

Glutathione S-Transferases: Gene Structure and Regulation of Expression

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ABSTRACT: The current knowledge about the structure of GST genes and the molecular mechanisms involved in regulation of their expression are reviewed. Information derived from the study of rat and mouse GST Alpha-class, Ya genes, and a rat GST Pi-class gene seems to indicate that a single *cis*-regulatory element, composed of two adjacent AP-1-like binding sites in the 5'-flanking region of these GST genes, is responsible for their basal and xenobiotic-inducible activity. The identification of Fos/Jun (AP-1) complex as the *trans*-acting factor that binds to this element and mediates the basal and inducible expression of GST genes offers a basis for an understanding of the molecular processes involved in GST regulation. The induction of expression of Fos and Jun transcriptional regulatory proteins by a variety of extracellular stimuli is known to mediate the activation of target genes via the AP-1 binding sites. The modulation of the AP-1 activity may account for the changes induced by growth factors, hormones, chemical carcinogens, transforming oncogenes, and cellular stress-inducing agents in the pattern of GST expression. Recent observations implying reactive oxygen as the transduction signal that mediates activation of *c-fos* and *c-jun* genes are presently considered to provide an explanation for the induction of GST gene expression by chemical agents of diverse structure. The possibility that these agents may all induce conditions of oxidative stress by various pathways to activate expression of GST genes that are regulated by the AP-1 complex is discussed.

KEY WORDS: *c-fos* and *c-jun* genes, AP-1 binding site, transduction signal, oxidative stress, antioxidant defense.

I. INTRODUCTION

The glutathione *S*-transferases (E.C.2.5.1.18) are a family of enzymes that catalyze the nucleophilic attack of the sulfur atom of glutathione on the electrophilic center of a variety of chemical compounds (for a review on enzyme structure, catalytic activities, and nomenclature see Reference 1). These enzymes, which have evolved together with glutathione in the aerobic organisms, are abundant and widely distributed in most forms of life: animals, plants, insects, parasites, yeast, fungi, and bacteria.² In almost all the different organisms in which it has been found, the glutathione *S*-transferase (GST) activity comprises a number of isoenzymes with broad substrate specificities.³ The various GST isoenzymes have generally been found to occur as homodimers and heterodimers of subunits ranging in size from 17 to 28 kDa.¹ The GSTs differ in their expression from one tissue to another

and they may be activated by inducers of drug metabolism.³

The GSTs are believed to play an important role in the protection of cellular macromolecules from attack by reactive electrophiles. By catalyzing glutathione (GSH) conjugation with electrophilic compounds, many of which are produced during xenobiotic metabolism, the GSTs function as an intracellular detoxication system of mutagens, carcinogens, and other toxic compounds. In addition GSTs, via their GSH-dependent peroxidase activity, may play an important role in protecting tissues from endogenous organic hydroperoxides produced during oxidative stress (for reviews on the role of GSTs in detoxication see References 4–8).

The GSTs, in addition to their enzymatic activities, bind with high affinity a variety of hydrophobic compounds such as heme, bilirubin, hormones, and drugs, which suggests that

they may serve as intracellular carrier proteins for the transport of various ligands (for a review see Reference 9). A marked increase in GST activity has been observed in tumor cells resistant to anticancer drugs. This has raised the question of a possible role of GST in the development of drug resistance in chemotherapy.⁴

In recent years, molecular biology studies have been carried out on rat, mouse, and human GST genes encoding cytosolic isoenzymes (for a review see Reference 10). The scope of this review is to present the current knowledge about the structure of GST genes and the molecular mechanisms involved in the regulation of their expression.

II. GST GENE STRUCTURE

Mammalian GSTs have been thoroughly investigated and the knowledge about isoenzyme occurrence in the different tissues is rapidly expanding. The most extensive studies of GST isoenzymes have been initially carried out in the rat liver where the multiple GST forms have been characterized.³ GSTs have also been identified from mouse and human tissues. These studies have shown that the cytosolic rat, mouse, and human GSTs are homodimers or heterodimers of subunits that have been classified into three groups: alpha, mu, and pi; a fourth GST activity, membrane bound, is the microsomal GST.^{1,3}

In recent years, because of construction and characterization of cDNA clones, there has been a rapid increase in the structural information available about glutathione transferases and their genes. Sequence analysis of cDNA clones has revealed that alpha and mu GST subunits are encoded by multigene families and has enabled evolutionary studies between different members of these families. In addition, because specific GST subunits are induced by the administration of various xenobiotics and are differentially expressed in various tissues, the cloned cDNA sequences have been instrumental for the study of the inducible and tissue specific expression of GST genes.¹⁰

A. GST Alpha Gene Family

The cDNA clones complementary to the mRNAs encoding the alpha GST subunits from rat, Ya (GST1-1)¹ and Yc (GST2-2),¹ have been constructed and analyzed by a number of laboratories.¹¹⁻¹⁷ These studies have shown that the Ya and Yc mRNA sequences are 75% identical in the protein-coding region.¹⁷ DNA sequence analysis of different rat liver Ya mRNA cDNA clones has revealed the existence of two highly homologous Ya subunits.¹¹⁻¹⁵ The sequences of the two 222 amino acid subunits differ in eight amino acids and the corresponding mRNAs present divergence in the 3'-noncoding regions. The sequence of a mouse Ya subunit, derived from a genomic clone, was found to encode a 223 amino acid polypeptide highly homologous (94%) to one of the rat liver Ya mRNA species detected by cDNA cloning.¹⁸ Two alpha-class GST cDNA clones, rbGST α I and rbGST α II, isolated from rabbit lung and liver encoding polypeptides of 223 and 221 amino acids, respectively, were found to be 78% identical to each other in amino acid sequence and more closely related (>80% identity) to human Ha subunit than to rat and mouse alpha subunits.¹⁹ The nucleotide sequence of human GST cDNA clones encoding alpha subunits was reported²⁰⁻²⁴ and the Ha genes have been assigned to chromosome 6.²² A close relationship was observed between the human Ha subunit and the rat Ya and Yc subunits as well as their cDNA sequences (~80%).^{20,21} Southern blot hybridization of rat and mouse genomic DNAs, using a Ya cDNA probe, have indicated that Ya subunit is encoded by a multigene family.²⁵ In mouse genome, all detectable copies of Ya-equivalent mouse class-alpha genes have been located on mouse chromosome 9²⁵ and mapped on this chromosome in the vicinity of the d locus.^{26,27} Southern blot hybridization studies of rat genomic DNA, using 5' and 3' regions of Ya and Yc cDNA probes, have indicated the presence of multiple Ya and Yc genes in the rat genome, which was further confirmed by isolation of unique genomic fragments from a rat genomic library.²⁸

The Ya structural genes isolated from rat²⁹ and mouse^{18,30} span about 11 kb and contain

seven exons. In addition to a high homology between the mouse and rat Ya gene mRNA coding sequences, an extensive sequence conservation (70 to 80%) was observed in the 50 to 200 bp of introns at the exon-intron junctions as well as in the region beyond the cleavage-polyadenylation site of pre-mRNA of the two genes.¹⁸ A recently isolated and sequenced human GST Ha₁ subunit gene has a similar structure with the mouse and rat Ya genes: it spans a region of ~12 kb and contains seven exons.³¹ Because the 5'-flanking region of the human GST Ha₁ gene has not yet been characterized further upstream than the 300 bp of the proximal promoter, it is not known whether it contains *cis*-acting regulatory elements that would mediate a tissue-specific or inducible expression of this gene. Such regulatory elements have been located in the 5'-flanking regions of rat³² and mouse Ya gene^{33,34} and their structure and function will be discussed in following chapters.

B. GST Mu Gene Family

The cDNA clones, corresponding to subunits Yb₁, Yb₂, and Yb₃ of the rat GST Yb family have been isolated and characterized.³⁵⁻³⁹ A sequence comparison among these Yb cDNA clones shows about 80% identity in their protein coding region and a very high divergence in the 3'-nontranslated mRNA regions.³⁸ There is little sequence homology between Yb cDNAs and the Ya and Yc nucleotide sequences and no cross-hybridizations have been observed between the two GST families under moderately stringent conditions.^{14,16,37}

Southern blot hybridizations of rat genomic DNA with Yb cDNA probes present a pattern of complexity consistent with the presence of a multigene Yb family in the rat genome.³⁷ The isolation and characterization of three Yb genes, Yb₁, Yb₂, and Yb₄, have been described.^{39,40} The Yb₄ gene encodes a putative Yb₄ subunit that has not yet been purified from any rat tissue and there is no evidence that the Yb₄ gene is expressed at all. The Yb₃ gene has not yet been isolated but, through the use of the Yb₃ cDNA clone, it was demonstrated that

this gene is expressed primarily in the brain and very little in the liver.⁴¹ The structure of rat GST Yb₁, Yb₂, and Yb₄ genes is similar: they span about 5 kb, contain eight exons, and three out of seven introns are conserved to the extent of more than 88% nucleotide identity.³⁹ This latter observation has led to the assumption that a gene conversion mechanism may have played a role in the evolution of GST Yb genes.³⁹ Gene conversion within the germ-line was proposed as a mechanism important to the evolution of any gene family that maintains microdiversity in a set of otherwise very similar and functional molecules.⁴² The sequence identity in the introns of gene families is considered an evidence of the occurrence of gene conversion. Evidence was reported that germ-line gene conversion also occurs in the homologous region of two human GST mu-class genes.⁴³ The nucleotide sequences subject to gene conversion that include both intron and exon regions were also conserved between man and rat and, in the case of one human GST *mu* gene, were found to be capable of modulating promoter activity in transient-expression assays.⁴³ Thus, the unusual conservation across species of the intron sequences in gene conversion in the GST *mu* gene family seems to be due to the presence of regulatory elements within them.

Genetic loci for six human GSTs, GST1 through GST6, have been described.⁴⁴⁻⁴⁶ Three of them, GST1, GST4, and GST5, were shown to encode class-mu isoenzymes,^{44,46-48} whereas GST2 and GST3 represent Ha or alpha and pi subunit, respectively (for a nomenclature of human GSTs see Reference 49). The GST1 is a polymorphic locus for which three alleles have been described: a null allele, GST1-1, and GST1-2. Approximately 50% of the human population is homozygous for the null allele.^{45,50} This "deficiency" has been correlated with a lack of GST activity on *trans*-stilbene oxide and a possible increase in lung cancer incidence among smokers.^{51,52} The mRNAs corresponding to GST1-1 and GST1-2 alleles have been cloned.^{53,54} and their cDNAs were found to differ by a single base pair in the protein coding region to encode at residue 172 an asparagine in GST Ψ ⁵⁵ or a lysine in GST μ ⁴⁷

subunits. Genomic DNA hybridizations using a panel of DNAs from mouse-human somatic cell hybrids show that the Hb (*mu*) genes of human GSTs are located on three separate chromosomes: 1, 6, and 13. The GST μ (Ψ) gene, whose expression is polymorphic in the human population, was assigned to chromosome 13.⁵⁶ It was proposed that the GST μ (Ψ) gene was created by a transposition or recombination event and that the null phenotype may have resulted from a lack of DNA transposition as well as from a deletion of an inserted gene.⁵⁶ Additional human mu-class GST cDNA clones have been isolated: one encoding a muscle-specific GST subunit⁵⁷ and another expressed in brain and testis.⁵⁸ Presently, because regulatory 5'-flanking regions of GST mu-class genes have not yet been analyzed for tissue specificity or xenobiotic inducibility, there is no information about the regulation of expression of this GST subunit gene family.

