Adrenocorticotrophic-hormone-dependent regulation of a μ -class glutathione transferase in mouse adrenocortical cells

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Three different forms of glutathione transferase (GST) have been resolved in the two mouse adrenal tumour cell lines Y1 and Kin 8. Two of these belong to the μ and π classes respectively. The third form is so far unidentified. In the Y1 cells, the levels of the μ form (mGTmul) and the unidentified form, are both down-regulated in the presence of adrenocorticotrophic hormone (ACTH) while the π form is unaffected. The Kin 8 cell line is derived from Y1 cells and harbours a defect in the cyclic AMP (cAMP)-dependent protein kinase, making it refractory to cAMP-dependent regulation of several enzymes. The GST levels in this cell line were unaffected by ACTH. Also, the steady-state levels of mGTmul mRNA were much lower in Y1 cells treated with forskolin (which activates adenylate cyclase) compared with

control cells, but there was no difference in mGTmul mRNA levels between control and forskolin-treated Kin 8 cells. This indicates that the ACTH-dependent regulation of the μ class GST is pre-translational and that a functional cAMP-dependent protein kinase is required for the regulation. We have further shown that the difference in mRNA steady-state levels between control and forskolin-treated Y1 cells is abolished when transcription is inhibited by actinomycin D. In light of the stability of mGTmul mRNA, it would appear most likely that actinomycin D inhibits the transcription of short-lived factors which regulate the turn-over of mGTmul transcripts in response to changes in intracellular cAMP levels.

INTRODUCTION

The multifunctional glutathione transferase (GST) enzymes are involved in the metabolism of a range of xenobiotics as well as endogenous substances, and are abundant in most forms of aerobic species and tissues investigated so far. The cytosolic forms of the enzymes can be divided into four different classes, α , μ , π and θ , according to primary structure, substrate specificities and immunological properties (Mannervik, 1985; Mannervik and Danielsson, 1988). They are involved in the detoxification of electrophilic xenobiotics by conjugating glutathione to the substrate, thereby rendering it less reactive, more water-soluble and easier to excrete (Pickett and Lu, 1989). A range of endogenous molecules, some of which may arise as a consequence of lipid peroxidation of biological membranes, have been found to be substrates for GSTs, and a protective role for these enzymes against harmful endogenous molecules has been suggested (Mannervik, 1987; Ketterer et al., 1989). In addition, the GSTs function as intracellular binding proteins and have the capacity to bind both endogenous substances, such as steroid and thyroid hormones, bilirubin, haem and bile acids, and xenobiotics (Smith and Litwack, 1980; Homma et al., 1986; Listowsky et al., 1988).

The GSTs can be induced by xenobiotics, e.g. phenobarbital, β -naphthoflavone, 3-methylcholanthrene and *trans*-stilbene oxide (Chassaud, 1979; Guthenberg et al., 1980; Ding and Pickett, 1985; Rushmore et al., 1993). In addition, we and others have shown earlier that the levels of GSTs can be influenced by both peptide and steroid hormones (Igarashi and Satoh, 1989; Mankowitz et al., 1990; Staffas et al., 1992), which may be another indication that these enzymes are important for endogenous cell metabolism. In the rat, GSTs belonging to the μ class are regulated by pituitary hormones (Mankowitz et al., 1990, 1991a). In the liver, sex differences in the expression of

various isoenzymes can be attributed to the sexually dimorphic secretion patterns of growth hormone (Staffas et al., 1992). In the rat adrenal gland, we have shown that adrenocorticotrophic hormone (ACTH), in addition to controlling the steroidogenic enzymes, also regulates the levels of several forms of GSTs (Mankowitz et al., 1990). The effect of ACTH on both steroid hydroxylases and GSTs is mediated by cyclic AMP (cAMP) (Simpson and Waterman, 1988; Mankowitz et al., 1991b). In contrast to the steroid hydroxylases, however, the GSTs are down-regulated by ACTH. The reciprocal regulation may thus suggest a function for the GSTs during periods of low pituitary ACTH production and low adrenal steroid hydroxylase activities, such as that which occurs during fetal development (Lund et al., 1988; Tangalakakis et al., 1989; Conley et al., 1992a,b).

