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Decrease in Class Pi Glutathione Transferase mRNA Levels by Ultraviolet Irradiation of Cultured Rat Keratinocytes

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The effect of ultraviolet (UV) B irradiation on pi class glutathione transferase (GST-P) gene expression was examined in cultured rat keratinocytes. Immunoblotting demonstrated GST-P to be the major GST form in the cells, and it was significantly decreased following irradiation. Northern blot analysis revealed that the mRNA decreased to 10–25% of the initial value 24 h after irradiation at a dose of 40 mJ/cm². No remarkable changes were observed at earlier time points. Hydrogen peroxide treatment enhanced GST-P mRNA expression, with a 70% increase at 250 μM concentration. Alterations in possible trans-acting factors were examined to clarify the mechanism of repression by UV irradiation. c-Jun mRNA was induced 3.5-fold at 4 h after irradiation, but by 24 h fell to a lower level than that observed initially. c-Fos mRNA was increased 10-fold at 1 h but was completely suppressed at 12 and 24 h. Thus, the changes of c-Jun and c-Fos mRNA differed from that of GST-P mRNA. The level of mRNA for silencer factor-B was decreased to less than 10% at 12 h. UV irradiation of cells transfected with the chloramphenicol acetyltransferase (CAT) reporter gene containing enhancer (GPE I) or silencer regions of the GST-P gene did not suppress CAT activity. Although basal expression of the GST-P gene was mainly dependent on GPE I, altered expression of *c-jun*, *c-fos* and other genes coding for factors possibly trans-acting on GPE I did not appear to be responsible for the decreased GST-P mRNA levels.

Key words: Glutathione transferase — Ultraviolet irradiation — Keratinocyte — Skin cancer

Glutathione transferases (GSTs) are a family of multifunctional dimeric enzymes that catalyze the conjugation of glutathione to electrophilic xenobiotics, including carcinogens, and also exhibit glutathione peroxidase activity toward lipid hydroperoxides.[1,2] Thus, GSTs are considered to play an important role in detoxification of carcinogens, thereby inhibiting carcinogenesis.[3] The rat pi class GST, GST-P, is specifically expressed in chemically induced hepatic preneoplastic and neoplastic lesions.[4] The pi class GSTs in other species, such as human GST-π (GST P1-1 in the new nomenclature[5]), are also increased in a wide range of carcinomas.[6] Because of the involvement of GSTs in detoxification of anti-cancer drugs as well as carcinogens, the increased expression of pi class forms in cancer cells has been suggested to be responsible for the drug-resistant nature of the cells.[7] Indeed, either inhibition of their activities or suppression of their expression has been reported as an effective approach to overcome drug resistance.[8]

The rat GST-P gene has a strong enhancer element, GPE (GST-P enhancer element) I, located 2.5 kb upstream from the transcription initiation site.[9] Since the enhancer consists of two palindromic phorbol 12-*O*-tetradecanoate 13-acetate-responsive element (TRE)-like sequences, its expression has been suggested to be partly regulated by the oncogene products c-Jun and c-Fos.[10,11] The gene also possesses silencer regions located between nucleotides −396 and −140, and binds at least three nuclear trans-acting factors.[12] One of them, silencer factor B (SF-B), was cloned and found to be similar or identical to liver activator protein (CCAAT/enhancer binding protein β or interleukin 6-dependent DNA binding protein).[13] In a previous study, we immunohistochemically demonstrated that the localization of GST-P is separate from those of c-Jun and c-Fos in most tissues, although GST-P is coexpressed with the oncogene products in the skin epidermis.[14]

With the continuous erosion of the stratospheric ozone layer, the risk of exposure to cytotoxic ultraviolet (UV) has been increasing.[15] UV irradiation of experimental animals is known to induce DNA damage with eventual cancer formation in the skin and it also triggers the expression of various cellular proteins that participate in DNA repair, transcription, and other functions.[16,17] Affected proteins include the oncogene products, c-Jun and c-Fos, collagenase, metallothionein and interleukin 6. The induction of collagenase and metallothionein is reported to be mediated by the action of transcription factor c-Jun/c-Fos heterodimer.[18] Although UV irradiation is suggested to release active oxygen species,[19,20] its

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effects on GST expression remain to be clarified. Thus, UV-irradiated skin keratinocytes may provide a good system to examine whether GST-P expression is regulated by these oncogene products. It is also of interest to know whether UV influences the expression of GST forms closely linked to carcinogenic processes.