C. GST PI Genes

cDNA clones encoding rat^{59,60} and human⁶¹ GST pi subunits have been isolated as well as the corresponding rat⁶² and human⁶³ structural genes. Both rat GST-P and human GST- π gene span about 3 kb, contain seven exons, and encode a 210 amino acid protein. Unlike other classes of GST subunits that are encoded by gene families, the pi-class subunits of both species are expressed from a single gene.^{62,63} The rat genome was also found to contain a number of processed-type pseudo genes.⁶²

D. Microsomal GST Genes

The cDNA clones of rat and human microsomal GST enzymes encoding a 154 amino acid protein have been isolated.⁶⁴ The two microsomal GSTs exhibit 95% similarity in amino acid sequences to each other and only limited sequence homology (from amino acids 57 to 63) to the cytosolic GST isoenzymes.^{64,65} This leads to the assumption that this form of GST is most likely a result of convergent rather than divergent evolu-

tion.⁶⁶ The microsomal GST is a single-or low-copy gene in both rat and human genomes and, by genomic blot hybridizations of DNA from a panel of mouse-human somatic cell hybrids, the human microsomal GST gene was assigned to chromosome 12.^{64,66}

III. REGULATION OF GST GENE EXPRESSION

A. Induction of GSTs in Animal Tissues

The cytosolic GSTs are a class of enzymes that are inducible by drugs, xenobiotics, food additives, and natural dietary components. Studies on the induction of cytosolic GSTs activities have been carried out by administration of various xenobiotics, 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), phenobarbital, 3-methylcholanthrene, *trans*-stilbene oxide, and many other compounds, to rats and mice followed by analysis of the enzyme levels in the different organs.⁶⁷⁻⁷¹ It was observed that the xenobiotics induce severalfold the activity of some GST subunits and that the increase in GST Ya subunit activity in rat liver is paralleled by an increase in the translational activity of the respective mRNA.⁷²⁻⁷⁵ The availability of cDNA cloned sequences of GST mRNAs and their use as specific probes in RNA blot hybridization and nuclear run-on experiments have enabled to demonstrate that the changes in the steady-state levels of rat liver GST Ya and Yb mRNAs induced by phenobarbital and 3-methylcholanthrene⁷⁶ are due to the transcriptional activation of the respective GST genes.⁷⁷ From similar experiments, the tissue-specific induction of murine class alpha, mu, and pi GST mRNAs by BHA was found to be due to increased rates of transcription.⁷⁸ The effects of inducers of drug metabolism such as BHA, phenobarbital, cafestol palmitate, 3-methylcholanthrene, *trans*-stilbene oxide, and ethoxyquin on the levels of the different mouse and rat hepatic GST isoenzymes indicate a differential gene regulation⁷⁹ and have allowed the detection of new inducible alpha- and mu-class GST subunits.^{80,81}

B. Induction of GSTs as a Mechanism of Chemoprotection

Induction of GSTs and other Phase II* drug-metabolizing enzymes,⁸² glucuronosyl transferases, and NAD(P)H: quinone reductase by a wide variety of structurally unrelated compounds is considered a major mechanism of protection against chemical stress and carcinogenesis (for reviews see References 83 and 84). In fact, a correlation exists between GST induction and a lower incidence of experimental cancer in rodents.⁶⁸⁻⁷¹ The induction of protective GST activity was found to be associated with an increase in RNA levels and enhanced rates of enzyme synthesis.⁷⁵ These observations, which suggest the important role of GST induction in the prevention of chemical carcinogenesis, have given the incentive for an extensive screening for the anticarcinogenic or chemoprotective activity of different natural and synthetic chemical agents^{85,86} (for a review see Reference 83). In this respect, GST induction was instrumental in the detection of a variety of natural and synthetic compounds able to confer protection against the effects of carcinogens in laboratory animals. The structural diversity of these inducers of GSTs and other drug-metabolizing Phase II enzymes is remarkable and includes different classes of chemical agents such as planar aromatic compounds (flavonoids, polycyclic aromatic hydrocarbons, and azo dyes), phenolic antioxidants, coumarins, aromatic isothiocyanates, thiocarbamates, 1,2-dithiol-3-thiones, barbiturates, indoles, etc.^{83,84} The puzzling feature of these chemical agents is that, despite their structural heterogeneity, they all share the ability to elevate in animal cells the activities of Phase II enzymes that inactivate the reactive electrophilic forms of carcinogens. It was observed, however, that a class of chemicals, the planar aromatic compounds (polycyclic aromatic hydrocarbons, azo dyes, flavonoids, dioxins) are inducers of Phase I enzymes (e.g., cytochromes P-450) as well as Phase II enzymes, whereas the

other types of chemoprotective agents are relatively selective inducers of Phase II enzymes.^{83,84} According to their enzyme induction patterns, the chemical inducers have been classified by Talalay et al.⁸⁴ into bifunctional inducers (planar aromatic compounds) that induce both Phase I and Phase II enzymes and monofunctional inducers (diphenols, thiocarbamates, isothiocyanates, 1,2-dithiol-3-thiones) that selectively induce only Phase II enzymes.

The molecular mechanisms by which the planar aromatic compounds induce the activity of Phase I cytochrome P₁-450 enzymes, which introduce by oxidation or reduction functional groups into xenobiotics, are well established (for reviews see References 87-90). Planar aromatics bind with high affinity to the cytosolic *Ah* receptor. Following a temperature-dependent activation step, the liganded *Ah* receptor is translocated into the nucleus where, by interacting with specific enhancer sequences, it induces the expression of selected cytochrome P₁-450 genes. The inducible expression of cytochromes P₁-450 by planar aromatics is mediated by multiple copies of xenobiotic responsive elements (XRE) containing the core sequence 5'-T-GCGTG-3' and located in the 5'-flanking regions of their genes.^{91,92} Because the induction of Phase II enzymes by planar aromatic compounds was observed to occur only in mouse strains and cultured hepatocytes with a functional *Ah* receptor, the regulation of these enzymes has been initially assumed to occur through the same mechanism as that regulating the genes of cytochrome P₁-450.⁸⁸ However, early results implying a direct participation of *Ah* receptor in induction of Phase II enzymes have not been convincing.⁹³⁻⁹⁵

Because GST expression is rapidly lost in cultured hepatocytes, Talalay et al.^{84,96-98} have concentrated their studies on the regulation of NAD(P)H:quinone reductase, an enzyme activity that is coordinately induced with GST in animal tissues and is still expressed in a number of hepatoma cell lines. Some quinone reductase

* Enzymes of xenobiotic metabolism have been classified into two groups⁸²: Phase I enzymes (e.g., cytochromes P₁-450) that introduce by oxidation or reduction functional groups into chemical compounds and Phase II enzymes that conjugate the functionalized compounds with endogenous ligands (e.g., glutathione, glucuronic acid).

inducers identified in cell cultures were also tested as inducers of quinone reductase and GST in mouse tissues. A systematic analysis of the induction of quinone reductase by planar aromatics (β -naphthoflavone, azodyes, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) and monofunctional inducers (diphenols, aromatic diamines, 1,2-dithiol-3 thione) was carried out by Talalay and collaborators⁹⁶⁻⁹⁸ using a murine hepatoma cell line (Hepa 1c1c7) and two mutants defective in either *Ah* receptor function (BP^{rc1})^{99,100} or in cytochrome P₁-450 activity, (c1).¹⁰¹ From these experiments, it was concluded that the signal for quinone reductase induction depends on the oxidative lability of the inducers⁹⁶ and that this induction by the redox labile compounds does not depend on a functional *Ah* receptor or cytochrome P₁-450 activity.⁹⁷ In contrast, induction of quinone reductase by planar aromatic compounds requires both a competent *Ah* receptor and a functional cytochrome P₁-450 enzyme.^{97,98} These observations have been corroborated also for the induction of GST expression from experiments in which the GST activity was monitored in parallel with that of quinone reductase and cytochrome P₁-450 in genetically defined mouse strains with high (C57BL/6J) and low (DBA/2J) affinity *Ah* receptors. From this apparently coordinate induction of Phase I and Phase II enzymes by planar aromatic compounds, Talalay et al.⁸⁴ have proposed the metabolic cascade model described in Figure 1. According to this model, the mechanism of Phase II enzyme induction by planar aromatics includes two steps: (1) induction of cytochromes P₁-450 via the *Ah* receptor and (2) conversion of the planar aromatics by the cytochrome P₁-450 activity into oxidation-reduction labile species, diphenols, aminophenols, quinones, which in turn signal the induction of Phase II enzymes. This model implies a unified mechanism for induction of Phase II enzymes by all classes of chemical compounds. The induction appears to depend on a redox signal generated by the oxidation-reduction lability of the inducing agents. The validity of this model for the GST induction by planar aromatics was recently supported by molecular studies on the regulation of GST Ya gene expression.^{102,103}

The model presented in Figure 1, however, does not exclude the possibility that complexes of planar aromatic compounds with *Ah* receptor could bind to specific regulatory sequences to activate directly the transcription of Phase II enzyme genes by a mechanism similar to that described for cytochromes P₁-450 genes.⁸⁸ Such a mechanism, which would require the presence of XRE sequences in the regulatory regions of the genes encoding Phase II enzymes, was also proposed.⁹⁷ According to this, poorly or nonmetabolizable bifunctional inducers such as TCDD¹⁰⁴ are expected to activate Phase II enzyme genes exclusively via the *Ah* receptor/XRE binding mechanism, whereas the metabolizable bifunctional inducers (e.g., β -naphthoflavone, 3-methylcholanthrene) could act also via the metabolic cascade, whereby the induced cytochromes P₁-450 activity converts these compounds into redox labile electrophilic metabolites resembling monofunctional inducers. The experimental evidence obtained for quinone reductase induction in Hepa1c1c7 wild type murine hepatoma and *Ah* receptor (BP^{rc1}) or cytochrome P₁-450 (c1) defective mutant cells by Prochaska and Talalay⁹⁷ support their model. Thus, monofunctional inducers increase quinone reductase activity to the same extent in mutant as in wild-type cells, whereas bifunctional inducers β -naphthoflavone and TCDD were inactive in the *Ah* receptor defective cells. In c1 mutant cells, however, the bifunctional inducers β -naphthoflavone and TCDD were found to be very weak inducers (1.2- to 1.6-fold) of quinone reductase activity. This finding is somehow difficult to explain because in the cytochrome P₁-450-defective c1 cells the bifunctional inducers (β -naphthoflavone, TCDD) should be able to induce quinone reductase via an *Ah* receptor/XRE-binding mechanism.