Although mechanisms of positive cAMP-dependent regulation of adrenocortical gene expression have been defined (Simpson et al., 1990; Parker and Schimmer, 1993), less is known about negative regulation of gene expression by cAMP in these cells. In order to further characterize and study the mechanism of the ACTH-dependent regulation of GSTs in the adrenal, we have looked for an appropriate model cell line. In the present investigation we have characterized ACTH-dependent regulation of GSTs in a mouse adrenal tumour cell line, Y1. We demonstrate that the level of a μ class isoenzyme is decreased by ACTH and most likely involves the regulation of short-lived factors which control the turnover of the GST transcript.

EXPERIMENTAL

Cells and culture conditions

The mouse adrenal tumour cell lines, Y1 (Yasumura et al., 1966; Schimmer, 1979) and Kin 8 (Williams and Schimmer, 1983;

Abbreviations used: ACTH, adrenocorticotrophic hormone; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione transferase; cAMP, cyclic AMP; cAPK, cAMP-dependent protein kinase.

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Wong et al., 1992; Olson et al., 1993), were cultured in Dulbecco's modified Eagle medium containing 10% (v/v) fetal calf serum. The cells were treated with either 100 nM ACTH (Synachten, Ciba-Giegy AG, Basel, Switzerland) or 10 μ M forskolin for the indicated times before harvesting the cells. In some experiments, the transcription inhibitor actinomycin D (5 μ M) was included.

Determination of enzymatic activities

Cells were rinsed with PBS and harvested by scraping in ice-cold 0.25 M sucrose using a rubber policeman. The cell suspensions were sonicated and then kept at -20°C until use. Cytosol was prepared by centrifugation at 100 000 *g* for 1 h. GST activity was measured spectrophotometrically with 1-chloro-2,4-dinitrobenzene (CDNB) or 1,2-dichloro-4-nitrobenzene (DCNB) as substrates, as described (Habig et al., 1974).

Affinity chromatography and h.p.l.c. analysis

The cytosols were purified by affinity chromatography on GSH-Sepharose as described by Simons and Vander Jagt (1977), together with rat GST 1-1 as an internal standard. The elution buffer contained 100 mM Tris/Cl, 5 mM hexyl-glutathione and 5 mM glutathione, pH 9.1. The eluate was concentrated on Millipore immersible cx-10 filters.

H.p.l.c. analysis was performed using a reverse-phase column (Dynamax 300 A, C4-83-503-C5; Rainin Instruments, Woburn, MA, U.S.A.) on a Gilson h.p.l.c. system. The method is a modified version of the one described by Ostlund-Farrants et al. (1987). The gradient was run with 0.1% (v/v) trifluoroacetic acid in water (A) and acetonitrile (B) as follows: 5 min with 35% B, a linear gradient increasing to 50% during the course of 20 min, and finally a linear gradient increasing to 60% during a period of 7 min. GST subunits were detected and quantified on the basis of their A_{214} .

Antibodies

Rat GST 3-3 was purified and antibodies raised in rabbits as described by Mannervik et al. (1985).

SDS/PAGE and immunoblot analysis

Electrophoresis was performed on SDS/15% (w/v) polyacrylamide gels by the method of Laemmli (1970). The separated proteins were transferred electrophoretically to nitrocellulose membrane filters with 0.2 μ m pores. The nitrocellulose sheets were then blocked with 3% (w/v) BSA for at least 2 h, washed and incubated with primary and secondary antibodies, and visualized essentially according to the manufacturer's description of the electrochemiluminescence method (Amersham International, U.K.).