In the present study, we investigated the effects of UV irradiation on GST-P mRNA levels in cultured rat keratinocytes in comparison with alterations in the expression of c-*jun* and c-*fos*. In addition, assessment of the influence of UV on reporter gene control by GST-P gene enhancer and silencer regions was conducted to cast further light on the mechanisms of action.

MATERIALS AND METHODS

Reagents Hydrogen peroxide was purchased from Wako Pure Chemical Co. (Osaka). Fetal calf serum (FCS) was from Irvine Scientific Co. (Santa Anna, CA). Chloramphenicol was from Sigma Chemical (St. Louis, MO). [α-^{32}P]dCTP (3,000 Ci/mmol) was from New England Nuclear (Boston, MA). All other reagents were of analytical grade.

Cell culture FRSK, a cell line derived from epidermal keratinocytes of fetal rats, obtained from the American Type Culture Collection (Rockville, MD), was grown in Eagle's minimal essential medium (MEM) supplemented with 10% (vol/vol) FCS at 37°C in an atmosphere of 5% CO_2/95% air.

Source of UVB UVB irradiation of subconfluent FRSK cells was carried out with an M-DMR-80 (Clinical Supply Co., Ltd., Tokyo) as reported previously.[21] The UVB dose was measured with an IL-1700 radiometer (International Light Inc., Newburyport, MA) fitted with a UVB detector (peak sensitivity at 305 nm).

Hydrogen peroxide treatment A 30% hydrogen peroxide solution in water was diluted to final concentrations of 50, 250, 500 and 1000 μM with MEM containing 10% FCS immediately before use. Subconfluent FRSK cells were washed twice with 10 m*M* sodium phosphate buffer, pH 7.4, containing 0.15 *M* NaCl, prior to hydrogen peroxide exposure for 6 h. The cells were harvested for RNA preparation immediately after hydrogen peroxide treatment.

Cell viability Cell viability was assayed by the trypan blue-dye exclusion method. Viabilities were approximately 70% for cells irradiated at a dose of 40 mJ/cm^2 and 95% for cells treated with 1000 μM hydrogen peroxide.

cDNA probes The GST-P cDNA clone (pGP5)[22] was a kind gift from Dr. M. Muramatsu, Saitama Medical School, Saitama. Rat c-*jun* cDNA (pRJ101)[23] was kindly donated by Dr. M. Sakai, Hokkaido University School of Medicine, Sapporo. v-*fos* cDNA (pfos-1)[24] was obtained from the Japanese Cancer Research Resources Bank. SF-B cDNA[13] and β-actin cDNA were also used as probes.

Extraction and analysis of RNA Total cellular RNA was extracted from cells by the method of Chirgwin *et al.*[25] Samples containing 10 or 20 μg of RNAs were electrophoresed on 1.5% formaldehyde-agarose gels and the bands were transferred to nitrocellulose filters. Blots were sequentially hybridized with ^{32}P-labeled cDNA probes as previously described.[26] Results of autoradiography were quantified with a densitometer (Model-Pan, Jookoo Co., Tokyo) and the values were expressed relative to the respective initial values.

Immunoblot analysis SDS-polyacrylamide gel electrophoresis was performed with 12.5% polyacrylamide gels by the method of Laemmli[27] and immunoblotting was carried out using anti-GST 1-2 (alpha-class), 3-4 (mu-class) or P (7-7) antibodies by the method of Towbin *et al.*[28] These antibodies were raised in rabbits as described previously.[4] GST 1-1 and 3-3 were purified from rat livers and GST-P from hyperplastic hepatic nodules as reported earlier.[4]

Assay of GST activity The cells were disrupted in a solution of 10 m*M* Tris-HCl, pH 7.8, containing 0.2 *M* NaCl by sonication for 60 s using a model UR-20P sonifier (Tomy Seiko Co., Tokyo), and centrifuged at 16,000*g* for 15 min. Samples of the resulting supernatants were used for the assay of GST activity. Total GST activity was assayed using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate by the method of Habig *et al.*[29] The activity of GST-P was determined by activity inhibition assay with the anti-GST-P antibody as described previously.[30]