In a search for a common chemical signal among the various monofunctional inducers, a remarkable similarity between the structural features of substrates for GSTs and those required for Phase II enzyme induction was noticed.¹⁰⁵ Many inducers contain, or acquire by metabolism, the structure of a Michael reaction acceptor characterized by olefinic or acetylenic bonds

INDUCERS OF PHASE I AND II

INDUCERS OF PHASE II

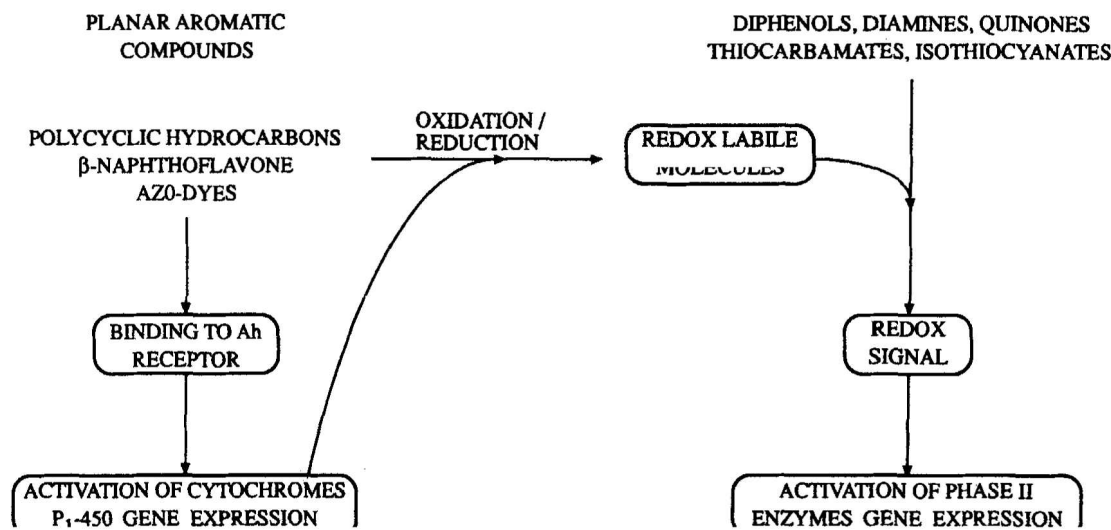


FIGURE 1. The relation between the mechanisms of induction of Phase I and Phase II drug-metabolizing enzymes. (Adapted from Talalay, P., De Long, M. J., and Prochaska, H. J., *Cancer Biology and Therapeutics*, Cory, J. G. and Szentivanyi, Eds., Plenum, 1987, 197.)

that are rendered electrophilic by conjugation with electron-withdrawing groups.¹⁰⁵ One of the basic structural elements of GST substrates is a carbon-carbon double bond activated by an adjacent electron-withdrawing carbonyl group. The glutathione conjugation catalyzed by the GST appears to be a thiol addition to an α,β -unsaturated carbonyl compound that involves a nucleophilic attack on the electrophilic β carbon similar to a classic Michael reaction.¹ Both induction and substrate activity therefore appear to require the presence of an electrophilic Michael acceptor function. Thus, many Phase II enzyme inducers such as α,β unsaturated esters, aldehydes, ketones, lactones, quinones, nitro alkenes, sulfones, ethacrynic acid, etc. are also substrates for GSTs. The potency of inducers of quinone reductase was found to parallel their efficiency as substrates for GSTs.¹⁰⁶ These findings suggest that the GSTs or their subunits may participate in the mechanism of Phase II enzyme induction by the electrophilic compounds.¹⁰⁶ This relation may only indicate, however, that both processes require the presence of electrophilic centers.

C. GST Ya Genes

1. Induction of GST Ya Gene in Hepatoma Cells

Because expression of GSTs is normally extinct in cultured cells, the two differentiated rat hepatoma cell lines, FAO and H4II,¹⁰⁷ which still express a large number of liver-specific functions including phenobarbital and 3-methylcholanthrene-inducible forms of cytochromes P₁450,¹⁰⁸ have proven instrumental for the study of endogenous GST Ya gene induction. In these cells, it was possible to show by RNA blot analysis using GST Ya cDNA cloned sequences, the induction of GST Ya mRNA by phenobarbital and planar aromatic compounds (3-methylcholanthrene and β -naphthoflavone),³⁴ as well as by the oxidation labile *tert*-butyl hydroquinone.¹⁰⁹ A number of inducers of chemical stress such as arsenite, arsenate, and heavy metals (Zn, Cd, Pb), that cause the synthesis of heat-shock proteins and hydrogen peroxide were also found to induce increased levels of GST Ya mRNA in rat hepatoma H4II cells¹¹⁰ (Figure 2). A variable 2- to 8-fold increase in

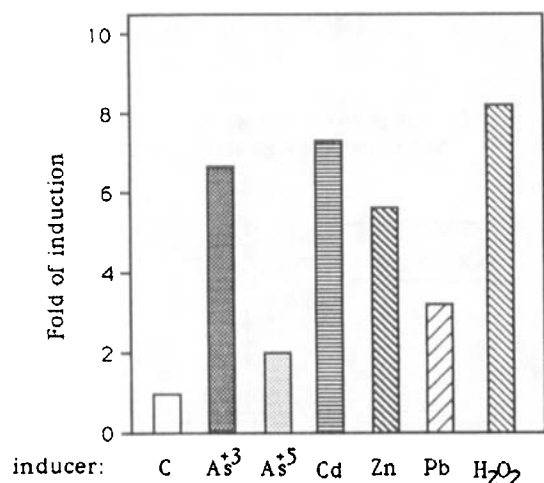


FIGURE 2. Induction of GST Ya mRNA by stress-inducing agents. Total RNA extracted from hepatoma H4II cells, untreated (C) or exposed for 3 h to 25 μ M sodium arsenite (As³⁺), 50 μ M sodium arsenate (As⁵⁺), 5 μ M cadmium chloride (Cd), 200 μ M zinc chloride (Zn), 100 μ M lead nitrate (Pb), or 500 μ M hydrogen peroxide (H₂O₂), was fractionated by agarose/formaldehyde gel electrophoresis and analyzed by RNA blot hybridization with a ³²P-labeled Ya cDNA probe. The autoradiogram was quantitated by densitometry. (From Pinkus, R., M. Sc. thesis, Feinberg Graduate School, Weizmann Institute of Science, Rehovot, 1992.)

GST Ya mRNA levels was observed for the different chemicals. The induction of GST Ya mRNA by *tert*-butyl hydroquinone reaches a maximal level after 4 to 8 h of cell exposure to the xenobiotic and requires on-going protein synthesis because it is completely inhibited by the presence of cycloheximide (Figure 3). This suggests that induction of GST Ya mRNA by the electrophilic inducer may involve the synthesis of a regulatory protein responsible for the transcriptional activation of the GST Ya gene.

2. Cis-regulatory Elements for Basal and Inducible Expression

Studies on the regulatory elements controlling the basal and xenobiotic-inducible expression of GST Ya subunit genes have been conducted on the highly homologous rat²⁹ and mouse^{18,33} GST Ya gene. The 5'-flanking regions of these genes have been screened for *cis*-regulatory elements with respect to tissue-specific and inducible expression. The functional

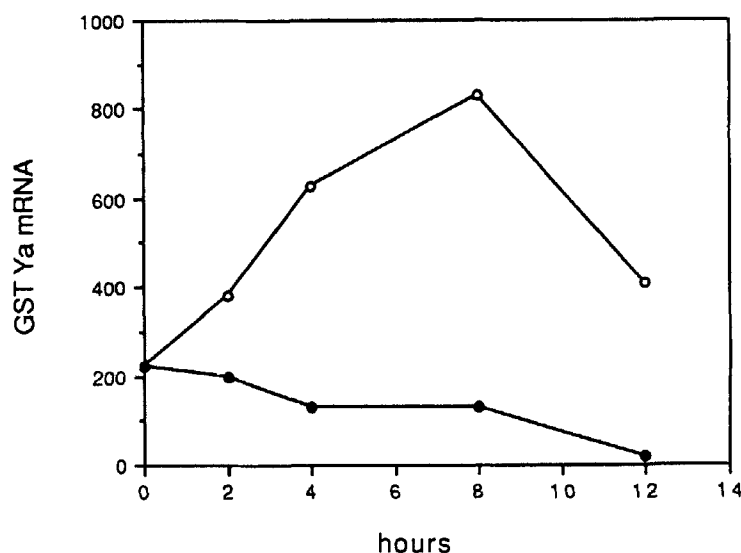


FIGURE 3. Effect of cycloheximide on the induction of GST Ya mRNA by *tert*-butylhydroquinone. Total RNA, extracted from hepatoma H4II cells exposed to 30 μ M *tert*-butylhydroquinone in the absence (open circles) or presence (closed circles) of 60 μ g/ml cycloheximide, was fractionated by agarose/formaldehyde gel electrophoresis and the GST Ya mRNA levels measured by RNA blot hybridization with a ³²P-labeled Ya cDNA probe. The hybridization autoradiogram was quantitated by densitometry. (From Bensimon, A., Ph.D. thesis, Feinberg Graduate School, Weizmann Institute of Science, Rehovot, 1992.)

analysis of the 5'-flanking region was carried out by measuring its ability to promote expression of the bacterial chloramphenicol acetyl transferase (CAT) reporter gene. Chimeric genes, constructed through the ligation of DNA fragments from the 5'-flanking region to a minimal promoter of the respective GST genes fused to the CAT coding sequence, were transfected for transient expression into human, rat, or mouse hepatoma cells. Generally, the CAT activities directed by the different 5'-flanking sequences have been monitored before and after exposure of the transfected cells to different classes of chemical inducers.

The 5'-flanking region of the mouse GST Ya gene contains a single positive regulatory element responsible for the basal as well as the inducible expression of this gene by xenobiotic compounds.¹⁰² The regulatory element, located between nucleotides -754 and -714 upstream from the initiation of transcription start, was found to be responsive to both planar aromatic (β -naphthoflavone, 3-methylcholanthrene, TCDD) and to electrophilic (*tert*-butylhydroquinone, -dimethylfumarate, and *trans*-4-phenyl-3-buten-2-one) compounds.¹⁰²

The finding of a common responsive element in the mouse Ya gene for both planar aromatic and electrophilic inducers raised the question of the different mechanisms proposed for the GST gene activation by the two classes of inducers.⁹⁷ First, the absence of a XRE sequence from the 41 bp (-754 to -714) Ya gene regulatory element excludes a mechanism of induction involving direct binding of an Ah receptor-aromatic compound complex similar to the transcriptional activation of cytochrome P₁-450 genes.⁸⁸ Second, transfection experiments of 5'-flanking region Ya-cat fusion gene constructs into murine hepatoma cell mutants defective in either the Ah receptor (BP₁c1 mutant), or in the cytochrome P₁-450 gene (c1 mutant) have shown that induction by planar aromatic but not by electrophilic inducers required a functional Ah receptor and cytochrome P₁-450 activity.¹⁰² From this, it was concluded that the planar aromatic compounds have to be metabolized by the cytochrome P₁-450 enzymes to acquire the electrophilic properties responsible for GST Ya gene induction. These findings support the metabolic

cascade model proposed by Prochaska and Talalay^{84,97} for Phase II enzyme induction by planar aromatic compounds (see Figure 1). The presence of an electrophilic center or an oxidation lability, which was described as a characteristic feature of the monofunctional inducers of Phase II enzymes,¹⁰⁵ thus appears to be an exclusive requirement for the activation of the regulatory element of mouse Ya gene.¹⁰² Because of this exclusive activation by an electrophilic or redox signal, the -754 to -714 regulatory sequence of the mouse Ya gene was referred to as an electrophile-responsive element, EpRE.¹⁰² An EpRE-containing 41 bp oligonucleotide (-754 to -714) ligated to the -187 site of the Ya gene promoter was found to confer upon it an increase in basal activity and inducibility by a wide range of chemical agents (Figure 4). The basal activity augmented synergistically with the number of EpRE copies.¹⁰²

The fact that TCDD, which is considered a non- or slow-metabolizable compound,¹⁰⁴ has induced CAT activity (albeit only by a factor of 1.5- to 1.8-fold) via the same EpRE regulatory element as the other planar aromatics¹⁰² is puzzling. It may be assumed that a limited metabolism of TCDD is sufficient to produce the redox signal required for Ya gene induction or, alternatively, that such a signal may be acquired by a pathway independent of TCDD metabolism.

