance in a chronic lymphocytic leukemia (CLL) patient [19]. In previous work, we reported an increased level of GSTπ enzyme activity, protein content and transcript level in an EA-resistant cell line [20]. It has also become evident that EA has the potential to induce a broader cellular stress response. Because of our previous analyses, DDH, an aldoketoreductase that provides a further example of an EA-inducible phase II detoxification enzyme, was also included in the present study. We now present data that support the concept that multiple mechanisms may contribute to the enhanced expression of GSTπ following drug exposure.

MATERIALS AND METHODS

Materials

Cell culture media and reagents were obtained from Gibco-BRL (Grand Island, NY). [^{35}S]Methionine–cysteine mixture (*trans*-label), [α-^{32}P]dCTP and [α-^{32}P] UTP were purchased from ICN Radio Chemicals (Costa Mesa, CA). [5,6-^{3}H]Uridine was purchased from New England Nuclear (Boston, MA). All other materials were purchased from Sigma (St. Louis, MO).

Cell culture

The EA-sensitive human colon carcinoma cell line (HT4-1) and the EA-resistant cell line (HT6-8) were

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‡ Abbreviations: GST, glutathione *S*-transferase; EA, ethacrynic acid; GSH, glutathione; 4-TU, 4-thiouridine; DDH, dihydrodiol dehydrogenase; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; and γ-GCS, γ-glutamylcysteine synthetase.

grown as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 2 mM L-glutamine, 50 U of penicillin/mL, 50 μg of streptomycin/mL and 10% heat-inactivated fetal bovine serum (FBS). The EA-resistant cell line was generated without ethyl methanesulfonate pretreatment, as described before [20], and was maintained with continuous exposure to 72 μM EA. The human ovarian carcinoma cell line (SKOV3) was grown in α-MEM supplemented with 15% FBS. The human prostatic carcinoma cell line (DU145) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, and 10% FBS.

Determination of protein half-life

Pulse-chase. HT6-8 cells were maintained in the presence of EA during the course of experiments. HT4-1 and HT6-8 cells were grown to ~60% confluence. Cells were washed in PBS and left in methionine-free medium for 2 hr. Fifty μC/mL of [^{35}S]methionine–cysteine mixture was added to cells and incubated for 1 hr. Cells were washed in PBS and placed in fresh complete medium. Cell pellets were isolated at different times up to 12 hr and stored at −70° until further analysis.

Chemical block. Cells were grown as above and treated with 150 μg/mL cycloheximide for 1 hr to block protein synthesis. The end of 1 hr was taken as zero time, and cells were isolated at different times and stored at −70° until further analysis.

Preparation and analysis of samples. Thawed cells were resuspended in 10 mM Tris–HCl, pH 7.8, containing protease inhibitors and sonicated on ice for 30 sec. Suspensions were centrifuged at 10,000 *g* for 20 min, and the supernatants were spun at 100,000 *g* for 1 hr to isolate cytosolic fractions. Protein concentrations were estimated by the Bradford method [21]. One-hundred micrograms of protein from each sample was separated on a 12% polyacrylamide gel and blotted onto nitrocellulose membrane [22, 23]. GSTπ protein was identified by western blot analysis. For quantitation, in the pulse-chase method, bands from membranes representing different time points were cut out, and the amount of radioactivity incorporated was counted in a scintillation counter. In the case of the chemical block method, samples were analyzed by two-dimensional gel electrophoresis [24] using the pI range of 4 to 6.5. Second dimension and western blotting were performed as described above. Western blots were stained with GSTπ antibodies followed by ^{125}I conjugated secondary antibodies and exposed to film. To quantitate the results, autoradiograms were scanned by use of an Ultroscan XL laser densitometer (Pharmacia LKB Biotech., Uppsala, Sweden).