DNA transfection and chloramphenicol acetyltransferase (CAT) assay CAT reporter constructs attached to various portions of regulatory elements of the GST-P gene[9, 12] were kindly donated by Dr. M. Imagawa, Faculty of Pharmaceutical Sciences, Osaka University, Osaka. ECAT included the enhancer, GPE I, and silencer regions of the GST-P gene, spanning −2,900 through +59 with respect to the cap site, and 1CAT and 4CAT spanned −2,200 to +59 and −400 to +59, respectively, both lacking GPE I but possessing the silencer regions. 4(ΔGPS1)CAT encompassed −400 to +59 with a deletion of sequences (from −276 to −262) corresponding to the silencer regions. 5CAT and Δ56CAT spanned −136 to +59 and −55 to +59, respectively. The 5CAT included the TRE sequence located between −61 and −56. Transfection of the respective CAT constructs into FRSK cells was performed by the calcium phosphate coprecipitation method.[31] Briefly, 2×10^5 cells in MEM containing 10% FCS were plated onto 60 mm dishes. Five hours after the addition of 10 or 20 μg of DNA, cells were treated with 15% (vol/vol)

glycerol and the medium was changed to MEM containing 10% FCS. After 48 h incubation, cells were exposed to UVB at a dose of 40 mJ/cm^2. Cells were incubated for a further 36 h, then harvested and lysed by several cycles of freezing and thawing. The CAT activity of the cell lysates was determined according to the method of Gorman *et al.*[32)]

RESULTS

Alterations in GST-P and its mRNA levels by UVB irradiation and hydrogen peroxide treatment GST forms expressed in FRSK cells were examined by immunoblotting with anti-GST 1-2, GST 3-4 and GST-P antibodies. As shown in Fig. 1 (lane 2), GST-P was the major form with other class forms not being detected. Activity inhibition assay demonstrated that GST-P accounted for more than 95% of the total GST activity assayed with CDNB (data not shown). Cells harvested 36 h after UV irradiation at 40 mJ/cm^2 demonstrated a decrease in GST-P protein by immunoblotting (Fig. 1A, lane 3), as compared with non-irradiated cells (lane 2). No expression of alpha or mu class GST subunits could be detected after irradiation (Fig. 1B and C).

When cells were exposed to increasing doses of UV, and total RNA was prepared 24 h after irradiation, northern blot analysis revealed a dose-dependent decrease in GST-P mRNA level to 10–25% of the initial value at a dose of 40 mJ/cm^2, whereas β-actin mRNA levels were not significantly altered (Fig. 2). To examine the effect of hydrogen peroxide treatment on GST-P mRNA levels, cells were exposed to up to 1000 μM for 6 h. In contrast to UV irradiation, this treatment enhanced GST-P mRNA expression with a 70% increase at a concentration of 250 μM (Fig. 3).

Altered levels of mRNAs for factors transacting on the GST-P gene Since the GST-P gene has been suggested to be positively regulated at least partly by the transcription factor AP-1 (c-Jun/c-Fos heterodimers), and negatively by SF-B, the effects of UVB on the expression of these transcription factors were examined and compared with

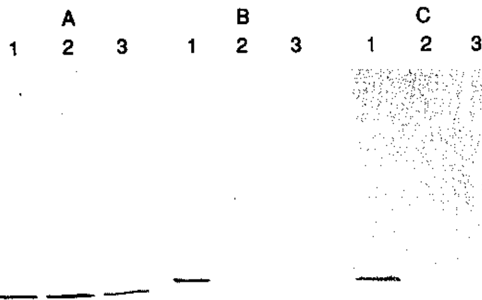

Fig. 1. Immunoblot analysis of GST subunits in UVB-irradiated rat keratinocytes. For SDS-polyacrylamide gel electrophoresis, purified GST or protein samples (100 μg) from 16,000*g* supernatants were applied to each lane. A, B and C, immunoblots with anti-GST-P (7-7), GST 1-2 and GST 3-4 antibodies, respectively. Lane 1, mixture of 0.5 μg each of GST 1-1, GST 3-3 and GST-P; lanes 2 and 3, protein samples from non-irradiated cells and from cells 36 h after UV irradiation at 40 mJ/cm^2, respectively.