Deletion analysis studies of the 5'-flanking region of a rat GST Ya gene²⁹ have revealed a *cis*-regulatory element between nucleotides -722 to -682 very similar in structure to the EpRE of the mouse Ya gene.¹¹¹ Because of its activation by a phenolic antioxidant such as *tert*-butylhydroquinone this regulatory element was defined as an antioxidant-responsive element (ARE), although it was found to be equally well induced by the planar aromatic β -naphthoflavone.^{103,112} A consensus core sequence 5'-puGTGACNNNGC was proposed for ARE.¹¹² In contradistinction with the mouse Ya gene, the 5'-flanking region of rat GST Ya gene was found to include, between nucleotides -908 to -899, a XRE sequence identical to that found in the planar aromatic-responsive cytochromes P₁-450 genes.^{111,113} Insertion of this XRE sequence in front of the minimal rat GST Ya promoter resulted in a construct that was exclu-

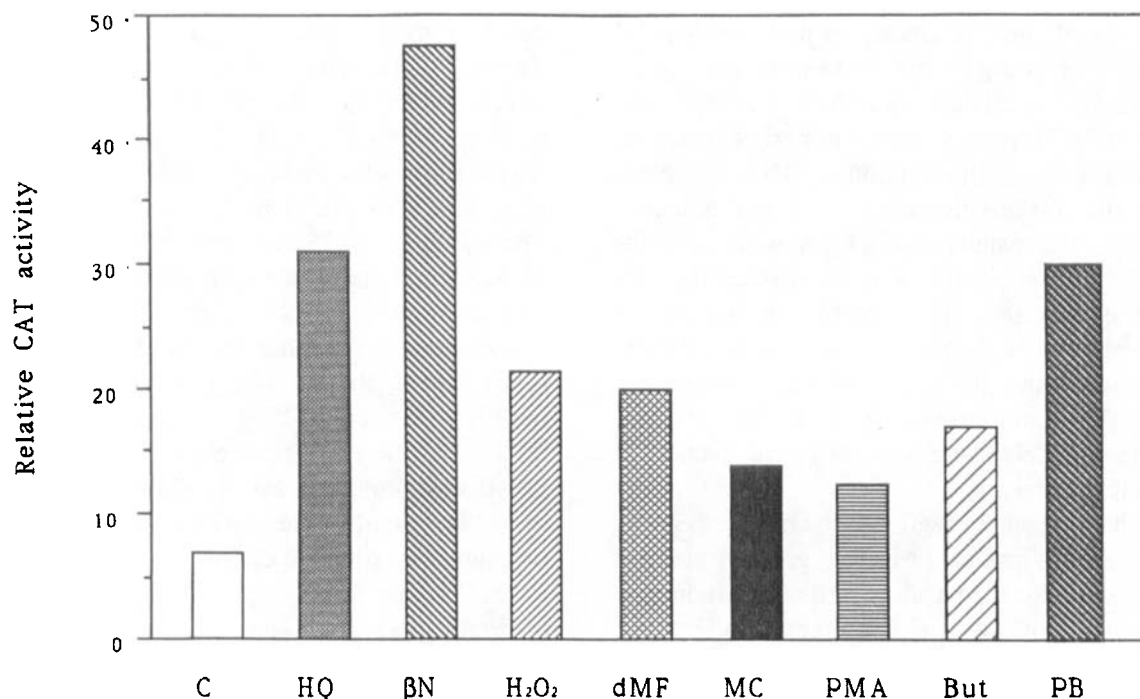


FIGURE 4. Induction of EpRE Ya-cat gene construct by chemical agents. Hepatoma HepG2 cells transfected with EpRE Ya-cat plasmid DNA were untreated (C) or exposed for 16 h to 30 μ M *tert*-butylhydroquinone (HQ), 50 μ M β -naphthoflavone (β N), 500 μ M H₂O₂; 30 μ M dimethylfumarate (dMF), 50 μ M 3-methylcholanthrene (MC), 100 ng/ml PMA, or 6 h to 2 mM phenobarbital (PB). CAT activities were measured in cell extracts and normalized to endogenous β -galactosidase activity expressed from a cotransfected RSV-gal plasmid. (Adapted from References 102, 120, and 103.)

sively responsive, about 3-fold, to planar aromatic inducer β -naphthoflavone. The presence of XRE (−908 to −899) upstream the ARE (−722 to −622) in the 5′-flanking region of the rat GST Ya gene, however, does not seem to contribute additively to the maximal responsiveness of this gene to planar aromatic inducers, because a deletion of the XRE did not affect the levels of basal or inducible expression of this gene.^{111,113} The XRE sequence was, however, indispensable for the activation of the rat Ya gene promoter constructs in HepG2 cells by a poorly metabolizable inducer such as TCDD. Surprisingly, the same XRE-Ya gene promoter construct was not inducible by the planar aromatic compounds when transfected in cytochrome P₁-450 enzyme-defective c1 hepatoma cells,¹⁰³ despite the fact that these cells are considered to have a normal Ah receptor.

3. Tissue-specific Regulatory Elements

The GST Ya subunit is a protein expressed preferentially in the liver and, to a smaller extent, also in the kidney. Therefore, in addition to DNA sequences that control the response of xenobiotics the Ya gene expression may also involve tissue-specific regulatory elements recognized by specific *trans*-acting factors. Several regulatory elements, presumably involved in the constitutive and hepatocyte-specific expression, have been identified in the 5′-flanking regions of mouse and rat GST Ya genes by nuclear protein-DNA interactions.^{34,113}

A putative binding site for HNF-1, a transcription factor involved in the control of expression of various liver specific genes,^{114–116} was detected at position −860 from the start of transcription of the rat GST Ya gene.^{111,113} An addi-

tional binding site for a hepatocyte-enriched factor, HNF-4,¹¹⁷ was detected between nucleotides -810 to -720.¹¹³ Binding experiments with rat liver nuclear extracts and DNase footprinting analysis of 5' upstream sequences of the mouse GST Ya gene, have enabled the detection of recognition sites for the ubiquitous NF-1 factor^{118,119} at positions -776 and -135 from the start of transcription.^{34,102} Because these putative regulatory sites have been detected in the GST Ya genes by binding of proteins from crude nuclear extracts and the respective purified factors have not yet been shown to interact with the detected sequences, the identity of the regulatory factors cannot be conclusive.

4. Transactivation of the EpRE of GST Ya Gene

The electrophile-inducible enhancer (EpRE) of the mouse GST Ya gene was found to contain a 9 bp direct repeat sequence, TGACA(A/T)(A/T)GC, spaced by 6 bp¹²⁰ (Figure 5A). The two adjacent 9 bp motifs are actually variants of the consensus Activator Protein-1 (AP-1) binding site, TGACTCA, originally identified as the phorbol 12-myristate 13-acetate (PMA) responsive element.¹²¹⁻¹²³ The inducible transcription enhancer AP-1 binding site, observed in the promoter region of several PMA-inducible genes,¹²¹⁻¹²³ was shown to be regulated by the binding of protein products of *c-fos* and *c-jun* genes forming the Fos/Jun heterodimeric (AP-1) complexes (for review see Reference 124). The functional role of the AP-1 like sequences in EpRE was analyzed by ligation of synthetic oligonucleotides representing each of these sequences at the -187 site of GST Ya gene promoter fused to the CAT coding sequence. The constructs were transfected for expression into HepG2 hepatoma cells. It was demonstrated that, separately, the first AP-1-like site (TGACATTGC) did not confer basal or inducible activity, whereas the second AP-1-like site (TGACAAAGC) increased to a small extent the basal activity (~4-fold) and conferred only a low level of inducibility (~1.5-fold). At close distance, however, the two AP-1-like binding sites increased considerably the basal activity and con-

ferred xenobiotic inducibility.¹²⁰ From these observations, which are illustrated in Figure 5B, it appears that the two AP-1-like sequences act synergistically to form the xenobiotic inducible EpRE of GST Ya gene. The synergistic effect of multiple AP-1-like sequences on Ya gene expression is demonstrated in Figure 5C, which compares the CAT activities expressed from minimal promoter Ya-cat constructs containing one, two, or five AP-1-like TGACAAAGC sequences, respectively. In the absence of inducer, there is an increase in the level of CAT expression that correlates with the number of AP-1-like sites. About 20- and 50-fold increases in basal activity were observed for constructs containing two and five AP-1-like sequences, respectively, when compared with a single one. The inducibility, however, was observed to decrease with the increase in basal activity and the induction ratio conferred by five is lower than that conferred by two AP-1-like sequences¹²⁰ (see Figure 5C). These observations have suggested that multiple copies of AP-1 sites may reduce the sensitivity to the small increases in abundance or activity of the binding factors caused by the xenobiotic induction.

It was reported that the AP-1 binding site is recognized by the same transcription factor, AP-1 complex, before and after PMA induction.¹²² A point mutation at position 1 of AP-1 site that decreases AP-1 complex binding and abolishes both basal and inducible activity of the AP-1 binding site¹²² is expected to affect similarly the activity of the AP-1-like site of the EpRE. Indeed a single base conversion T→A at position 1 of the AP-1-like motif TGACAAAGC of the EpRE completely abolished the basal and inducible activity of the site independent of the number of the mutant AGACAAAGC copies.¹²⁰

Protein-DNA binding experiments have indicated an increase (5- to 10-fold) in EpRE footprinting activity in nuclear extracts from hepatoma cells exposed to *tert*-butylhydroquinone.¹⁰² Several lines of evidence have indicated that the AP-1 complex is, indeed, the EpRE recognition factor whose abundance or activity is increased after treatment of hepatoma cells with electrophilic agents. Protein-DNA binding experiments using gel-shift assays have shown that *in vitro*, synthesized c-Fos and c-Jun proteins inter-

act cooperatively with the EpRE. These experiments have indicated that, similar to an AP-1 site, the formation of a gel-retarded protein-DNA complex by EpRE requires both Fos and Jun proteins¹²⁰ (Figure 6A). The transactivation of the EpRE by Fos and Jun proteins was demonstrated by cotransfection of an EpRE Ya-cat construct with c-Jun and c-Fos expression vectors into undifferentiated embryonal carcinoma F9 cells. In these cells, which lack endogenous AP-1 activity,^{125,126} the EpRE had no basal or inducible activity. A 100-fold increase in the EpRE basal activity was observed only in the presence of both exogenous c-Jun and c-Fos.¹²⁰ These experiments indicate that activation of EpRE cannot be achieved by c-Jun homodimers alone and requires the Fos/Jun heterodimeric complex (Figure 6B). In a titration experiment, a steady increase in EpRE activity in F9 cells is proportional to the amounts of exogenous c-Fos and c-Jun expression vectors introduced in these cells (Figure 7).