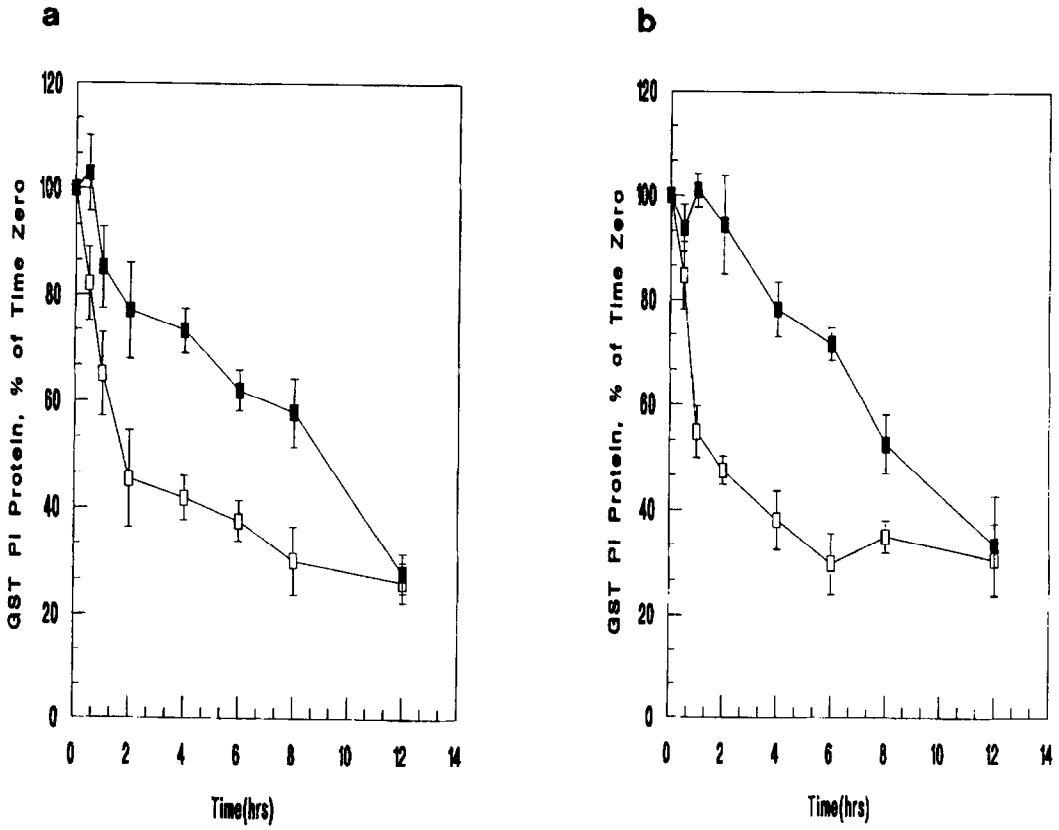

Fig. 1. Determination of GSTπ protein half-life by pulse-chase (a) and translation block methods (b). Cells were treated as described in Materials and Methods and harvested at various time points. Cytosolic lysates were prepared, separated on acrylamide gels, blotted and immunostained with GSTπ antibodies (1:10,000). Data were analyzed as described in Materials and Methods. Key: (□) HT4-1 cell line; and (■) HT6-8 cell line. Values are means ± SD (N = 4).

Determination of transcript half-life

4-TU labeling and separation of newly synthesized RNA. Cells that were ~60% confluent were placed in uridine-free prewarmed medium containing 100 μM 4-TU and 5 μCi/mL [^{3}H]uridine according to the method of Woodford *et al.* [25]. After a 1-hr incubation in the presence or absence of 72 μM EA, the medium was aspirated and cells were washed with cold PBS. Total RNA was extracted using guanidinium isothiocyanate, and newly synthesized RNA was separated by phenylmercury affinity chromatography. This method is primarily suitable for those transcripts whose half-life is longer than the labeling period. This is the case for the transcripts covered by this study.

Northern blot analysis. This was performed following the protocol of Johnson *et al.* [26]. After determination of radioactivity, purified newly synthesized RNA and total RNA from which newly synthesized RNA was purified were fractionated on a 1% agarose gel and transferred to the Magna-NT membrane; ethidium bromide stained 28S and 18S RNA bands served as control for equal gel loading. The immobilized membrane was hybridized to DNA probes that were ^{32}P-labeled by using a random-primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). After washing, the membranes were exposed to X-ray film. Quantification of the autoradiograms was carried out by densitometric scanning of the appropriate signals. The half-life of transcripts was calculated as described previously [26].