Preparation of RNA and Northern blot analysis

Total RNA was prepared from Y1 and Kin 8 cells by extraction with guanidinium thiocyanate followed by centrifugation in caesium chloride according to a standard protocol (Sambrook et al., 1989). Formaldehyde/agarose gel electrophoresis of RNA and Northern blotting were performed essentially as described (Sambrook et al., 1989) but modified to reduce the formaldehyde content of the gel and to include staining of the nylon-filter with Methylene Blue before hybridization (Herrin and Schmidt, 1988; Chomczynski, 1992a,b). The nucleotide sequences of two highly similar murine μ class GST cDNAs, mGTmu1 and mGTmu2 (Townsend et al., 1989), were aligned and two synthetic oligonucleotides were synthesized that should be able to distinguish

between the respective mRNAs in Northern blot analysis, based on nucleotide divergence in the alignment. The oligonucleotide mu1 (5'-TGAAAATATAGGTGTTGCGATGTA-3') is designed to detect mRNA for mGTmu1 whereas the oligonucleotide mu2 (5'-TGCAAAGATTGGCTTGGAGAGGAA-3') is designed to detect mRNA for mGTmu2 (Townsend et al., 1989). Sequence analysis indicates that mGTmu1 is most closely related to rat GST subunit 3 and mGTmu2 to rat GST subunit 4 (Townsend et al., 1989). The oligonucleotides were end-labelled with [γ - ^{32}P]ATP using T4 polynucleotide kinase and used in Northern analysis. The filters were hybridized with the labelled oligonucleotide probes in 20 mM sodium phosphate, pH 7.0 containing 5 $\times$ SSC, 7% (w/v) SDS, 10 $\times$ Denhardt's solution, and 500 μ g of tRNA/ml. The hybridization temperature was 50 $^{\circ}\text{C}$ for mu1 and 60 $^{\circ}\text{C}$ for mu2. Following hybridization, the filters were washed for 3 $\times$ 30 min with 3 $\times$ SSC, 5% SDS, 10 $\times$ Denhardt's in 10 mM sodium phosphate, pH 7.0. The filters were normalized by hybridization of the filters with a cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which had been labelled by the random hexamer method using [α - ^{32}P]dCTP. Filters were stripped between hybridizations by heating the filters to 60 $^{\circ}\text{C}$ for 30 min in 96% (w/v) formamide, 10 mM Tris, 10 mM EDTA, pH 7.0.

Protein and DNA determinations

DNA determinations were performed on the Y1 and Kin 8 cell suspensions by fluorimetric measurements according to Labarca and Paigen (1980). Protein concentrations were determined in the 100 000 *g* cytosols by the method of Lowry et al. (1951).

RESULTS

CDNB and DCNB activities in Y1 cells

Initially, we investigated the possible presence of enzymatic activities in mouse adrenocortical Y1 cells towards two well characterized substrates for GSTs, CDBN and DCNB.

After 48 h in culture, Y1 cells were treated with ACTH-containing medium for 24, 48, 72 or 96 h before harvesting. New ACTH-containing medium was applied to the cells every 24 h. In response to ACTH, the treated cells showed the characteristic changes in morphological appearance (Yasumura et al., 1966). Cells were scraped off the plates and GST activities towards CDBN and DCNB were measured in the cytosol. The amount of cytosolic protein/ μ g of DNA was unaffected by ACTH treatment (results not shown) and, therefore, all values are given as per mg of cytosolic protein. The activity towards CDBN in cytosolic preparations from control cells varied between 0.6 and 2.4 μ mol/min per mg of protein and, with DCNB as substrate, between 20 and 57 nmol/min per mg of protein. The values are within the normal activity range for these substrates in cytosolic preparations from mouse organs (Warholm et al., 1986; Gupta et al., 1990). An increase in control activity between 24 and 72 h was commonly observed and might reflect the variation in cell density during the experiment. Treatment of the Y1 cells with 100 nM ACTH gave, irrespective of the control level, a decrease in the specific activities towards both substrates. With CDBN as substrate, the maximum effect was obtained after 72 h, i.e. a 40% decrease in activity. A representative experiment is illustrated in Figure 1(a) and the mean values from several experiments, presented as percentage of control, are illustrated in Figure 1(b). The effect of ACTH treatment on the activity towards DCNB was more pronounced. In this case, the decrease in enzyme activity after 96 h of treatment was 57%, as presented in Figures 1(c) and 1(d). The substrate DCNB is a more specific