D. GST Pi Genes

The regulatory mechanisms involved in the expression of GST subunits of the pi-class were studied using isolated genes from rat, GST P,⁶² and man, GST π .⁶³ The GST pi-class is of particular interest because its expression has been associated with carcinogenesis¹²⁷⁻¹³¹ and drug resistance.¹³²⁻¹³⁵ Placental GST-P (or GST 7-7), the only GST of the pi-class in rats, is absent from the normal rat hepatocytes and is specifically induced at an early stage of chemical hepatocarcinogenesis.¹²⁷⁻¹³¹ The GST-P becomes expressed constitutively and is present at high levels in preneoplastic hepatocytes and hepatocellular carcinomas and appears to be a valuable marker for hepatocellular carcinogenesis in rat.¹²⁷⁻¹³¹ The progress from GST-P-negative normal adult rat liver to highly positive preneoplastic and neoplastic liver, observed by using anti-GST-P antibody for the detection of the protein, was shown to be paralleled by the relative abundance of the mRNA.¹³⁶ Thus, GST-P mRNA, which is absent from normal as well as the regenerating rat liver, becomes highly abundant in hyperplastic nodules, chemically induced hepatocellular carcinomas, and cell lines derived from hepatomas.^{136,137}

The high incidence of derepression of the GST-P gene expression in the preneoplastic lesions and hyperplastic nodules,¹³⁰ irrespective of the type of carcinogen used as inducer, has suggested that the mechanisms regulating the expression of this gene may be closely related to the neoplastic transformation of the rat liver.¹³⁸ The GST-P gene appears to be responsive to *ras* oncogenes, as an increase in GST-P expression was observed in rat liver epithelial cells following transformation with *N-ras* gene¹³⁶ and *v-H-ras* or *v-raf* genes.¹³⁹ In these cells, an increase in GST-P mRNA levels was also observed following the expression of an inducible *Ha-ras* gene introduced by transfection.¹⁴⁰

The GST-P gene expression appears to be developmentally regulated. The relative high levels of GST-P mRNA observed in the fetal liver decrease rapidly after the first week of postnatal development to become undetectable in the adult liver.¹⁴¹ In contrast, the GST-P mRNA levels were found to increase in the adult brain.¹⁴¹ It should be observed that the GST-P mRNA is normally present in a wide range of tissues such as kidney, lung, testis, heart, spleen, and placenta.¹⁴² The GST-P, in contrast to other GSTs (e.g., GST Ya subunit), is not inducible in the normal liver by short-term administration of drugs, carcinogens, or tumor promoters. Exceptionally, lead nitrate was observed to induce GST-P in rat liver.^{137,143} Hepatocytes in primary culture, however, exhibit changes in GST gene expression: the expression of class-alpha GST decreases, whereas that of class-mu GST and GST-P are markedly increased.¹⁴⁴⁻¹⁴⁷ Hepatocytes, on continuous culture in media containing serum, were found to express low levels of GST-P mRNA, which were greatly increased by induction with aflatoxin metabolites¹³⁶ or phenobarbital.¹⁴⁵ In addition, hepatocytes cultured in medium supplemented with insulin accumulated GST-P mRNA in the first 24 h and this effect was blocked by glucocorticoid dexamethasone.¹⁴⁶ Recent data show that GST-P is significantly induced in serum-free primary cultures of rat hepatocytes by epidermal growth factor (EGF) or insulin.¹⁴⁸ This suggests that the spontaneous induction of GST-P observed in hepatocyte cultures containing serum may be due to the presence of hormones and growth factors in the added serum. The inducible effects of EGF

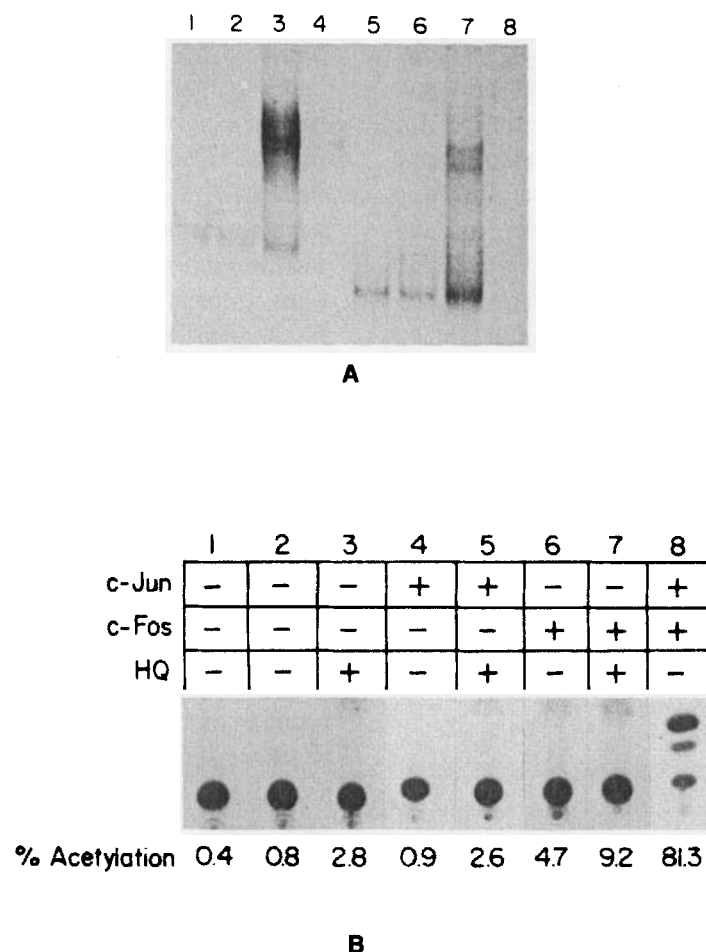


FIGURE 6. Binding and transactivation of EpRE by Fos and Jun proteins. **(A)** Gel shift assay for binding of *in vitro* synthesized Fos and Jun proteins. c-Jun (lanes 1 and 5), c-Fos (lanes 2 and 6), c-Jun + c-Fos (lanes 3 and 7), and c-Jun + c-Fos in the presence of 100 × molar excess of unlabeled EpRE oligonucleotide (lanes 4 and 8). Lanes 1 to 4 represent binding to a ³²P-labeled AP-1 consensus oligonucleotide and lanes 5 to 8 represent binding to the ³²P-labeled EpRE oligonucleotide. **(B)** Transactivation of EpRE by Fos and Jun. Plasmid constructs -187 Ya-cat (lane 1) and EpRE Ya-cat were cotransfected with various combinations of 5 µg RSV-c-Jun and RSV-c-Fos into embryonal carcinoma F9 cells followed by exposure for 16 h to 30 µM *tert*-butylhydroquinone (HQ) were indicated. (Experimental details are in Friling, R. S., Bergelson, S., and Daniel, V., *Proc. Natl. Acad. Sci. U.S.A.*, 89, 668, 1992.)

and insulin on GST-P gene expression in primary hepatocytes were shown to be associated with an enhanced expression of *c-jun* and *c-fos*.¹⁴⁸ This finding supports an involvement of Fos and Jun transcription factors in the regulation of GST-P expression and is consistent with the increased

levels of *c-jun* mRNA that accompany that of GST-P mRNA in rat liver preneoplastic foci, hyperplastic nodules, and hepatocellular carcinomas.¹⁴⁹

The molecular mechanisms controlling the expression of GST-P during hepato carcino-

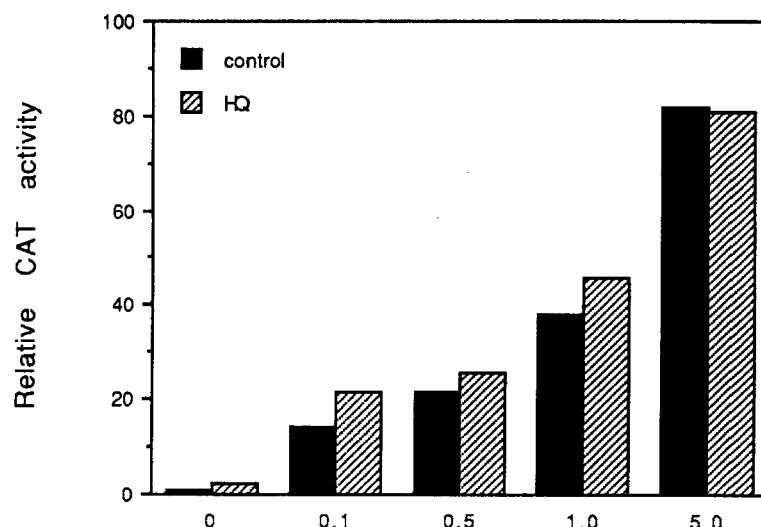


FIGURE 7. Increase in EpRE activity as a function of c-Fos/c-Jun. The EpRE Ya-cat construct was cotransfected together with increasing amounts (0.1 to 5 μg) of RSV-c-Jun + RSV-c-Fos into F9 cells in the absence (control) or presence of 30 μM *tert*-butylhydroquinone (HQ). Because RSV promoter is not induced by HQ, the marginal activation observed in the presence of HQ is due to the induction of an endogenous activity in F9 cells. (Experimental details as in Friling, R. S., Bergelson, S., and Daniel, V., *Proc. Natl. Acad. Sci. U.S.A.*, 89, 668, 1992.)

genesis have been investigated extensively by Muramatsu and colleagues.^{150–153} The analysis of the 5'-flanking sequence of the rat GST-P gene has revealed a number of *cis*-acting regulatory DNA elements: in the proximal promoter, at position –61 from the initiation of transcription site, a consensus sequence AP-1 binding site TGACTCAGC together with a GC box, at position –40, contribute to the basal level of GST-P gene expression.^{150–152} A stretch of DNA sequences containing negative elements acting as a silencer to repress gene expression was detected at position –400 upstream the initiation of transcription site.^{154,155} A DNA element, GPEII at position –2.2 kb, containing two SV-40-like and one polyoma-like enhancer sequences and, at position –2.5 kb, an enhancer termed GPEI was found to mediate the PMA inducible expression of the GST-P gene.^{150–152} The GPEI inducible enhancer of GST-P is similar in structure to the EpRE inducible enhancer described in mouse GST Ya gene¹²⁰; it contains two adjacent AP-1-like binding sites that separately have no activity but that act synergistically to confer

a strong transcriptional enhancing activity on a GST-P minimal promoter.¹⁵³ The GPEI enhancer was shown to bind a HeLa cell nuclear factor that was identified as the AP-1 binding activity by gel-shift analysis using competitor oligonucleotides containing a consensus AP-1 site sequence or GPEI site point mutants that abolish binding.¹⁵¹ In addition, the GPEI, because of the synergistic effect of its two adjacent AP-1-like binding sites, exhibited a relatively high activity in F9 cells despite the low levels of Fos and Jun proteins in these cells. Nevertheless, the GPEI enhancer activity was further stimulated in F9 cell by the Fos/Jun heterodimeric complex obtained from cotransfection with both *c-fos* and *c-jun* expression vectors.¹⁵³

The silencer region of GST-P, located between nucleotides –396 to –140, was demonstrated to include multiple *cis*-elements that act cooperatively to exert a negative effect on gene expression.¹⁵⁴ At least three nuclear *trans*-acting factors were found to bind to the GST-P silencer region. One of them, silencer Factor B (SF-B), was cloned and found to be similar or

identical to an IL-6 inducible *trans*-activator LAP/IL6-DBP.¹⁵⁵

The GST- π gene-encoding human GST-pi subunit was sequenced^{63,156} and the 5'-flanking region was analyzed for DNA elements that regulate its expression.¹⁵⁷⁻¹⁵⁹ These studies bring evidence that the control of expression of GST- π differs markedly from that of its rat GST-P analog subunit.¹⁵⁷ GST- π activity is absent from normal human hepatocyte and, unlike rat GST-P, is not expressed in hepatocellular carcinoma.¹³⁷ GST- π levels are increased in human malignant disease and in some human tumor cell lines that have acquired drug resistance to antitumor drugs.^{131,134,137,160} The expression of GST- π , however, unlike that of its rat counterpart GST-P subunit, is not elevated in human cells expressing *ras* (c-Ha-*ras* or N-*ras*) oncogenes and there is no correlation between expression of activated *ras* and the expression of GST- π mRNA.¹⁵⁷ In addition, treatment of HeLa cells, HepG2 cells, or a lung carcinoma line GLC8 with PMA failed to alter the steady state levels of endogenous GST- π mRNA.¹⁵⁷ The difference between the control of expression of the pi-class GSTs in rat and man, which may account for the different patterns of expression of these enzymes in the hepatocellular carcinomas of the two species, is probably due to a structural difference in the regulatory 5'-flanking region of the two genes. Thus, the GPEI enhancer, located 2.5 kb upstream the transcription start site of the rat GST-P gene, which is responsible for the inducible expression of this gene by PMA¹⁵⁰ and probably for the responsiveness to *ras* oncogene, is absent from the same position in the human GST- π gene¹⁵⁸ as well as from the entire 6 kb of 5'-flanking region of this gene.¹⁵⁷ Both rat GST-P and human GST- π genes contain a consensus sequence AP-1 binding site in the proximal promoter at position -60 that is not inducible by PMA but is essential for basal level of promoter activity.^{150,158} The human GST- π gene contains an additional positive *cis*-acting DNA element downstream from the transcription start site between nucleotides +8 and +72 that is required for the maximal basal activity of the promoter.¹⁵⁹ A closer examination of these sequences reveals the presence of an additional AP-1 binding site,

TGAGTACGC, between nucleotides +35 and +41 that is absent from the rat GST-P gene.