Nuclear run-on assay

Nuclear isolation. HT4-1 and HT6-8 cells were washed in cold PBS and homogenized. Nuclei were isolated via sequential centrifugation, initially through 30% sucrose at 1,000 *g;* this pellet was then centrifuged through a 2 M sucrose buffer at 101,800 *g* for 1 hr. The resulting nuclei (pellet) were used immediately in a nuclear run-on assay [27].

Nuclear run-on assay. Nuclei (10^8) and 200 μCi of [α-^{32}P]UTP were incubated in a reaction mixture at room temperature for 10 min. Following incubations with DNase I and proteinase K, the above ^{32}P-transcripts were isolated in the aqueous phase of a phenol:chloroform extraction. The radiolabeled transcripts were precipitated onto HAWG 0.45 μm filters using 10% trichloroacetic acid. Following quantitation of radioactivity incorporation, the transcripts were eluted from the filters in a 10% SDS buffer, treated with proteinase K, removed into the aqueous layer of a phenol:chloroform extraction, and precipitated in 75% EtOH overnight. Band intensity was accurately quantitated using an LKB Ultrascan XL enhanced laser densitometer. Actin was used as a control probe, and the rate of GSTπ transcription was expressed as a function of actin.

Hybridization. GSTπ cDNA was immobilized to Zeta-probe nylon membranes. These membranes were hybridized to the ^{32}P-labeled transcripts for 24–48 hr according to manufacturer's instructions. Following three washes at room temperature, the membranes were air dried and quantitated via scintillation counting.

RESULTS

The degradation of GSTπ protein in EA-sensitive and -resistant cell lines is shown in Fig. 1. The half-life of GSTπ enzyme was estimated to be 1–2 hr in the HT4-1 cell line and 8–9 hr in the HT6-8 cell line, measured by either the pulse-chase or the cycloheximide block method. Densitometric scanning of autoradiograms from western blots using ^{125}I-labeled secondary antibody of cycloheximide-treated samples following the two-dimensional separation of proteins permitted quantification of the results. These data were consistent with those obtained by other techniques (data not shown).

The half-life of the GSTπ transcript was calculated as 4.1 hr in HT4-1 cells and 8.4 hr in HT6-8 cells (Table 1). When SKOV3 and DU145 cell lines were treated with 72 μM EA for 24 hr, the half-life of this transcript was also found to be increased 2-fold. For comparison, the half-life of DDH was also increased about 6-fold in HT6-8 cells. Previously, we reported the protein half-life of this enzyme to be 2–4 hr in HT6-8 cells; the enzyme is below detection limits in HT4-1 cells [28], although it is induced significantly (mRNA, enzyme protein and activity) by either acute or chronic EA exposure. The fact that DDH induction could not be detected reflects the low basal expression in these cell lines. The methodology used here was not sensitive enough to allow detection after acute EA exposure.

To investigate the acute effects of EA on GSTπ transcript half-life, HT4-1 cells were treated with 72 μM EA for varying lengths of time. As shown in Fig. 2 and Table 2, there was a time-dependent increase in the half-lives of both GSTπ and DDH. At 6 hr, both transcript half-lives were increased slightly, and at 24 hr, the values were increased to approximately the same as those in HT6-8 cells. Thus, short-term effects are important to the inductive effect, while chronic exposure to EA has an impact in maintaining the acquired resistance.

For comparison, the half-lives of several other transcripts are shown in Table 3. G3PDH transcript half-life was decreased in HT6-8 cells compared with HT4-1 cells and showed no change between control cells and EA-treated cells in either SKOV3 or DU145 cell lines. The half-lives of β-actin and β-tubulin transcripts in HT6-8 were decreased slightly in contrast to untreated cells. Treatment of SKOV3 and DU145 cells with 72

Table 1. Half-lives of GSTπ and DDH transcripts in HT, SKOV3 and DU145 cell lines

Cell lines	EA*	Half-lives (hr) GSTπ	DDH
HT 4-1	–	4.1 ± 1.6†	2.3 ± 0.8
HT 6-8	+	8.4 ± 1.4	13.8 ± 3.1
SKOV3	–	4.4 ± 0.7	ND‡
	+	8.6 ± 1.8	ND
DU145	–	3.8 ± 1.1	ND
	+	6.4 ± 1.4	ND

Cells were incubated with 100 μM 4-TU and 5 μCi/mL [^{3}H]uridine for 1 hr in the presence or absence of 72 μM EA. Total RNA and newly synthesized RNA were prepared and fractionated on 1% agarose gel. For details, see Materials and Methods.