Figure 1 Specific activity towards CDNB and DCNB of GSTs in Y1 cells

The cells were either untreated (■ or black bars) or treated with ACTH (□ or white bars) for the times indicated in the Figures. GST activities were measured in cytosolic preparations using either CDNB (a and b) or DCNB (c and d) as substrates. Figures (a) and (c) show representative experiments, and (b) and (d) show the means $\pm$ S.D. ($n = 3$) for 24, 48 and 72 h and the means ($n = 2$) for 96 h, presented as percentages of the respective control values.

substrate than CDNB for the μ class of GSTs (Mannervik and Danielsson, 1988). Thus these results provided an early indication that Y1 cells may contain μ class GSTs which are regulated by ACTH.

H.p.l.c. quantification of GST isoenzymes in Y1 cells

We next determined which isoenzymes of GSTs are present in Y1 cells and which of these may respond to ACTH, to account for the observed decrease in enzymatic activity.

When cytosol from Y1 cells was affinity-purified on GSH-Sepharose and then analysed by h.p.l.c., three different peaks were detected (Figure 2a). Two of these, peaks 1 and 3, comigrated with purified enzyme standards from mouse liver and corresponded to the μ and π forms respectively (results not shown). The second peak did not correspond to any of the mouse liver GST standards. The μ form was the most abundant GST in the untreated Y1 cells.

After treatment of the Y1 cells with ACTH, changes in the cytosolic isozyme patterns were obtained (Figure 2b). After 96 h of treatment, there was a considerable decrease in the levels of the μ form and of the unidentified peak, while the π form was unaffected. Figures 3a–3c show the quantification by h.p.l.c. of the three GST forms in cytosolic preparations from cells cultured in the absence or presence of ACTH for various periods of time. Purified rat GST 1-1 was used as an internal standard during the affinity purification and h.p.l.c. analysis. For all isoenzymes, an initial increase was observed after 48 h in control cells. Thereafter, the levels decreased to the initial control value and were maintained at this level throughout the rest of the experiment. Similarly, initial increases in the enzyme levels after 24 h of ACTH treatment were observed for all isoenzymes. Thereafter, decreases were observed for the μ and unknown forms in ACTH-treated cells compared with untreated cells, resulting after 96 h in enzyme levels which were 26 and 28% of the control levels

respectively. The π form was unaffected by ACTH-treatment of the cells.

H.p.l.c. quantification of GST isoenzymes in Kin 8 cells

A mutant subline of the Y1 cells, Kin 8, with a defect in the regulatory subunit of the cAMP-dependent protein kinase (cAPK) (Williams and Schimmer, 1983; Wong et al., 1992; Olson et al., 1993) was used in order to evaluate the possible involvement of this enzyme in the regulation of GSTs by ACTH. The Kin 8 cells were treated and kept in culture under the same conditions as the Y1 cells. The Kin 8 cells also contained three forms of GSTs, as judged by h.p.l.c. analysis after GSH-affinity purification of cytosol (Figure 4a). The retention times of the three peaks were identical to those in preparations from Y1 cells. As was the case in Y1 cells, the μ form was most abundant in the Kin 8 cells. In contrast with the observation in Y1 cells, however, ACTH treatment was without effect on the h.p.l.c.-profile of GSTs in preparations from Kin 8 cells, irrespective of the time of treatment (Figures 4b and 5a–5c). Activities towards CDNB and DCNB in cytosolic preparations of Kin 8 cells were measured and varied over the ranges 0.8–3.3 and 43–109 nmol/min per mg of protein respectively. In accordance with the h.p.l.c. analysis, ACTH treatment of the Kin 8 cells had little or no effect on these activities (results not shown).