IV. THE ROLE OF FOS/JUN (AP-1) COMPLEX IN THE REGULATION OF GST GENE EXPRESSION

A. Induction of AP-1 Activity by Chemical Agents

Recent studies have revealed that transcriptional regulatory proteins Fos and Jun, the protein products of *fos* and *jun* protooncogenes, are induced by environmental signals and function in signal transduction processes (for reviews, see References 124 and 161). They play a role in coupling short-term extracellular signals to changes in gene expression in the nucleus by interacting with specific DNA-binding sites in target genes. In addition, *fos* and *jun* are cellular "immediate-early" genes and, as such, are rapidly induced in resting cells by mitogens, which suggests that they may be involved in the initiation of cell cycling.¹⁶²⁻¹⁶⁴ In the majority of cell types, the basal level of expression of *fos* and *jun* genes is relatively low. However, the rapid and transient induction of expression of Fos- and Jun-related proteins by a great variety of extracellular stimulatory agents that promote cell proliferation, differentiation, and neuronal excitation enables these proteins to modulate their DNA-binding activities and to mediate specific alterations in gene expression.^{162,165-167} Following induction, the synthesized Fos and Jun protein products are translocated into the nucleus where they form homodimeric and heterodimeric complexes through a structure known as the leucine zipper.¹⁶⁸ These associations are not limited to c-Fos and c-Jun but are also observed among other members of the Jun (Jun B,¹⁶⁹ Jun D¹⁷⁰) and Fos (Fos B,¹⁷¹ Fra 1,¹⁷² Fra 2¹⁷³) families. The protein dimers that constitute the AP-1 complex activate transcription of target genes containing AP-1 binding sites having the consensus DNA sequence TGACTCA in their promoters (Figure 8). While c-Jun homodimers were reported to be able to bind to AP-1 sites

and activate transcription,¹²⁶ the Fos proteins are active only as heterodimers with any of the Jun proteins. The cooperative formation of heterodimers between Jun and Fos proteins significantly enhances both AP-1 binding and transcriptional activation of AP-1 responsive genes.^{169,174-179}

The AP-1 binding site, originally identified in the control regions of viral and cellular genes whose transcription is induced by treatment of cells with tumor-promoting phorbol ester PMA,^{122,123,180-182} is present in a large number of genes, many of which may not be induced by treatment with this agent.^{150,158,183,184} The expression of *fos* and *jun* is induced by treatment of cells with a wide range of stimulatory agents, which in addition to phorbol ester PMA, include serum,¹⁶³ growth factors,^{164,185-187} calcium ionophore,^{188,189} activation of potassium channels or membrane depolarization,^{190,191} heat shock,¹⁹² neurotransmit-

ters,^{193,194} H₂O₂,^{195,196} UV,^{195,197} and X-ray¹⁹⁸ radiation (for reviews see References 124, 166, 167). In addition, AP-1 activity is also induced by neoplastic transformation^{199,200} as well as the transient expression of transforming oncogenes such as *ras*, *src*, and *mos*.^{201,202}

Recent studies indicate that Fos and Jun transcription factors are also involved in the cellular response to chemical stress. Their induction by a variety of structurally unrelated chemical agents mediates the activation of GST Ya gene.^{203,204} Thus, in addition to phenobarbital,²⁰³ dioxin (TCDD),²⁰⁵ and tumor promoter PMA, chemical compounds such as planar aromatic hydrocarbons (e.g., β -naphthoflavone, 3-methylcholanthrene), *tert*-butylhydroquinone, *trans* 4-phenyl 3-buten-2-one, and H₂O₂ all induce an increase in AP-1 binding activity²⁰⁴ (Figure 9).

The inducible expression of AP-1 binding activity was shown to involve an activation of

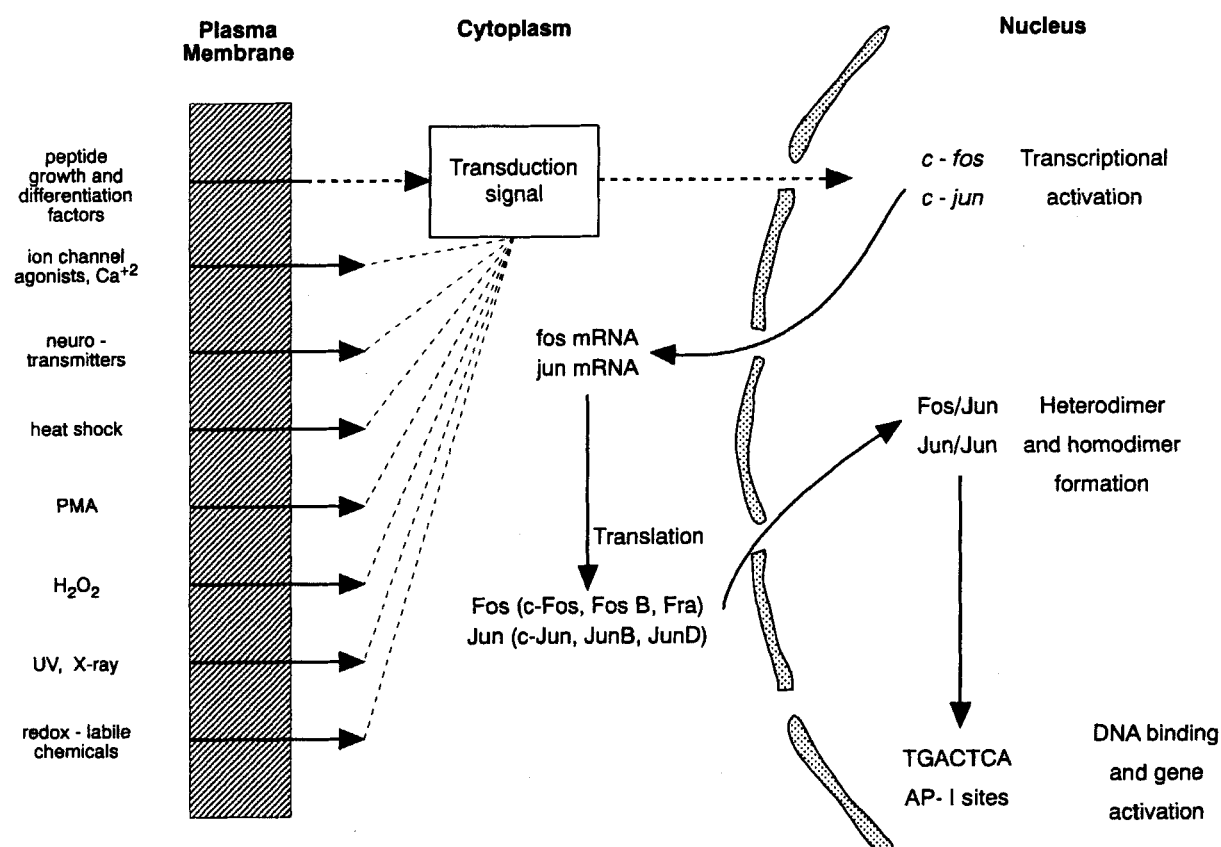


FIGURE 8. Scheme for the induction of *c-fos* and *c-jun* gene expression by various extracellular stimuli. The synthesized Fos/Jun (AP-1) complexes regulate the expression of target genes containing AP-1 sites.

preexisting transcription factors, such as Fos/Jun complex and serum responsive factor (SRF), followed by induction of *c-fos* and *c-jun* expression.¹⁶⁷ Although the initial step of activation of *c-fos* and *c-jun* gene transcription is a

expression of GST genes offers a mechanistic approach for an understanding of the molecular processes involved. The modulation of the AP-1 activity should account for the changes induced by growth factors, hormones, chemical carcino-

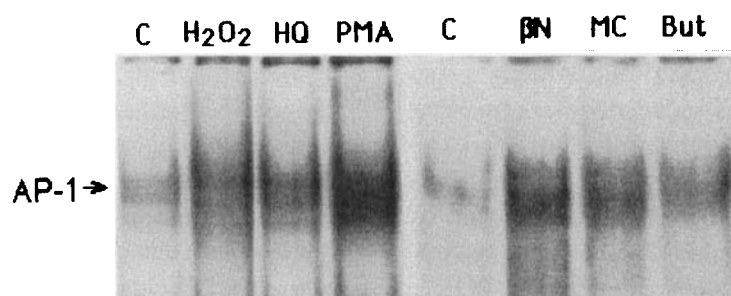


FIGURE 9. Induction of AP-1 binding activity by various inducers of GST Ya gene. Nuclear extracts were prepared from HepG2 cells untreated (C) or exposed for 3 h to 30 μ M *tert*-butylhydroquinone (HQ), 100 ng/ml PMA, 500 μ M H_2O_2 , 50 μ M β -naphthoflavone (β N), 50 μ M 3-methylcholanthrene (MC), or 30 μ M *trans*-4 phenyl-3-buten-2-one (But) and were analyzed for AP-1 binding activity by electrophoretic mobility shift using a 32 P-labeled AP-1 oligonucleotide probe. (Data adapted from Daniel, V., Bergelson, S., and Pinkus, R., *Structure and Function of Glutathione S-Transferases*, Tew, K. D., Pickett, C. B., Mantle, T. J., Mannervik, B., and Hayes, J., Eds., CRC Press, Boca Raton, FL, 1993 (in press).)

translation-independent event, the subsequent increase in AP-1 activity was found to be dependent on *de novo* protein synthesis.^{200,201} The requirement for new c-Fos protein synthesis, which was reported to be a prerequisite for gene activation through the AP-1 binding site,²⁰¹ is probably due to the short half-life of c-Fos mRNA and c-Fos protein. In line with these observations, the inhibition by cycloheximide of GST Ya gene induction by *tert*-butylhydroquinone in H4II hepatoma cells, described in Figure 3, can be explained by a block in the synthesis of the AP-1 complex and a lack of activation of GST Ya gene via the EpRE site. Indeed, gel-shift experiments with nuclear extract from hepatoma cells exposed to GST Ya inducer show an inhibition of the induction of the AP-1 binding activity by the presence of cycloheximide.²⁰⁴

The finding that the AP-1 activity is the *trans*-acting factor that regulates the basal and inducible

gens, transforming oncogene expression, and cellular stress-inducing agents in the pattern of GST expression. The stimulation of AP-1 activity by chemical carcinogens and transforming oncogenes is probably an important event in the neoplastic process and may account for the altered patterns of gene expression in malignant cells. Thus, the GST-P expression in the early stages of chemical hepatocarcinogenesis in rats was observed to be associated with the increased expression of *c-fos*¹⁴⁸ and *c-jun*.¹⁴⁹ In addition, GST-P appears in cells transformed with *ras* oncogenes^{136,139} in which the AP-1 activity is the major transcription factor whose activity is augmented by the transforming oncogenes.²⁰⁶ The induction of *c-fos* and *c-jun* by EGF and insulin observed in a number of cells²⁰⁷⁻²⁰⁹ may explain the enhancement of GST-P expression by these effectors in primary cultured rat hepatocytes.¹⁴⁸