* Cells were treated with 72 μM EA for 24 hr except for the HT6-8 cell line, which was exposed continuously to 72 μM EA.

† Each value is the mean ± SD from three experiments.

‡ ND: not detectable.

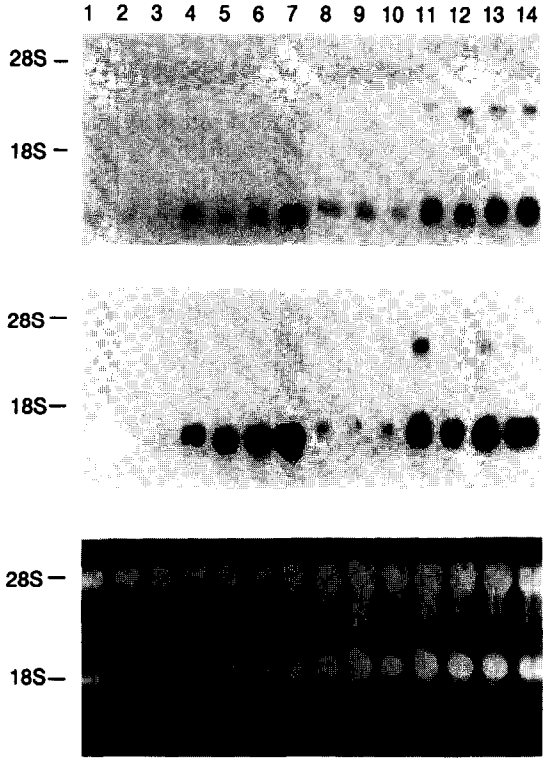

Fig. 2. Northern blot analysis of GSTπ and DDH in the HT4-1 cell line treated with EA. Analysis was performed using GSTπ cDNA (upper panel) and DDH cDNA (middle panel) as probes. Lanes 1–6, purified newly synthesized RNA from HT4-1 cells treated with 72 μM EA for 0, 2, 6, 12, 24 and 48 hr and lane 7 from HT6-8 cells. Lanes 8–14, total RNA from those corresponding to lanes 1–7. Ethidium bromide stained 28S and 18S RNA bands are shown as control for equal gel loading (lower panel).

μM EA for 24 hr did not increase the half-life of the β-actin transcript. Two bands were detected when the RNA transferred Magna-NT membrane was hybridized with either γ-GCS or β-tubulin cDNA (Fig. 3). The upper and lower band sizes of γ-GCS transcript are 4800 and 4000 bp, and those for β-tubulin transcript are 3400 and 1950 bp, respectively. For γ-GCS, both a heavy and a light subunit are known to exist, and these two transcripts may represent these two subunits. For tubulin, more than one transcript can result from splicing of the nuclear RNA, and it is probable that the larger labeled band represents the latter. The half-lives of both bands for γ-GCS transcript were increased coordinately in HT6-8 cells. Unlike γ-GCS, the half-life of the upper band of the β-tubulin transcript was longer than the lower. However, there were no differences between HT4-1 and HT6-8 cell lines, and no change when cells were treated with 72 μM EA for 24 hr in either SKOV3 or DU145 cell lines.

Table 2. Effect of EA on GSTπ and DDH transcript half-lives in the HT4-1 cell line

Incubation time (hr) with 72 μM EA	Half-lives (hr)	
	GSTπ	DDH
Control	4.1	1.8
2	3.4	1.7
6	5.8	2.4
12	6.7	11.8
24	8.1	11.4
48	7.9	11.7

Cells pre-exposed to 72 μM EA for various time length were incubated with 4-TU and [^{3}H]uridine for 1 hr in the presence of 72 μM EA. Subsequent procedures were the same as those described in the legend of Table 1.