Immunoblot experiments with Y1 and Kin 8 cells

Immunoblot experiments of cytosolic preparations from Y1 and Kin 8 cells with antibodies towards rat GST 3-3 (which is the rat form most homologous to the mouse μ form present in these cells; see below) confirmed the results obtained by h.p.l.c. Irrespective of the duration of ACTH treatment of Y1 cells, the amount of enzyme detected was lower in the treated cells than in

Figure 2 H.p.l.c. pattern of GST subunits in cytosol from Y1 cells

The cells were either untreated (a) or treated with ACTH for 96 h (b). The cytosol was purified by affinity chromatography as described in the Experimental procedures section, before analysis by reverse-phase h.p.l.c. The subunits were detected via A_{214} .

control cells (Figure 6a). In contrast, the enzyme levels in the Kin 8 cells were unaffected by ACTH treatment (Figure 6b).

Northern blot analysis

To determine which μ class GSTs are present in Y1 and Kin 8 cells, we designed oligonucleotides to specifically detect two previously characterized murine homologues of rat subunits 3 and 4 respectively (Townsend et al., 1989). As shown in Figure 7(a), the probe specific for mGTmu1 (similar to rat subunit 3) detected a message of 1.5 kilobases in total RNA preparations from both Y1 and Kin 8 cells. In contrast, hybridization of the filter with the probe specific for mGTmu2 (similar to rat subunit 4) did not detect any specific mRNA (Figure 7b; overexposed

Figure 3 Quantification of GST subunits separated by h.p.l.c. of affinity-purified cytosol from Y1 cells

The peak areas from the h.p.l.c. chromatograms were integrated. Each panel shows control levels (black bars) and the effect of ACTH treatment on the levels of the different subunits (white bars), for the times indicated. Figure (a) shows the variations in the μ form, (b) in the unknown form and (c) in the π form.

blot to show background hybridization). In murine tissues known to express the mGTmu2 mRNA (Townsend et al., 1989), the mGTmu2 probe was able to detect a message of the correct size (results not shown). It cannot be excluded, however, that a more sensitive assay would be able to detect mGTmu2 mRNA in Y1 cells. A labelled cDNA encoding GAPDH was used as a hybridization probe to normalize for differences in the loading of RNA on the gels (Figure 7c).

We also evaluated whether the decrease in enzymatic activity and μ class isoenzyme levels observed after ACTH treatment of Y1 cells could be accounted for by a cAMP-dependent decrease in steady-state levels of mRNA for mGTmu1. In order to mimic the mechanism by which ACTH increases cAMP levels, we used

Figure 4 H.p.l.c. pattern of GST subunits in cytosol from Kin 8 cells

The cells were either untreated (a) or treated with ACTH for 72 h (b). The cytosol was purified by affinity chromatography as described in the Experimental procedures section, before analysis by reverse-phase h.p.l.c. The subunits were detected via A_{214} .

forskolin, an agent that activates adenylate cyclase and thereby increases the intracellular concentration of cAMP. As shown in Figure 7, forskolin treatment of Y1 cells led to a time-dependent decrease in the levels of mGTmu1 mRNA (Figure 7a). Forskolin treatment of Kin 8 cells had no effect on the levels of mGTmu1 mRNA, demonstrating the requirement of a functional cAPK for the ACTH and forskolin effects. Forskolin treatment of Y1 and Kin 8 cells had no effect on the message levels for GAPDH (Figure 7c).

Effect of actinomycin D on mGTmu1 mRNA levels in Y1 cells

To address the possible mechanism responsible for the cAMP-dependent decrease in mGTmu1 mRNA, we examined the effect

Figure 5 Quantification of GST subunits separated by h.p.l.c. of affinity-purified cytosol from Kin 8 cells

The peak areas from the h.p.l.c. chromatograms were integrated. Each panel shows the control levels (black bars) and the effect of ACTH treatment (white bars) on the levels of the different subunits, for the times indicated. Figure (a) shows the variations in the μ form, (b) in the unknown form and (c) in the π form.

