The human glutathione S-transferase P1-1 gene: modulation of expression by retinoic acid and insulin

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Glutathione S-transferases (GSTs) are a group of enzymes which play an important role in the detoxication of xenobiotics. It is shown that the expression of human glutathione S-transferase P1-1 (GSTP1-1) is suppressed by retinoic acid (RA) as the result of decreased transcription from its gene, *GSTP1*. Chloramphenicol acetyltransferase (CAT) assays indicate that the effect of RA on the transcription of a *GSTP1* promoter–*CAT* fusion gene is mediated by the region −99 to +72 of *GSTP1*. A consensus activator protein 1-binding site, located at nucleotide position −59 to −65 of *GSTP1*, is suggested to be responsible for RA repression. This effect of RA on *GSTP1* expression is mediated by the human β-type RA receptor, hRARβ, but not the chicken retinoid X receptor, cRXR. The retinoid X receptor does not augment the action of hRARβ on *GSTP1*. In addition, it is shown that GSTP1-1 expression is enhanced by insulin as a result of increased transcription of *GSTP1*. Assay of CAT activity indicates that the effect of insulin on the transcription of *GSTP1* is also mediated by the region −99 to +72 of *GSTP1*. Comparison with sequences of other insulin-responsive genes, suggests that insulin enhancement of *GSTP1* expression is effected by an eight-base-pair sequence, 'CCCGCGTC', located at +48 to +55 in intron 1 of the gene. These results are discussed in relation to the increased expression of GSTP1-1 in many tumour cells.

INTRODUCTION

Glutathione S-transferases (GSTs) (EC 2.5.1.18) are a group of multifunctional dimeric enzymes involved in the detoxication of numerous carcinogenic, toxic or pharmacologically active electrophiles by catalysing the conjugation of these electrophiles to glutathione (Jakoby, 1978; Ketterer et al., 1988; Coles and Ketterer, 1990). The cytosolic forms of GST subunits can be grouped into four evolutionary classes, namely Alpha, Mu, Pi and Theta, based on their primary structures, and their kinetic and immunological properties (Mannervik and Danielson, 1988; Meyer et al., 1991).

The Pi-class GST from rat, GST 7-7, is of particular interest as it is associated with chemically induced hepatic preneoplastic foci, hyperplastic nodules and hepatocellular carcinoma (Satoh et al., 1985; Sugioka et al., 1985a,b). The gene encoding GST subunit 7 has been studied by Muramatsu and colleagues, who have identified multiple regulatory elements (Okuda et al., 1987, 1989, 1990; Sakai et al., 1988; Imagawa et al., 1991a,b). In particular, it has been shown that the transcription of subunit 7 is induced by phorbol 12-myristate 13-acetate (PMA) and the *ras* oncoprotein (Sakai et al., 1988). Recently, it has been demonstrated that the expression of GST 7-7 is enhanced in the presence of epidermal growth factor and insulin (Hatayama et al., 1991) and suppressed by the glucocorticoid hormone dexamethasone (Sakai et al., 1992).

The human Pi-class GST, GSTP1-1 (formerly GST π, Mannervik et al., 1992) has been associated with malignancy and the development of chemotherapeutic drug-resistance (Batist et al., 1986; Deffie et al., 1988). We have previously sequenced the gene encoding human GSTP1-1 including its promoter region (Cowell et al., 1988) and demonstrated that the consensus activator protein 1 (AP1)-binding site (−59 to −65) is essential for its promoter activity and that an intronic sequence (+8 to +72), which up-regulates the transcription of *GSTP1*, is integral to the promoter (Xia et al., 1991a,b). In this paper we report that the transcription of *GSTP1* is negatively modulated by retinoic acid (RA) and positively modulated by insulin.