To investigate the transcription rate of GSTπ in HT4-1 and HT6-8 cell lines, nuclear run-on experiments were carried out. As shown in Fig. 4, when the GSTπ cDNA immobilized membranes were hybridized to the ^{32}P-labeled transcripts and followed by quantitation via scintillation counting, the GSTπ/actin ratios of HT6-8 and HT4-1 cells were 0.70 ± 0.04 and 0.35 ± 0.10, respectively, indicating an approximately 2-fold increase in transcription rate in the HT6-8 cell line.

DISCUSSION

Understanding how detoxification enzymes such as GST are regulated in response to chemical insults is integral to understanding acute and chronic stress response. EA, a Michael-reaction acceptor enhances the expression of a number of detoxification enzymes by an as yet undefined mechanism(s). Clarifying how elevated GSTπ activity may be induced and maintained in the presence of EA is particularly interesting inasmuch as EA is both a GSTπ inhibitor and inducer [15, 16]. The HT6-8 cell line exhibits 2-fold resistance to EA, and correlates with a 2- to 3-fold elevation in GSTπ activity and protein amount [20]. The present data would support the conclusion that elevated GSTπ transcript is a composite of increased transcriptional rate and a stabilization of enzyme protein and mRNA. It should be noted that the aggregate of changes for each of these parameters is greater than the eventual induced level of GSTπ protein. The specific reasons for this are not clear. However, there is no paradigm that protein synthesis must be linearly related to protein or transcript half-life or transcriptional rate. Indeed, it is possible that specific feedback mechanisms may take effect to control eventual enzyme activity. In addition, EA may have a direct effect upon protein synthesis, thereby limiting the eventual enzyme levels.

When two other cell lines, SKOV3 and DU145, were treated with EA, a similar effect was seen for GSTπ transcript half-life, although their baseline expression of DDH was too low to detect any effect. The effect on transcript half-life for both GSTπ and DDH was coordinately time dependent (Table 2), suggesting that some aspect of the cellular pharmacokinetics of the drug may be involved. Interestingly, the half-life of the GSH biosynthetic enzyme, γ-GCS, was also increased in the HT6-8 cell line (Table 3), suggesting a compensatory enhancement of other enzymes involved in GSH metabolism. The absence of any general up-regulating effects on the other "housekeeping genes," such as G3PDH, β-actin and β-tubulin, argues against the possibility that this effect is a general consequence of EA toxicity. The half-life of DDH transcript was also enhanced in the HT6-8 cell line as well as EA-treated HT4-1, SKOV3 and DU145 cells. Such results suggest that EA may se-

Table 3. Half-lives of G3PDH, β-actin, γ-GCS and β-tubulin transcripts in HT, SKOV3 and DU145 cell lines

		Half-lives (hr)					
				γ-GCS†		β-Tubulin†	
Cell lines	EA*	G3PDH	β-Actin	(a)	(b)	(a)	(b)
HT4-1	–	23.0 ± 1.1‡	9.2 ± 0.9	8.5 ± 3.0	8.5 ± 2.4	15.9 ± 8.9	11.1 ± 4.6
HT6-8	+	11.7 ± 1.9	7.9 ± 1.5	14.6 ± 1.5	14.5 ± 3.2	12.9 ± 8.1	8.7 ± 3.4
SKOV3	–	3.9 ± 1.9	2.1 ± 0.8	ND§	ND	3.8 ± 2.4	2.1 ± 0.9
	+	4.6 ± 0.4	2.7 ± 1.0	ND	ND	5.9 ± 3.1	5.4 ± 2.4
DU145	–	10.7 ± 2.9	4.0 ± 0.7	ND	ND	22.9 ± 16.9	5.4 ± 0.6
	+	11.2 ± 3.8	5.6 ± 1.1	ND	ND	24.9 ± 19.3	11.8 ± 8.1

Cell treatments and subsequent procedures were the same as those described in the legend of Table 1.
* Cells were treated with 72 μM EA for 24 hr except for the HT6-8 cell line, which was exposed continuously to 72 μM EA.
† There were two bands hybridized with γ-GCS or β-tubulin cDNA, an upper band (a) and a lower band (b).
‡ Each value is the mean ± SD from three experiments.
§ ND: not detectable.

lectively affect enzymes of thiol-metabolism and detoxification, both of which could be considered to be part of a cellular defense system.