of treating the cells with an inhibitor of transcription. However, such attempts to measure mRNA stability after inhibiting RNA synthesis were unsuccessful because the inhibitors were actually found to block the decrease in mRNA accumulation that occurred after elevations in intracellular cAMP concentrations. A Northern blot of RNA from Y1 cells treated for 12 h with forskolin in the presence or absence of actinomycin D is shown in Figure 8. Forskolin treatment of the cells resulted in the expected decrease in mRNA for mGTmu1, whereas actinomycin D alone had a very modest effect on mGTmu1 message (Figure 8a), indicating that the mRNA is very stable (half-life > 24 h, results not shown). When both forskolin and actinomycin were

Figure 6 Immunoblot analysis of GST μ in cytosol from Y1 and Kin 8 cells

The Y1 (a) and Kin8 (b) cytosols were derived from cells either untreated (—) or treated (+) with ACTH for the times indicated in the Figures. Samples were subjected to SDS/PAGE, transferred electrophoretically to nitrocellulose paper and treated with antibodies towards GST subunit 3, as described in the Experimental procedures section.

Figure 7 Northern blot analysis of total RNA from Y1 and Kin 8 cells

Kin 8 and Y1 cells were treated for 12 or 24 h in the presence (+) or absence (—) of 10 μ M forskolin. Cells were harvested, and total RNA was prepared and size-fractionated by formaldehyde-agarose electrophoresis. After transfer of the RNA to a nylon filter, it was sequentially hybridized to 32 P-labelled probes to detect mRNAs for (a) mGTmu1, (b) mGTmu2 and (c) GAPDH by autoradiography. Figure (b) is overexposed to show background hybridization.

added together, the forskolin-dependent decline in mGTmu1 mRNA was abolished. When the same filter was hybridized to the GAPDH cDNA (Figure 8b), no decrease in GAPDH mRNA was observed in response to forskolin and actinomycin D caused only a minor decrease in the message levels (half-life > 24 h, results not shown). This is consistent with published data on GAPDH mRNA, which is a very stable transcript (Fujimoto et al., 1992). These data would suggest that on-going transcription

Figure 8 Effect of actinomycin D on the forskolin-induced decrease in mGTmu1 mRNA

Y1 cells were cultured in the presence or absence of 10 μ M forskolin and in the presence or absence of 5 μ M actinomycin D for 12 h. Northern blot analysis of total RNA from these cells was performed using 32 P-labelled probes to detect mRNA for mGTmu1 (a) or GAPDH (b) on the same filter. The filter was completely stripped of probe between hybridizations.

is required for the cAMP-dependent decrease in mGTmu1 mRNA. Given the stability of the mGTmu1 mRNA, it is not likely to be caused by inhibition of transcription of the mGTmu1 gene *per se*.

DISCUSSION

In the present investigation, we have characterized at least two different forms of GSTs in the mouse adrenal tumour cell lines Y1 and Kin 8. The two forms, belonging to the μ and π classes respectively, are present in both cell lines. A third form detected in the two cell lines did not co-migrate with any of the available standards on h.p.l.c. and is so far uncharacterized. In the mouse, three main forms of GSTs belonging to the α , μ and π classes respectively have been detected in mouse liver (Warholm et al., 1986), whereas several other α and μ forms have been identified in extrahepatic organs and in induced liver (Townsend et al., 1989; Gupta et al., 1990; McLellan et al., 1991; Medh et al., 1991; Awasthi et al., 1993). The as yet unidentified GST form in Y1 and Kin 8 cells could be identical to one of the forms detected in certain extrahepatic mouse organs (Medh et al., 1991; Awasthi et al., 1993). However, upon SDS/PAGE analysis of GSH-affinity-purified Y1 and Kin 8 cytosol, we detected only two bands (results not shown). This may indicate that the unknown form we found upon h.p.l.c. either co-migrates with one of the other forms during SDS/PAGE gel or is an oxidized or otherwise altered form of the μ or π isoenzymes with aberrant h.p.l.c. behaviour. The true identity of the protein in this peak will have to await further experiments, e.g. preparation of sufficient quantities for N-terminal sequencing.