The inhibitory effect of dexamethasone on the induction of GST-P expression in primary

cultured hepatocytes by insulin^{146,147} can be explained on the basis of the role played by AP-1 complex in the regulation of GST-P gene transcription. There appears to be a general mutual antagonism between activation by AP-1 and by glucocorticoid receptors of target genes that contain recognition sites for these factors.²¹⁰⁻²¹³ The AP-1 activity was found to be negatively regulated by protein-protein interaction with the glucocorticoid receptor that, in the presence of dexamethasone, inhibits both basal and inducible activity of the AP-1 complex.²¹⁰⁻²¹³

B. AP-1 Binding Sites Responsible for the Inducible Expression of GSTs

The AP-1 binding sites present a great variability in nucleotide sequence, a fact that raises the question whether particular AP-1 protein complexes may recognize specific variants of the AP-1 site.¹²⁴ The study of the inducible enhancer EpRE of the mouse GST Ya gene suggests that the deviation from a consensus AP-1 sequence may play a role in the modulation of gene expression. Each of the two AP-1-like binding sites that form the EpRE, when assayed separately, confer a lower basal activity to the GST Ya gene promoter than the consensus AP-1 sequence and show very little or no inducibility.¹²⁰ It should be observed that the AP-1 binding site, unlike other response elements, exhibits a considerable basal activity prior to stimulation. This activity varies among cultured cell lines and is due to the presence of certain amounts of AP-1 complex even before induction. The lower basal activities of the AP-1-like sites of GST Ya EpRE, which correlates with their divergence from the consensus sequence TGACĀĤĤ < TGACĀAA < TGACTCA in this order (the dots indicate departure from the consensus), may suggest a lower affinity of the AP-1 complex for the AP-1-like site. Two adjacent AP-1-like sites, however, act synergistically to form an EpRE enhancer inducible by a variety of chemical agents (see Figure 5). The inducibility of the EpRE is higher than that of a single consensus sequence AP-1 site.¹²⁰ From this, it may be concluded that an enhancer composed of two adjacent AP-1-like binding sites, which have a low

affinity for the AP-1 complex but act synergistically, has the advantage to confer a higher sensitivity to the subtle changes in AP-1 complex activity induced by environmental signals. The importance of an inducible element with such a structure in the regulation of gene expression is indicated by the presence of similar enhancers in the PMA- and antioxidant-responsive regions of a number of genes.^{112,120,153,210,214} Figure 10 shows the regulatory elements composed of two adjacent AP-1-like sites that form the inducible enhancer of several Phase II xenobiotic-metabolizing enzyme genes (e.g., GST Ya genes from mouse¹²⁰ and rat,¹¹² GST-P from rat,¹⁵³ quinone reductase from rat²¹⁴) and proliferin.²¹⁰ The deviation of these AP-1-like sequences from the AP-1 consensus sequence is shown in Figure 11. It should be observed that in the GST-P gene, the two AP-1-like sites forming the GPE-1 enhancer are responsive to PMA induction, whereas a single-copy consensus sequence AP-1 site located in the proximal promoter region at position -60 of this gene as well as in that of human GSTπ gene confers only basal activity and has no inducibility.^{150,158,159}

C. The Transduction Signal

A characteristic of AP-1 transcription factor is the variety of apparently unrelated agents that can induce its DNA-binding activity. This characteristic is shared with another ubiquitous transcription factor, NF-κB (for a review, see Reference 215), and is an important feature of the transcription factor network that controls the cellular response to environmental signals. The mechanism by which transcription factor genes are induced by multiple cell stimuli is, however, not clear. It is tempting to assume that the various stimuli converge to a common intracellular messenger or transduction signal. Early observations have recognized the AP-1 binding site as a tumor promoter (PMA) response element. Because of the fact that PMA is a potent activator of protein kinase C,²¹⁶ a hypothetical pathway was proposed for the alteration of gene expression by this effector that assumed that a PMA-activated protein kinase C would modulate the activity of AP-1 binding factors by specific phosphorylations.^{122,180}

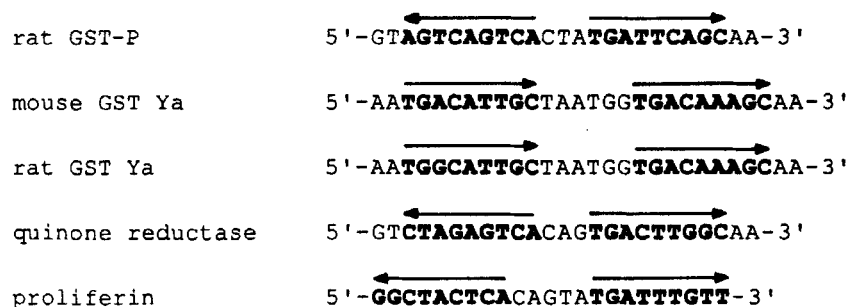


FIGURE 10. Regulatory elements composed of two adjacent AP-1-like sequences. GPE-1 in rat GST-P gene,¹⁵³ EpRE in mouse GST Ya gene,¹²⁰ ARE in rat GST Ya¹¹² and rat quinone reductase genes,²¹⁴ and proliferin gene.²¹⁰

AP-1 consensus	T G A G T C A
	C A
<hr/>	
GST Ya mouse	T G A C A T T G C
	T G A C A A A G C
GST Ya rat	T G G C A T T G C
	T G A C A A A G C
GST-P rat	T G A C T G A C T
	T G A T T C A G C
quinone reductase	T G A C T T G G C
	T G A C T C T A G
proliferin	T G A G T A G C C
	T G A T T T G T T

FIGURE 11. Comparison of the AP-1-like sequences described in Figure 10 with the consensus sequence AP-1 binding site. Dots indicate departures from consensus.

It should be noted that c-Fos and c-Jun proteins are known to be phosphorylated *in vivo*²¹⁷⁻²¹⁹ and a PMA-stimulated dephosphorylation of c-Jun at specific sites results in the activation of its DNA-binding function.²²⁰ The inhibition of PMA and H₂O₂ induction of *c-fos* and *c-jun* by protein kinase inhibitors^{221, 222} supports the assumption that the phosphorylation of certain transcription regulatory proteins (e.g., serum response factor, SRF, a transcription regulator of *c-fos* gene) is a key step in signal transduction. Although the activa-

tion of protein kinase C is an early event elicited in the initiation of cell proliferation by a number of growth factors and PMA,²¹⁶ however, there is no direct evidence that protein kinase C is involved in the induction of *fos* and *jun* gene expression. In fact PMA-or serum-induced phosphorylation of c-Fos protein was found to be independent of protein kinase C activation.²¹⁸ Moreover, in long-term PMA-treated cells in which protein kinase C is supposed to be down-regulated and the activation of *c-fos* by PMA is abol-

ished, H_2O_2 is found to induce *c-fos* gene as efficiently as in untreated cells.¹⁹⁶ These reports, therefore, do not support a universal role for protein kinase C as the second messenger in the multisignal transduction pathway responsible for the induction of AP-1 activity and suggest that other protein kinases may also be involved.

Recent observations point to reactive oxygen as the inducing signal of AP-1 activity.^{196,203,204,221–223} Reactive oxygen species known as pro-oxidants, such as superoxide anion, O_2^- , H_2O_2 , hydroxyl radical HO^\bullet , organic peroxides, and radicals, are produced endogenously by all aerobic cells as side products of a number of metabolic reactions.²²⁴ To prevent the oxidative damage caused by reactive oxygen species, cells have an elaborate antioxidant defense system consisting of nonenzymatic scavengers and quenchers known as antioxidants (e.g., GSH, β -carotene, vitamin C, α -tocopherol) as well as enzymatic systems, including GSH-peroxidases, superoxide dismutases, catalase, NAD(P)H:quinone reductase, GSSG reductase, NADPH-supply glucose 6-phosphate dehydrogenase, and conjugation enzymes, GSTs, and glucuronosyl transferases.²²⁵ The aerobic biological systems are characterized by a steady-state involving the formation of pro-oxidants and their consumption, at a similar rate, by the antioxidant defense system. "Oxidative stress" or pro-oxidant state results from an alteration of this pro-oxidant-antioxidant balance in favor of the pro-oxidants.^{225,226} In cellular pro-oxidant states, the intracellular concentration of reactive oxygen species is increased either because the cells overproduce them or because they are deficient in the ability to destroy them.

GSH, which is the most abundant intracellular thiol present in all eukaryotic forms of life,² plays a major role in maintaining the redox potential within cells, thereby protecting them against oxygen toxicity. GSH levels control the concentration of reactive oxygen species by direct reaction, scavenging, and via the GSH-peroxidase/GSH enzymatic system.² In this process, GSH is oxidized to GSSG and GSH-reductase, which catalyzes GSSG reduction by NADPH, functions in restoring the intracellular GSH:GSSG redox equilibrium. Because of the role of GSH in maintaining the intracellular redox potential, any increase in oxidant levels would result in a con-

sumption of GSH and low thiol levels. Thus, at any time, the intracellular thiol levels actually reflect a dynamic equilibrium between the GSH available and the amount of oxidants produced in the cell. A depletion of GSH, which reflects the increase in intracellular oxidant levels, was recently suggested to be involved in the signal transduction pathway that regulates expression of NF- κ B transcription factor.^{227,228}

There are a variety of agents and mechanisms that can induce pro-oxidant states, which include radiation (UV and X-ray), exposure to xenobiotics or their metabolites that can participate in redox cycling, incomplete oxygen reduction during cell respiration, inhibition of the antioxidant defense system and membrane perturbation (for a review see Reference 226). The cellular pro-oxidant states resulting from metabolic reactions in response to endogenous and exogenous inducing agents have been implicated in tumor promotion and a close correlation was observed between oxidative stress and chemical carcinogenesis.^{226,229} In this respect, it should be observed that the action of tumor promoters involves a modulation of expression of genes that regulate cell growth and differentiation. Evidence indicates that generation of active oxygen is one of the earliest events involved in the positive control of cell growth in response to growth factors or PMA.²²¹ It was also reported that active oxygens generated by xanthine/xanthine oxidase, H_2O_2 , and other oxidants could act as mitogenic stimuli in resting cells to induce DNA synthesis and transcriptional activation of immediate early genes *c-fos*, *c-jun*, and *c-myc*.^{196,221–223} Treatment of cells by H_2O_2 , which in the presence of transition metal ions is a producer of oxygen radicals,²³⁰ or by UV light activates *c-fos* and *c-jun* as well as AP-1-inducible genes such as collagenase and metallothionein.^{187,195,197} Similarly, H_2O_2 was found to induce GST Ya gene expression (see Figure 2) via the two adjacent AP-1-like sites that constitute the EpRE (see Figure 4) and the ARE¹¹² enhancers. An involvement of active oxygen in the phenobarbital induction of CAT activity from an EpRE Ya-cat gene construct and of AP-1 binding activity is supported by the inhibitory effect of the thiol compounds *N*-acetyl-L-cysteine and glutathione on this induction.²⁰³ This suggests that the phenobarbital induction of AP-1 activity, lead-

ing to the AP-1-mediated transcriptional activation of the GST Ya and quinone reductase genes,²⁰³ occurs at low thiol levels created by the generation of intracellular oxidants, because this induction can be prevented by increasing the intracellular thiol levels.

D. Inducers of GST as Producers of Oxidative Stress

The assumption that reactive oxygen is the transduction signal that mediates activation of *c-fos* and *c-jun* gene expression provides an explanation for the inducible expression of GST genes by chemical agents of diverse structure. These agents, which belong to many chemical classes,⁸⁴ may all induce conditions of oxidative stress by diverse pathways to activate the expression of GST and probably other Phase II enzyme genes that are regulated by the Fos/Jun (AP-1) complex. A close examination of the different classes of GST chemical inducers, indeed, reveals a variety of metabolic pathways and mechanisms by which these chemicals may disturb the cellular redox equilibrium toward a more oxidizing environment.