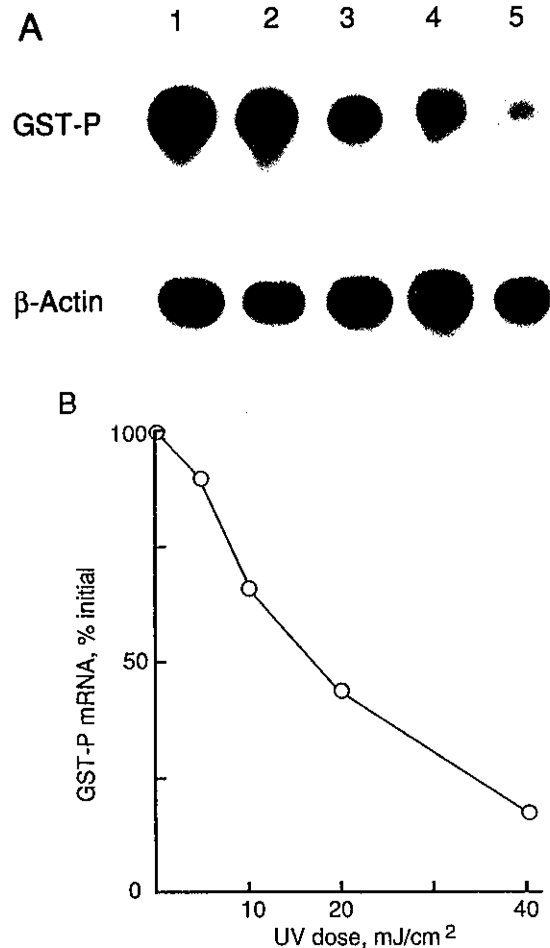

Fig. 2. Effects of increasing doses of UVB irradiation on GST-P mRNA and β-actin mRNA levels in keratinocytes. In A, expression of GST-P and β-actin mRNAs was examined by the northern blotting method as described in the text. Lanes 1, 2, 3, 4 and 5 each included 20 μg of total RNA isolated from cells irradiated at 0, 5, 10, 20 and 40 mJ/cm^2, respectively. RNA was extracted from the cells 24 h after the irradiation. In B, results in A were quantified by densitometry and GST-P mRNA values normalized to individual β-actin levels were expressed relative to the respective initial values.

the time course of alteration in GST-P mRNA levels. In the absence of UV irradiation, genes coding for these transcription factors were all expressed in the cells. In response to a dose of 40 mJ/cm^2 UV irradiation, a decrease of GST-P mRNA was evident at the time point of 24 h, with no remarkable changes being observed at earlier time points (Fig. 4). The c-Jun mRNA level was increased about 3.5-fold at 4 h as compared to the initial value, following a temporary reduction at 0.5 h, and then decreased at 24 h to below the initial level. c-Fos mRNA was induced 10-fold at 1 h, but was completely suppressed at 12 and 24 h. Thus, although the levels of c-Jun and c-Fos mRNA as well as GST-P mRNA were repressed at 24 h, alteration patterns of c-Jun or c-Fos mRNA levels differed from that of GST-P mRNA, suggesting that GST-P gene expression is not directly dependent on the levels of these transcription factors. The amount of mRNA for SF-B was decreased to less than 10% of the initial level at 12 h, exhibiting a similar alteration pattern to that of GST-P mRNA.

Effect of UVB on GST-P transcription To examine whether GST-P gene transcription and particular regulatory regions of the GST-P gene are involved in the decrease in GST-P mRNA caused by UVB irradiation, enhancer or silencer regions of the gene fused to the CAT reporter gene were transfected into FRSK cells and their CAT activities were compared before and after UV irradiation. Without UV treatment, the activity of ECAT, containing both GPE I and silencer regions, was 10-fold higher than that of 1CAT, which lacks the former (Fig. 5). Furthermore, deletion of silencer regions [4-(ΔGPS1)CAT and 5CAT] did not enhance the CAT activity, but rather repressed it as compared with 4CAT. Deletion of the TRE sequence near the cap site (Δ56CAT) completely repressed the activity. These re-