Upon treating the Y1 cells with ACTH we observed a down-regulation of GST activities towards the substrates CDNB and DCNB, as well as decreased protein levels of the μ and unknown forms of GSTs, whereas the π form was unaffected. This is in accordance with what we have seen earlier in the rat, where several of the μ forms are down-regulated by ACTH in the adrenal and by growth hormone in the liver (Mankowitz et al., 1990; Staffas et al., 1992).

Using primary cultures of rat adrenal cells we have previously shown that down-regulation of GST 4-4 is mediated by cAMP (Mankowitz et al., 1991b), which is the second messenger for ACTH-dependent regulation of intracellular events. Increased cAMP levels leads to the activation of a cAPK (Taylor et al., 1992). In order to investigate the possible involvement of cAPK in the regulation of GSTs, we used a cell line, Kin 8, which is a subline of the Y1 cell line harbouring a defect in this kinase

(Williams and Schimmer, 1983; Wong et al., 1992; Olson et al., 1993). The Kin 8 cells contained the same isoenzymes of GST as did the Y1 cells, but the levels were not affected by ACTH. This is an indication that the protein kinase is important for the regulation of GSTs by ACTH. The difference in the mechanisms between the positive cAMP-regulation of adrenocortical gene expression (Simpson et al., 1990; Parker and Schimmer, 1993) and this, more unusual, negative regulation is thus situated beyond the step involving activation of cAPK.

The Northern blot experiments show that the mRNA corresponding to the major μ form present in mouse liver [mGTmu1, similar to the rat 3-3 (Yb1) form] is also present in large amounts in the Y1 and Kin 8 cells. In contrast, the mRNA corresponding to mGTmu2 [similar to the rat 4-4 (Yb2) form] could not be detected. The latter form has been detected in several other extrahepatic mouse tissues (Townsend et al., 1989; Awasthi et al., 1993). Thus, in contrast to the regulation in rat adrenal, where the 4-4 form is the one most affected by ACTH, the μ form similar to rat 3-3 is the major form present and affected in Y1 cells. We can not exclude, however, the possibility that a more sensitive assay may be able to detect mGTmu2 mRNA in Y1 and Kin 8 cells. In particular, previous studies have shown the murine GST Yb₂ (mGTmu2) to elute after Yb₁ (mGTmu1) upon reversed-phase h.p.l.c. (Hayes et al., 1991), thus making it a candidate for the unidentified GST form in our h.p.l.c. analysis (see above). As pointed out above, further studies are needed to identify this form.

Previous studies have characterized GSTs in murine ovary and testis (Awasthi et al., 1993). Three μ -class isoenzymes were found to be present in these tissues and were shown to be formed through dimeric combinations of two subunits whose N-terminal sequences were similar to mGTmu1 and mGTmu 2 respectively (Townsend et al., 1989). Since these studies were performed with untreated mice, it would be of great interest in the future to determine whether the levels of the μ -class transcripts are regulated by the cognate trophic hormones for these steroidogenic tissues.

Our experiments show that the effect of forskolin, and thus cAMP, on the levels of the μ class GST in Y1 cells is pre-translational, as the steady-state levels of the mGTmu1 mRNA also decreased after this treatment. The mRNA stability experiments, where actinomycin D was used as an inhibitor of transcription, further show that the difference in mRNA levels between control and forskolin-treated cells is abolished when the inhibitor is present. The effect of forskolin is thus dependent on an on-going transcription. In light of the stability of the mGTmu1 transcript it would appear likely that the effect is not attributable to a decreased transcription of the mGTmu1 gene but rather to a change in mGTmu1 mRNA turn-over through post-transcriptional mechanisms. The loss of mGTmu1 mRNA in response to an elevation of intracellular cAMP levels could thus be mediated by short-lived factors or nucleases. Inhibition of the transcription of the genes encoding such factors could affect mRNA transport, processing or stability so as to increase the levels of the mGTmu1 transcripts. It will be important to complement the present study with investigations of the transcription rate of the mGTmu1 gene by nuclear run-on analysis to definitively rule out transcriptional regulation.