A great majority of GST and NAD(P)H:quinone reductase inducers are electrophilic compounds with structural features similar to those required to serve as substrates for glutathione transferases.¹⁰⁵ Many of the inducers are, in fact, classic Michael acceptors, that is, they contain an unsaturated bond rendered electrophilic by conjugation with an electron withdrawing group. Such electrophilic inducers may be expected to reduce the thiol (GSH) content of cells and induce pro-oxidant states in two ways: (1) by GST-mediated conjugation with GSH and (2) by nonenzymatic interaction with sulfhydryl group of GSH. Thus, it is possible that the close correlation observed between the structural features required for the inducible function, that is, induction of GSTs and NAD(P)H:quinone reductase gene expression and those required for compounds to serve as substrates for GSTs may reflect the involvement of the electrophilic compounds in GSH depletion via a GST-mediated conjugation. In fact, the potency of these chemicals as inducers parallels

their efficiency as substrates for GST.¹⁰⁶ The GSH depletion in animal tissues by substrates of GSTs has been reviewed extensively.²³¹ Following the depleted state, the rate of GSH synthesis was observed to increase.²³¹ Thus, diethyl maleate, an electrophilic GST substrate and inducer of NAD(P)H:quinone reductase,¹⁰⁵ at a concentration of 1 mM was found to cause a complete depletion of GSH and cellular death in human fibroblasts.²³² At a concentration of 0.1 mM, however, which does not cause cell death, after an initial decrease in GSH level for the first 3 h, diethyl maleate was found to induce a cystine-glutamate transport system and an enhanced GSH synthesis.²³² For some GST inducers such as 1,2-dithiole-3-thiones and isothiocyanates,^{86,105} the mechanism of production of an oxidative stress signal is not clear because there is no information concerning the reactivity of these compounds with SH groups, a GST-mediated conjugation with GSH, or other biological effects on the cellular systems. As an alternative mechanism, although the experimental evidence is lacking, one could speculate that some potent electrophilic inducers of GSTs^{86,105} may directly interact with an SH group of a regulatory protein involved in signal transduction.

A large number of xenobiotics that are inducers of GSTs and NAD(P)H:quinone reductase gene expression contain or acquire by metabolism quinoid structures. Quinones are among the most reactive and toxic electrophilic chemicals causing oxidative stress. A characteristic feature of the quinone moiety is its ability to undergo reversible oxidation-reduction cycles in which semiquinone intermediates are oxidized to quinones with concomitant reduction of O₂ to O₂⁻ (Figure 12). Quinones may undergo one-electron reduction by flavoenzymes, resulting in the formation of semiquinone radicals, or two-electron reduction by NAD(P)H:quinone reductase to form hydroquinones.²³³ By converting quinones to hydroquinones, which are less reactive species than the semiquinone radicals, the quinone reductase activity decreases the concentration of the quinone available for single-electron reduction and protects the cell against quinone toxicity.^{234–236} The semiquinone radicals auto-oxidize under aerobic conditions with the formation of

The tumor promoter PMA is also a membrane-active agent that, in addition to protein kinase C activation,²¹⁶ induces a chaotropic effect on plasma membrane conformation.²³⁹ It was suggested that perturbation of membrane conformation may render membrane lipids susceptible to auto-oxidation and activate phospholipase A₂ to release arachidonic acid from membrane phospholipids. The stimulation of arachidonic acid metabolism, which involves the increased formation of hydroxyperoxyarachidonic acid intermediates, may cause active oxygen production in PMA-treated cells.²²⁶

The mitochondrial cytochrome electron-transport chain, under normal physiological conditions, releases small amounts of active oxygen such as O₂⁻ and H₂O₂.²⁴⁰ When perturbed by inhibitors, uncouplers, and pseudosubstrates, however, it may produce increased amounts of active oxygen and cause oxidative stress. Heat shock inducers of AP-1 activity and GST Ya gene expression, arsenite and arsenate, act as inhibitors of mitochondrial respiration. Arsenite binds to SH groups to poison mitochondria and disrupt its function.²⁴¹ Arsenate is thought to act in more than one way: as a phosphate analog, it blocks oxidative phosphorylations in mitochondria²⁴² and it may disrupt mitochondrial functions after metabolic reduction to arsenite by cellular GSH.²⁴³

Recent evidence indicates that TCDD inhibits mitochondrial respiration, thereby inducing the formation of reactive oxygen molecules H₂O₂ and O₂⁻.²⁴⁴ The TCDD-induced oxidative stress, which is brought about by TCDD binding to cytochrome *b* and interference with electron-transferring components of the respiratory chain, also causes a partial uncoupling of oxidative phosphorylations. The simultaneous formation of both O₂⁻ and H₂O₂ in mitochondria in the presence of TCDD suggests the formation of the highly toxic radical HO[•]. This assumption is supported by the additive effect of SOD and catalase in protecting energy-linked respiration of mitochondria from TCDD-induced damage.²⁴⁴ This pathway of active oxygen production by TCDD may explain the toxicity of this compound, the induction of *c-fos* and *c-jun* gene expression, and increase in AP-1 activity,²⁰⁵ as well as the induction of GST Ya gene via the EpRE site,¹⁰² despite the fact that

TCDD is thought not to be metabolized by the cytochrome P₁-450 enzymes.¹⁰⁴

The mechanism by which phenobarbital may induce the formation of active oxygen is unknown, although a possible perturbation of the mitochondrial and microsomal cytochrome P₁-450 electron-transport chain was considered.²²⁶

E. Control of the "Oxidative Stress" Response

The cellular response to oxidative stress seems to involve the inducible expression of a battery of genes whose function is to counter the oxidative damage. Although the mechanism by which cells receive and respond to oxidative stress has not yet been clarified, the expression of the antioxidant enzymes induced by active oxygen appears to be under genetic control. Thus, in *S. typhimurium* and *E. coli*, the expression of nine of the proteins induced by oxidative stress is under the positive control of the *oxyR* gene.²⁴⁵ It was demonstrated that a direct activation by oxidation of Oxy-R protein is responsible for the transcriptional activation of oxidative-stress inducible genes.²⁴⁶ In this way, a transcription factor, OxyR, is both the sensor and the transducer of the oxidative stress signal.

In mammalian cells, recent evidence suggests that conditions of oxidative stress are responsible for the induction of *c-fos* and *c-jun* gene expression^{196,204} and the activation of NF- κ B transcription factor.^{227,228} The involvement of oxygen radicals as second messengers in the activation of transcription factors may be a general mechanism of regulation of gene expression in response to extracellular stimuli. Whether the regulatory proteins are activated by direct oxidation similar to the activation of OxyR or indirectly by modifications mediated by redox regulated enzymes has to be determined. The recently reported activation of a Src tyrosine kinase by oxidative stress is of considerable significance.²⁴⁷ The signaling cascade initiated by the UV-activation of this tyrosine kinase, followed by activation of Ha-Ras and Raf-1, was shown to increase the phosphorylation of c-Jun and its ability to activate transcription.²⁴⁷

Recent evidence suggests that xenobiotic-metabolizing enzymes of the Phase II group such as glutathione transferases, glucuronosyl transferases, NAD(P)H:quinone reductase, and the antioxidant enzymes, Cu/Zn superoxide dismutase, GSH-peroxidase, GSH-reductase, and the NADPH supplier glucose 6-phosphate dehydrogenase, are under a common genetic control and their levels may be coordinately regulated in response to oxidative stress. An increase in all these seven enzymatic activities was observed in the livers of mice carrying a radiation-induced homozygous deletion of 1.2 centimorgan on chromosome 7 that includes the albino (c) locus.²⁴⁸ The existence of a regulatory chromosome 7 gene was postulated whose protein product, a *trans*-acting negative effector, would control the battery of genes involved in cellular response to oxidative stress. The deletion of both allele copies of this gene releases the negative control and the "oxidative stress" enzymes become constitutively expressed. However, the gene encoding such a "master switch" regulator of oxidative stress response has yet to be identified.

V. OVEREXPRESSION OF GSTs IN ADAPTIVE RESPONSES TO CHEMICAL STRESS

Alterations in drug metabolism and the appearance of multidrug resistance are considered to be adaptive responses important for the carcinogenic process. According to this concept, the organism reacts to a cytotoxic environment with adaptive responses, which include acute transitory reactions (e.g., induction of drug-metabolizing enzymes, heat-shock proteins, DNA repair enzymes) followed by clonal development of new cell populations resistant to the cytotoxic agent (for a review see Reference 249). The hepatocyte nodules formed in the chemically induced neoplastic transformation of rat liver may be considered an adaptive physiological response to chemical stress.²⁵⁰ The clonal response of adaptation to environmental stress in the liver seems to be genetically programmed. The nodules have elevated levels of Phase II enzyme activities, glucuronosyl transferase, GSTs, NAD(P)H:quinone reductase,

epoxide hydrolase, γ -glutamyl transferase, and a decrease in cytochrome P₁-450 levels.²⁵¹ An increased expression of multidrug-resistance P-glycoprotein in liver nodules was also reported.^{252,253} The elevated levels of the Phase II enzymes in rat liver nodules are constitutive and are not dependent on the continuous presence of the drug to maintain the induced expression. Consistent with their high content of Phase II enzymes, liver nodules metabolize and excrete xenobiotic compounds in a more efficient way than normal liver cells and are more resistant to the cytotoxic effect of carcinogens.²⁴⁹ Using cDNA probes, it was demonstrated that the mRNAs specific for GST Ya and Yb subunits and quinone reductase were elevated in liver nodules and that Yb alone could be superinduced by 3-methylcholanthrene.²⁵⁴

Human tumor cells with primary or acquired multidrug resistance exhibit many of the resistance traits included in the resistance phenotype of liver nodules. For example, pi-class GST and the drug efflux pump P-170 glycoprotein are overexpressed in both xenobiotic resistant rat liver nodules and multidrug-resistant human breast MCF-7 cells.^{252,255} The alpha-class and pi-class GST isoenzymes were found to be overexpressed in tumor-cell lines resistant to alkylating agents as well as in drug-resistant tumor cells derived from patients treated with anticarcinogens.¹³²⁻¹³⁴ This apparent association of GST overexpression with drug resistance has given the incentive to experiments trying to provide direct evidence for the role of GSTs in the multidrug-resistance phenotype. In these experiments, transfection of alpha-class and pi-class GST expression vectors into yeast cells has resulted in a marked reduction of the cytotoxic effects of chorambucil and adriamycin.²⁵⁶ Similarly, transfection of a GST Ya expression vector into mammalian COS cells has enabled these cells to survive benzo(a)pyrene treatment.²⁵⁷ Other experiments, however, have shown that transfection of human alpha-class or pi-class GST expression vectors into human MCF-7 breast cancer cells did not confer resistance to the chemotherapeutic drugs.^{258,259} In addition, transfection of *mdr1* gene expression vector encoding P-glycoprotein into MCF-7 cells has produced a multidrug-resistant phenotype that was not further augmented by overexpression of GST π

gene.²⁵⁸ Thus, in multidrug-resistant MCF-7 cells, despite the association of increased GST pi and MDR P-glycoprotein activities, the resistant phenotype is primarily due to overexpression of the *mdr1* gene. These studies therefore do not show a consistent correlation between GST overexpression and resistance to anticancer drugs. The role of GST activity in drug resistance, particularly in human tumor cells exposed to chemotherapy, remains to be further clarified.

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