EXPERIMENTAL

Materials

All tissue-culture media and ingredients were from GIBCO BRL. RA, insulin, GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were obtained from Sigma Chemical Company. Acetyl-CoA was supplied by Boehringer–Mannheim Biochemicals. Deoxycytidine 5′-triphosphate ([α-^{32}P]dCTP, 500 Ci/mmol) was purchased from Amersham International. [^{14}C]Chloramphenicol (50 Ci/mmol) was obtained from DuPont (U.K.). Plasmid construct pSS0.2CAT, which contains the promoter region (−99 to +72) of *GSTP1*, has been described by Dixon et al. (1989). A human β-type RA receptor expression construct (hRARβ) and a chicken retinoid X receptor expression construct (cRXR) were gifts from Dr. P. Brickell, Department of Biochemistry and Molecular Biology, University College, London, U.K. pCH110, a β-galactosidase expression construct, was purchased from Pharmacia LKB.

Cell culture

All media were supplemented with 10% (v/v) foetal-calf serum and 10 μg/ml gentamicin. Human bladder carcinoma cells (EJ) (Knowles et al., 1980) were maintained in Dulbecco's modified Eagle's medium (DMEM). Human breast carcinoma cells

Abbreviations used: AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; RA, retinoic acid; RAR, retinoic acid receptor; hRARβ, human β-type RAR expression construct; RXR, retinoid X receptor; cRXR, chicken RXR expression construct; PMA, phorbol 12-myristate 13-acetate.

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(MCF7), a gift from Dr. P. Beverley of Imperial Cancer Research Fund, London, U.K. were cultured in RPMI 1640 medium. The simian virus 40-transformed human keratinocyte cell line (SVK14), a gift from Dr. T. Kamalaki, Department of Biochemistry, Charing Cross Hospital Medical School, London, U.K. was maintained in RPMI 1640 medium supplemented with 5 μg/ml hydrocortisone.

GST activity assay

GST activity was determined as described by Habig et al. (1974) using CDNB as substrate.

H.p.l.c. quantification of GSTP1-1 in cultured cells

Cultured cells were homogenized in 10 mM sodium phosphate, pH 7.0, 0.16 M KCl, 1 mM dithiothreitol and 25 μM phenylmethanesulphonyl fluoride. Cell extracts were prepared by centrifugation for 45 min at 45000 *g*. The supernatant was applied to an affinity column containing 0.5 ml of GSH–agarose. After washing with 10 ml of homogenization buffer, GST was eluted with 2.4 ml of 0.1 M Tris/HCl, pH 9.1 containing 5 mM GSH and 5 mM hexylglutathione. GST subunits were separated and quantified by reverse-phase h.p.l.c. as described by Meyer et al. (1989) except that a column of 5 μm particle size was used at a flow rate of 1 ml/min.

Transfection and chloramphenicol acetyltransferase (CAT) assay

Transfection was achieved by the calcium phosphate co-precipitation technique (Gorman et al., 1982), and carried out in DMEM for MCF7 and EJ cells and in Optimum 1 for SVK14 cells. Samples of pSS0.2CAT (5 μg), and/or RA receptor (RAR) expression constructs (1 μg) were used in each transfection. Cells were harvested 40–60 h after transfection and CAT assays were carried out as described by Gorman et al. (1982). pCH110 (1 μg) was co-transfected to assess the efficiency of each transfection (Petkovich et al., 1987).

RNA purification from cultured cells and Northern-blot analysis

Cultured cells (2×10^7) were lysed in 200 μl of 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$ and 0.5% (v/v) Nonidet P-40. The supernatant was extracted twice with 200 μl of Tris/HCl (pH 7.8)-buffered phenol and RNA was precipitated by addition of 2 vol. of ethanol at −20 °C for 2 h. Each loading used 20 μg of RNA. Northern-blot analysis was carried out using ^{32}P-labelled *GSTP1* cDNA as the probe.