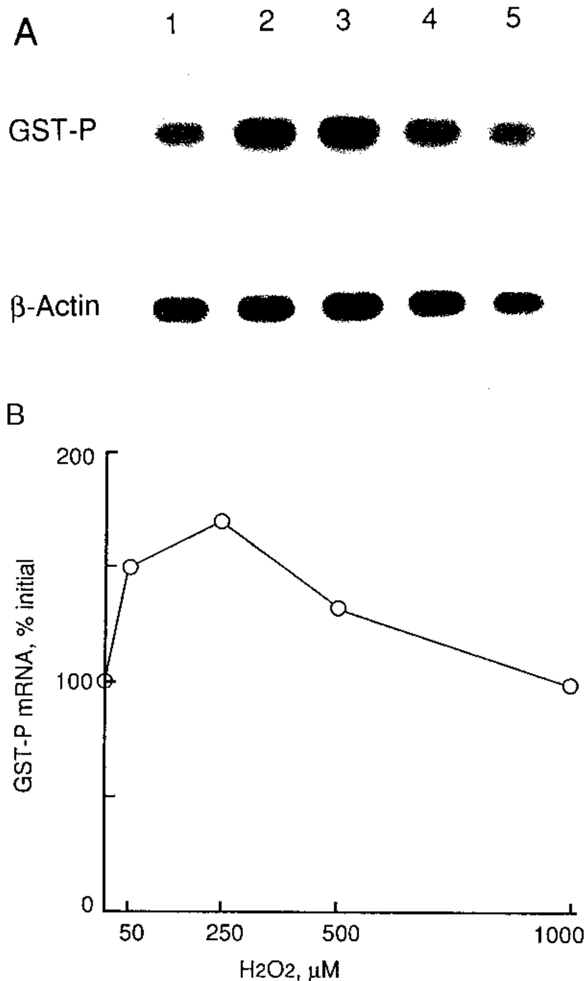

Fig. 3. Effect of hydrogen peroxide treatment on GST-P mRNA and β-actin mRNA levels in keratinocytes. In A, extraction of total RNA and northern blotting were carried out as described under Fig. 2. Lanes 1, 2, 3, 4 and 5 each included 10 μg of RNA isolated from cells treated for 6 h with 0, 50, 250, 500 and 1000 μM hydrogen peroxide, respectively. In B, results in A were quantified and GST-P mRNA values were expressed relative to the respective initial values as in Fig. 2.

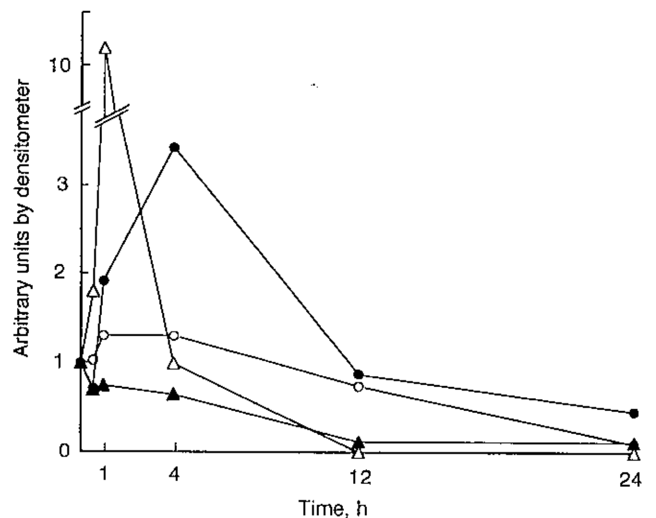

Fig. 4. Comparison of the effect of UVB irradiation on the kinetics of expression of c-Jun, c-Fos, SF-B and GST-P mRNAs. Cells were irradiated with UV at 40 mJ/cm^2, and total RNAs were prepared after 0, 0.5, 1, 4, 12 and 24 h. Equal amounts of RNA (20 μg) were electrophoresed, and analyzed by northern blotting using the respective cDNAs. Results of autoradiography were quantified by densitometry and the values normalized with respect to β-actin levels are shown relative to the respective initial values. ○ GST-P mRNA, ● c-Jun mRNA, △ c-Fos mRNA, ▲ SF-B mRNA.

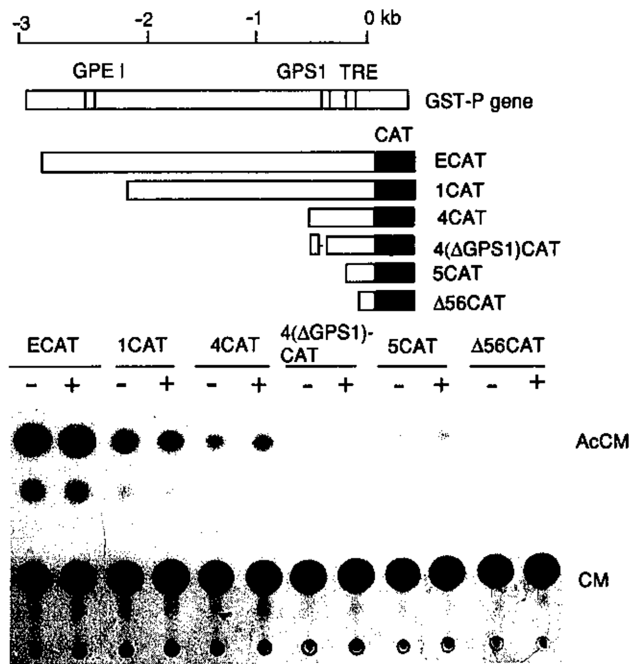

Fig. 5. Lack of effect of UVB irradiation on CAT activity in keratinocytes transfected with GST-P-CAT fusion genes. Cells were transiently transfected with reporter gene fragments containing the regulatory elements illustrated in the upper panel. At 48 h after transfection, UVB was irradiated at a dose of 40 mJ/cm^2. Cells harvested at 36 h after the irradiation were processed and assayed for CAT activity as described in the text. AcCM, acetylated form of chloramphenicol; CM, chloramphenicol. +, UV irradiated; −, not irradiated.