Because our data are most consistent with cAMP-induced destabilization of the mGTmu1 mRNA, it will also be important, if possible, to determine the chemical *in vivo* half-life of the message in the presence and absence of elevated cAMP levels through pulse-chase analysis using tritiated uridine. It is now well documented that mRNA degradation is an important point of control for the regulation of gene expression (reviewed by

Hentze, 1991). The control of transcript stability is thought to be achieved through an interplay between *cis*-acting elements within the mRNA and *trans*-acting factors. Such factors, in some instances including nucleic acid components, have been shown to regulate the turnover of a number of mRNAs (Hentze, 1991). Extra- and intracellular signals could thus control the stability of specific mRNA species by either controlling the accessibility of *cis*-acting sequences or by controlling the activity of *trans*-acting factors.

A vast array of signals have been shown to control mRNA stability, including glucocorticoids, iron, retinoic acid, phorbol esters and tumour necrosis factor- α (Paek and Axel, 1987; Klausner and Harford, 1989; Antras et al., 1991; Zhu et al., 1991; Stephens et al., 1992). In addition, the cAPK-dependent signal-transduction pathway has been implicated in the regulation of mRNA stability (for example, Fuh et al., 1989; Boggaram et al., 1989; Juliani et al., 1990; Chen et al., 1993). In most instances, cAMP appears to stabilize transcripts, and such regulation has previously been demonstrated in adrenocortical cells. Thus ACTH treatment of bovine adrenocortical cells in primary culture causes a 5-fold increase in the half-life of P450_{scc} mRNA (Boggaram et al., 1989). Destabilization of mRNA by cAMP has been shown in *Dictyostelium discoideum* (Juliani et al., 1990), but to our knowledge has not previously been demonstrated in adrenocortical cells. The mechanisms of cAMP-dependent control of mRNA stability remain largely to be elucidated. Recently, however, a cAMP-regulated RNA-binding protein that interacts with phosphoenolpyruvate carboxykinase mRNA was described (Nachaliel et al., 1993). The authors put forth a model in which the RNA-binding protein consists of a binding subunit and a regulatory component where the association of the subunits is regulated by the state of phosphorylation of the regulatory subunit. The binding subunit is thought to bind only in its uncomplex form and to then destabilize the message, possibly through the unwinding of double-stranded sequences within the mRNA (Nachaliel et al., 1993). Even though this is only a model and the functional consequences of these interactions need to be established further, it will be of great interest to investigate whether similar mechanisms may be operating in the cAMP-dependent control of μ -class GSTs in adrenocortical cells. The Y1 cell model described in this paper will then be of great value and we will initially investigate whether sequences of the mGTmu1 mRNA can confer cAMP-dependent destabilization of reporter mRNAs in Y1 cells.

In conclusion, this investigation shows that the murine Y1 cell line expresses the regulatory machinery for the down-regulation of certain GSTs by ACTH, a phenomenon that we have studied earlier in the rat adrenal. The hormonal regulation is thus conserved between these species, and this fact strengthens the hypothesis that the GSTs are of importance for the metabolism of endogenous substances. The biological role of the regulation is still unknown, but one possible function of varying GST levels may occur during the fetal period, when ACTH levels are fluctuating. It is known that other enzymes, i.e. the steroid-metabolizing cytochromes P-450, are regulated in the fetal adrenal in concert with varying ACTH levels in fetal plasma (Lund et al., 1988; Tangalikis et al., 1989; Conley et al., 1992a,b). Thus there appears to be a need to regulate the levels of the P-450 enzymes during fetal development. Because GSTs have a high capacity to bind steroids (Marayama and Listowsky, 1984; Homma et al., 1986; Abramowitz et al., 1988), one may speculate that the role of the ACTH-dependent regulation of GST expression is for these enzymes to metabolize or bind steroids during fetal periods when the presence of such compounds is not required. Further, because the ACTH-dependent regulation of

steroid hydroxylases is positive, a low concentration of ACTH would give rise to a low steroid production and an increased ability to metabolize or bind steroids by GSTs. This might thus be a way for the cell to effectively lower the steroid concentration under certain periods. This is of course a hypothesis that remains to be tested.

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