RESULTS

Negative regulation of *GSTP1* expression by RA

The effect of RA on the expression of GST activity was studied in SVK14 cells in which h.p.l.c. and Western-blot analyses show GSTP1-1 to be the only GST expressed. Cells were transfected with hRARβ, challenged with RA at different concentrations and GST activity towards CDNB was measured 60 h after RA stimulation. It was shown that RA repressed the endogenous GST activity in a concentration-dependent manner (Figure 1). To establish clearly that the expression of GSTP1-1 was under negative regulation by RA, SVK14 cells were transfected with either hRARβ or the control construct hRARβ^-, which contains the cDNA of human β-type RAR in the reverse orientation. Four hours after transfection, cells were challenged with 1 μM

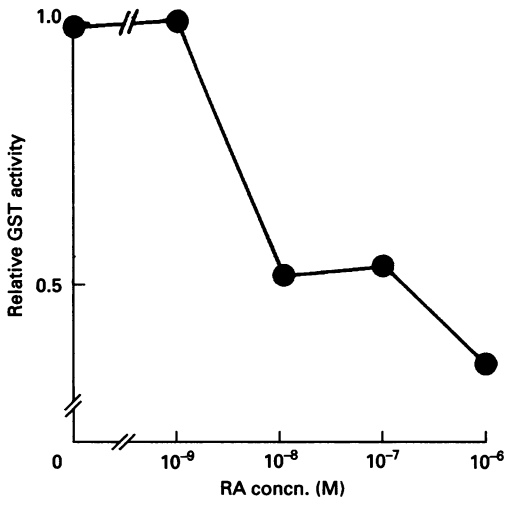

Figure 1 Effect of RA on GST activity in cultured SVK14 cells

SVK14 cells were transfected with hRARβ. Cells were cultured, 4 h after transfection, in RPMI 1640 medium supplemented with 2% (v/v) foetal-calf serum and treated with RA at different concentrations. Cells were harvested 60 h after RA stimulation and GST activity towards CDNB determined. The results presented are the means of three independent experiments.

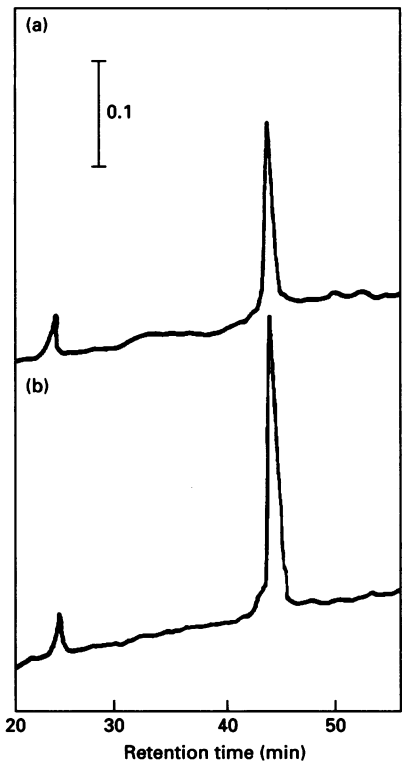

Figure 2 Analysis of RA repression of GSTP1-1 expression by reverse-phase h.p.l.c.

SVK14 cells were transfected with RAR expression plasmids. Cells were cultured, 4 h after transfection, in RPMI 1640 medium supplemented with 2% (v/v) foetal-calf serum and treated with 1 μM RA for 60 h. GSTP1-1 levels were quantified by reverse-phase h.p.l.c. as described in the Experimental section. Each experiment was performed in duplicate. (**a**) Cells transfected with hRARβ; (**b**) cells transfected with hRARβ^-.