sults suggest that GPE I plays the major role in governing basal expression in these cells. However, the activity of ECAT was not changed by UV irradiation (Fig. 5), although GST-P mRNA expression was repressed in these transfectants (data not shown). CAT activity was also not altered in cells harvested at 24 h after the irradiation (data not shown). UV irradiation did not alter the low CAT activities of 1CAT and other constructs.

DISCUSSION

Although expression of pi class GSTs is increased in many preneoplastic and neoplastic lesions[3] and UV irradiation is known to be associated with skin cancer formation,[16] the present study revealed that GST-P and its mRNA are decreased in cultured rat keratinocytes by UVB irradiation (Figs. 1 and 2). Such a decrease is analogs to the loss of pi class GSTs in human carcinomas after X-ray irradiation[33] and in rat preneoplastic lesions after clofibrate administration,[34] both inducing production of active oxygen species. However, GST-P mRNA was increased by hydrogen peroxide treatment (Fig. 3). Thus, GST-P expression seems to be repressed under oxidative stress conditions other than those associated with hydrogen peroxide, although the particular form(s) of active oxygen species involved remains to be clarified. Although alpha-class GST forms with high glutathione peroxidase activity are known to be induced in hepatoma cell lines by hydrogen peroxide or lipid hydroperoxides,[35] induction of these forms was not evident in FRSK cells. Expression of pi class GST is suggested to be dependent on the cell cycle in a human melanoma cell line[36] and UV irradiation is known to induce p53 protein in the epidermis,[37] leading to transient cell cycle arrest in the G1 phase.[38, 39] These findings raise the possibility that the decreased GST-P mRNA level in the present study may partly reflect altered cell cycle kinetics. Further studies are needed to clarify this.

Since induction of collagenase and metallothionein mRNAs following UV irradiation is reported to be a later event as compared with the rapid increase of c-Jun and c-Fos mRNA levels,[18] the time course of alteration in GST-P mRNA level was compared with those for the oncogene mRNAs. In spite of the transient increase of both c-Jun (3.5-fold) and c-Fos mRNA (10-fold) levels at 1 to 4 h, GST-P mRNA was decreased at 24 h (Fig. 4) and also at 48 h (data not shown). Complete suppression of c-Fos mRNA at 12 and 24 h (Fig. 4) raises the possibility that the loss or decrease of this factor might be relevant to the decrease of GST-P mRNA. However, this seems unlikely because the increase of c-Fos mRNA did not lead to a significant increase of GST-P mRNA. These results suggest that GST-P gene expression is not directly dependent on the levels of these transcription factors, consistent with our previous findings of their different localizations in many tissues other than skin epidermis.[14] Furthermore, the decrease in mRNA for SF-B (Fig. 4), identified as a negative regulator of the GST-P gene in some cell lines,[13] was opposite to what would be expected if it were responsible for the reduced GST-P mRNA. Although UV irradiation has been noted to suppress transcription of a number of genes,[19, 40] β-actin mRNA levels were not affected in the present case (Fig. 2), suggesting the lack of any general reduction of transcription under our experimental conditions.

UV irradiation of cells transfected with CAT constructs containing the GPE I or silencer regions did not suppress the respective CAT activities (Fig. 5). These results suggest that action on regulatory elements of the GST-P gene is not responsible for the decrease of mRNA levels due to UV irradiation, despite the fact that basal expression of the gene is mainly dependent on the presence of GPE I. Thus, decreased expression of *c-jun*, *c-fos* and other genes coding for factors possibly trans-acting

on GPE I did not appear to be involved in the decrease of GST-P mRNA. Koo *et al.* have reported that GST-P mRNA is partly regulated posttranscriptionally in the liver.[41]

In conclusion, we have observed a decrease of GST-P protein and its mRNA in rat keratinocytes exposed to UVB irradiation. Although c-*jun*, c-*fos* and GST-P gene were all expressed in these cells before irradiation, the results suggest that GST-P mRNA levels may not be directly regulated by these oncogene products.

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