The protein half-life experiments depicted in Fig. 1 illustrate first order rate kinetics in HT4-1 cells. For the cycloheximide block method, the HT6-8 cells could be construed as following zero order kinetics. Whichever technique is used, the differences between wild-type and resistant cells disappear at 12 hr. There is reported microheterogeneity for GSTπ [29]. It is possible that EA has a differential stabilizing effect on some transcript populations, presumably those with a shorter half-life. By 12 hr this effect essentially disappears.

Precisely how EA prolongs the half-lives of the GSTπ transcript and protein is not yet clear. There are several possibilities that might, in some degree, explain this mechanism: (i) EA can covalently bind to GSTπ [15, 16] and this binding in EA-resistant cells may protect the protein from intracellular degradation. Since the covalent binding is reversible in the presence of excess GSH [16], an overall reduction in GST enzyme activity would not necessarily be detected by subsequent measurements; (ii) the prolonged half-life of protein may, through some feedback mechanism, contribute to the stability of the transcript from which it was translated; and (iii) EA or an EA–protein conjugate may bind to a specific sequence within the GSTπ transcript and protect it from attack by cellular RNases or by other mechanisms. Whatever the actual mechanism may be, it seems apparent that chemically induced stress may effect a number of cellular adaptations, which contribute to the enhancement of detoxification capacity in a drug-stressed situation.

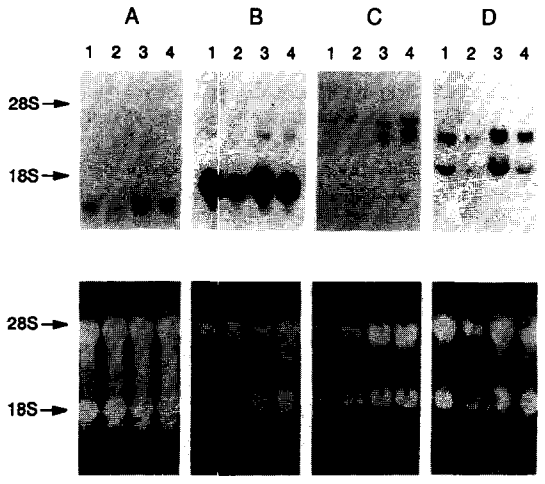

Fig. 3. Representative northern blot of G3PDH (A), β-actin (B), γ-GCS (C) and β-tubulin (D) (upper panel). Lanes 1 and 2 are purified newly synthesized RNA from HT4-1 and HT6-8 cells. Lanes 3 and 4 are total RNA from HT4-1 and HT6-8 cells. Ethidium bromide stained 28S and 18S RNA bands are shown as a control for equal loading (lower panel).

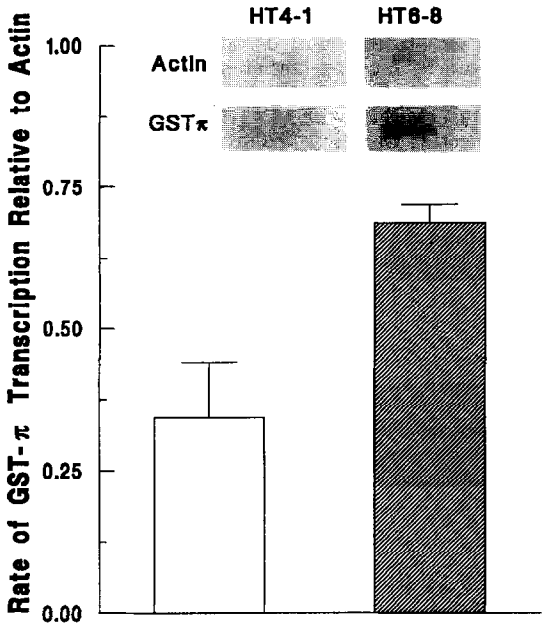

Fig. 4. Nuclear run-on assay for estimation of GSTπ transcriptional rate. Nuclei were incubated with 200 μCi of [^{32}P]UTP at room temperature for 10 min and followed by incubation with DNase I and proteinase K. The ^{32}P-transcripts were isolated and hybridized by slot blots with GSTπ or actin cDNAs (top of figure). For details, see Materials and Methods. Histogram bars are means ± SD of three experiments. Key: open bar, HT4-1, and shaded bar, HT6-8.

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