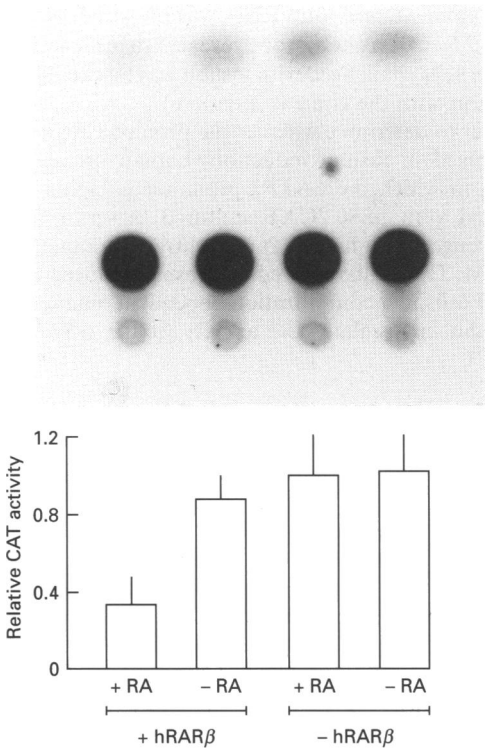

Figure 3 hRARβ-mediated RA repression of *GSTP1* promoter activity in EJ cells

pSS0.2CAT was co-transfected into EJ cells with or without hRARβ. Cells were cultured, 4 h after transfection, in medium containing 2% (v/v) foetal-calf serum with or without 1 μM RA. Cells were harvested 60 h after RA stimulation and CAT activities determined. Results presented are the means of three independent experiments.

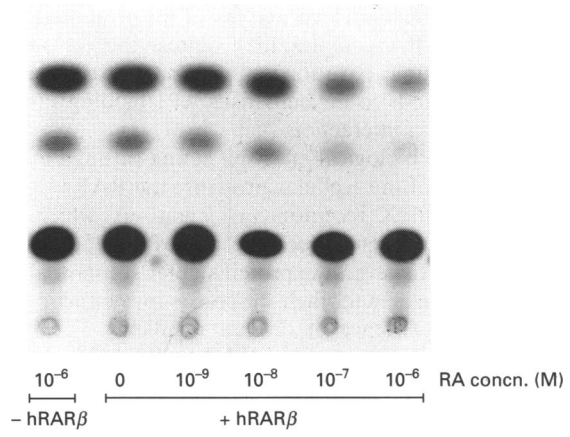

Figure 4 Repression of *GSTP1* promoter activity by RA

pSS0.2CAT was co-transfected with hRARβ into EJ cells. Cells were cultured, 4 h after transfection, in medium containing 2% (v/v) foetal-calf serum and stimulated with RA at different concentrations. Cells were harvested 60 h after RA treatment and CAT activities determined. Each experiment was performed in duplicate.

RA. Cells were harvested and the level of GSTP1-1 measured by reverse-phase h.p.l.c. analysis 60 h later. The results showed that the level of GSTP1-1 in cells transfected with hRARβ was 9.5 $\mu g/1 \times 10^8$ cells, whereas in cells transfected with hRARβ$^-$,

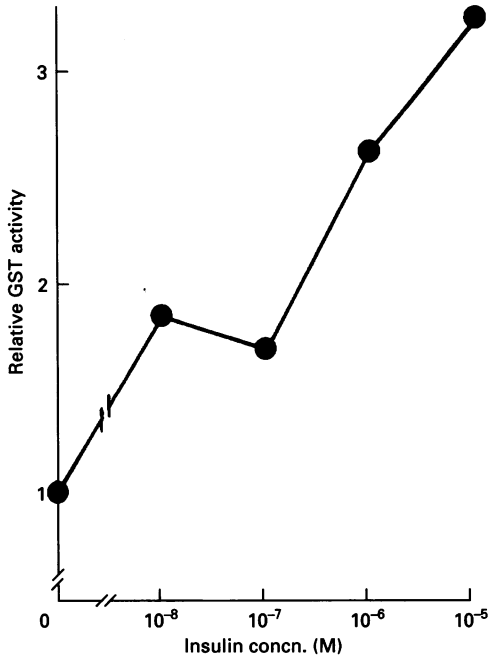

Figure 5 Effect of insulin on GST activity in cultured SVK14 cells

SVK14 cells were cultured in serum-free RPMI 1640 medium supplemented with 5 μg/ml hydrocortisone. Cells were harvested 40 h after 1 μM insulin treatment and GST activity towards CDNB determined. The results presented are the means of three independent experiments.

the level was 31 $\mu g/1 \times 10^8$ cells (Figure 2). This indicates that RA represses the expression of endogenous *GSTP1*.

Since the *GSTP1* promoter is highly active in EJ cells (Xia et al., 1991a), they were used to determine whether the DNA sequence in the promoter region of *GSTP1* was involved in the RA-dependent repression. When construct pSS0.2CAT, which contains the promoter region (−99 to +72) of *GSTP1* linked to the upstream of the CAT gene (Dixon et al., 1989), was transfected alone, or co-transfected with hRARβ, into EJ cells, it was shown that CAT expression was about 2-fold lower in the presence of both 1 μM RA and hRARβ ($P < 0.01$) than in their absence (Figure 3). The effect of RA concentrations on the level of CAT activity obtained from pSS0.2CAT co-transfected with hRARβ is shown in Figure 4. A decrease in CAT activity was observed at concentrations as low as 1×10^{-8} M and the repressive effect increased with the RA concentration. In a similar study, EJ cells were co-transfected with pSS0.2CAT and cRXR. The retinoid receptor had no effect on *GSTP1* promoter activity. Furthermore, co-transfection of cRXR with hRARβ and pSS0.2CAT failed to enhance the effect of RAR-mediated RA repression of *GSTP1* promoter activity.

Insulin induction of *GSTP1* expression

In order to study whether insulin could influence the expression of GSTP1-1, SVK14 cells were cultured in serum-free medium and treated with insulin at final concentrations ranging from 0 to 1×10^{-5} M. Cells were harvested, homogenized, centrifuged and the supernatant assayed for GST activity with CDNB 40 h after exposure to insulin. As shown in Figure 5, insulin increased GST activity up to 3-fold in a concentration-dependent manner. These results indicate that the increased GST activity is due to the induction of GSTP1-1.

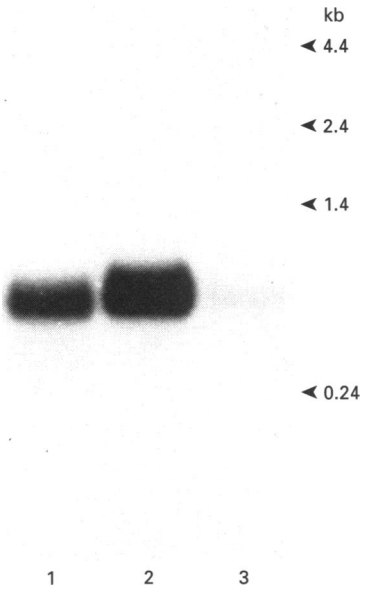

Figure 6 Northern-blot analysis of the induction of *GSTP1* by insulin

Total RNA from cultured SVK14 cells was prepared as described in the Experimental section. RNA from 3T3 cells was used as a negative control. The blot was probed with *GSTP1* cDNA. Each loading contained 20 μg RNA. Lane 1, SVK14 cells; lane 2, SVK14 cells challenged with insulin; lane 3, 3T3 cells.

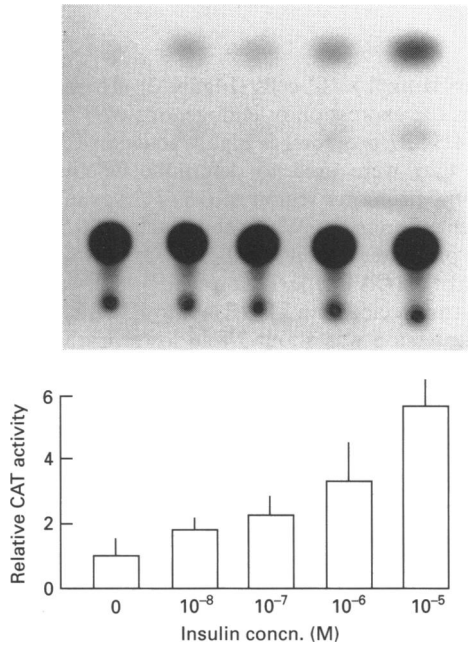

Figure 7 Induction of *GSTP1* promoter activity by insulin

pSS0.2CAT was transfected into MCF7 cells. Cells were cultured, 4 h after transfection, in serum-free medium and stimulated with insulin at different concentrations. Cells were harvested 48 h after insulin stimulation and CAT activities determined. Results presented are the means of three independent experiments.

To determine the effect of insulin on the transcription of *GSTP1*, SVK14 cells were cultured in serum-free medium and challenged with 1 μM insulin. Cells were harvested 48 h after insulin treatment, and total RNA was prepared. Northern blots using *GSTP1* cDNA as a probe revealed that the level of *GSTP1* mRNA in cells challenged with insulin was markedly increased in comparison with the control (Figure 6).

In order to determine whether the promoter region of *GSTP1* was involved in insulin induction, human breast cancer cells (MCF7), in which the *GSTP1* promoter is highly active, were transfected with pSS0.2CAT, cultured in serum-free medium and challenged with insulin at concentrations ranging from 0 to 1×10^{-5} M. The results showed that insulin induced CAT activity in MCF7 cells in a concentration-dependent manner, suggesting that insulin up-regulates the activity of the *GSTP1* promoter (Figure 7).

DISCUSSION

In this paper, we present evidence that RA represses both the transient expression of the CAT gene driven by the *GSTP1* promoter and the endogenous *GSTP1* expression. Chambon and his colleagues (Nicholson et al., 1990) have shown that the RA repression of the rat stromelysin gene is mediated by the AP1-binding site of the gene. However, there is no evidence that RARs bind directly to DNA in order to inhibit the activity of an AP1-binding site (Gudas, 1992). Comparison of the consensus AP1-binding site of the *GSTP1* promoter with that of the rat stromelysin gene reveals that the location and the sequence of the AP1-binding site of the two genes are very similar (TGACTCA at -58 to -65 for *GSTP1* and TGAGTCA at -65 to -71 for the stromelysin gene). Both elements are essential for their own promoter activities (Nicholson et al., 1990; Xia et al., 1991a,b). Therefore, it is likely that the consensus AP1-binding site of *GSTP1* is associated with the RAR-mediated RA repression. A similar phenomenon has been observed with the negative regulation of the collagenase gene by RA, which also occurs through an AP1-binding site (Lafyatis et al., 1990; Yang-Yen et al., 1991; Schüle et al., 1991). As the consensus AP1-binding site of *GSTP1* is essential for the basal level activity of the promoter, it appears that the RAR–RA complex may repress the transcription of *GSTP1* by blocking activation by an unknown positive regulatory factor, which binds to the consensus AP1-binding site, through a protein–protein interaction.

RA exerts its biological effect by acting through at least two distinct classes of intracellular proteins: the RARs (α, β and γ) and the retinoid X receptors (α, β and γ), which are both members of the steroid-receptor superfamily (Evans, 1988; Gudas, 1992). However, the proteins differ substantially in their primary structures and their response to synthetic retinoids, indicating the existence of distinct regulatory networks. It is interesting to note that, unlike hRARβ, cRXR failed to mediate the repression of *GSTP1* expression by RA. Schüle et al. (1991) have reported a similar phenomenon in which all three members of the RAR subfamily (α, β and γ), but not cRXR, can down-regulate the transcriptional activation of the human collagenase gene by *c-jun*. Recently, cRXR has been shown to interact directly with, and enhance, the DNA-binding activities of RARs, as well as receptors for vitamin D_3 and thyroid hormone (T_3) (Zhang et al., 1992; Kliewer et al., 1992). However, cRXR has no effect on the RAR-mediated RA repression of the *GSTP1* promoter, which occurs through a mechanism that does not involve the binding of RAR–RA complex to DNA.

Although the repression mechanism is not clear, the proposed co-localization of a sequence required for RAR–RA-dependent repression with a consensus AP1-binding site, which is essential for the basal level expression of *GSTP1*, is very interesting. The level of GSTP1-1 has been demonstrated to be elevated in many

tumours such as cervical and colon carcinomas (Sato, 1989; Tsuchida and Sato, 1992), and RA is known to inhibit cell growth and induce differentiation. Mutation of RARα has been shown to be associated with acute promyelocytic leukaemia (Borrow et al., 1990; de Thé et al., 1990; Alcalay et al., 1991), while RA treatment can prevent some human oral cancers (Garewal and Meyskens, 1992) and is an effective therapeutic agent for treating certain human cancers such as squamous cell carcinomas of cervix (Lippman et al., 1992b) and skin (Lippman et al., 1992a). Therefore, exploitation of the mechanism by which RA represses *GSTP1* expression via the consensus AP1-binding site will presumably open up an approach to understanding the mechanism which leads to an overexpression of GSTP1-1 in tumours and a potential linkage between the ability of RA to trigger cell differentiation and to block malignant progression.

In this paper, it is demonstrated that insulin induces *GSTP1* expression 3-fold. This finding is supported by the observation that the mRNA of *GSTP1* in SVK14 cells is elevated after insulin treatment and that insulin activates the promoter of *GSTP1* in a transient expression assay approx. 5-fold.

Insulin has been shown to regulate genes which encode proteins involved in a variety of biological phenomena. However, the mechanism by which insulin regulates gene expression is far from understood (O'Brien and Granner, 1991). The most studied mechanism is that by which insulin up-regulates the genes encoding c-Fos (Stumpo and Blackshear, 1986) and c-Jun (Mohn et al., 1990), which therefore affect the expression of numerous other genes with Fos/Jun (AP1)-responsive regulatory elements. Among these is the gene encoding rat GST 7-7, since Hatayama et al. (1991) demonstrated that the protein and mRNA of rat GST 7-7, which were undetectable in freshly isolated rat hepatocytes, were remarkably induced when primary cultured hepatocytes were treated with insulin. This enhanced transcription of the GST 7-7 gene by insulin is mediated by Jun and Fos proteins since the mRNA of *c-jun* and *c-fos* are increased as the result of insulin stimulation (Hatayama et al., 1991). This is consistent with the *ras*-inducibility of GST 7-7, as Burgering et al. (1991) have proposed that $p21^{ras}$ is an intermediate of an insulin signal-transduction pathway involved in the regulation of expression of genes, such as *c-jun* and *c-fos*. Furthermore, Sakai et al. (1992) have shown that Fos/Jun activation of GST 7-7 is mediated by its palindromic AP1-binding sites, called GPE1, located −2.5 kb upstream of *tsp* and that the glucocorticoid hormone, dexamethasone, completely inhibits the Fos/Jun activation. This is consistent with the finding that the glucocorticoid receptor antagonizes the function of Fos/Jun through protein–protein interaction (Schüle et al., 1990; Yang-Yen et al., 1990). However, the insulin-responsive pathway of *GSTP1* must differ from that of GST subunit 7, as it fails to respond to PMA, $p21^{ras}$, or Fos/Jun, and does not have a GPE1-equivalent site (Dixon et al., 1989; Morrow et al., 1990; Xia et al., 1991a). Thus the insulin induction of *GSTP1* would not be expected to be suppressed by glucocorticoid hormone and no significant difference in insulin responsiveness of GSTP1-1 expression was observed in SVK14 cells cultured in the presence or absence of hydrocortisone.

Sequence comparison has revealed that an eight-base sequence 'CCCGCCTC' is conserved in the 5′ flanking region of several genes which respond to insulin, including *c-fos*, *c-myc*, *p33* and the glyceraldehyde-3-phosphate dehydrogenase gene (Nasrin et al., 1990). A sequence CCCGCGTC, which differs by only one base from the consensus sequence, is located in intron 1 of *GSTP1* between +48 and +55, implying that the intronic sequence of *GSTP1* may be involved in mediating insulin induction of the gene. This region has been shown to be an integral part of the promoter and the proteins which bind to it appear to be titratable (Xia et al., 1991a). The equivalent region (+86 to +93; Okuda et al., 1987; Xia et al., 1991a) of the GST 7-7 gene has a nucleotide sequence 'CCCAGCTT', which differs by three bases from the consensus insulin-responsive element proposed by Nasrin et al. (1990). Therefore, although the response of these orthologous genes to insulin appears to be the same, the mechanisms involved are apparently distinct.

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