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Electrophile and antioxidant regulation of enzymes that detoxify carcinogens

(electrophile- and antioxidant-responsive elements/AP-1/phorbol ester-responsive element/chemoprotection/phase 2 enzymes)

TORY PRESTERA AND PAUL TALALAY*

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Contributed by Paul Talalay, June 5, 1995

ABSTRACT **Detoxication (phase 2) enzymes, such as glutathione *S*-transferases (GSTs), NAD(P)H:(quinone-acceptor) oxidoreductase (QR), and UDP-glucuronosyltransferase, are induced in animal cells exposed to a variety of electrophilic compounds and phenolic antioxidants. Induction protects against the toxic and neoplastic effects of carcinogens and is mediated by activation of upstream electrophile-responsive/antioxidant-responsive elements (EpRE/ARE). The mechanism of activation of these enhancers was analyzed by transient gene expression of growth hormone reporter constructs containing a 41-bp region derived from the mouse GST Ya gene 5′-upstream region that contains the EpRE/ARE element and of constructs in which this element was replaced with either one or two consensus phorbol 12-tetradecanoate 13-acetate (TPA)-responsive elements (TREs). When these three constructs were compared in Hep G2 (human) and Hepa 1c1c7 (murine) hepatoma cells, the wild-type sequence was highly activated by diverse inducers, including *tert*-butylhydroquinone, Michael reaction acceptors, 1,2-dithiole-3-thione, sulforaphane, 2,3-dimercapto-1-propanol, $HgCl_2$, sodium arsenite, and phenylarsine oxide. In contrast, constructs with consensus TRE sites were not induced significantly. TPA in combination with these compounds led to additive or synergistic inductions of the EpRE/ARE construct, but induction of the TRE construct was similar to that induced by TPA alone. Transfection of the EpRE/ARE reporter construct into F9 cells, which lack endogenous TRE-binding proteins, produced large inductions by the same compounds, which also induced QR activity in these cells. We conclude that activation of the EpRE/ARE by electrophile and antioxidant inducers is mediated by EpRE/ARE-specific proteins.**

Exposure of animal cells to low levels of mostly electrophilic compounds (oxidizable diphenols, quinones, Michael reaction acceptors, isothiocyanates, peroxides, vicinal dimercaptans, divalent mercury derivatives, and trivalent arsenicals) results in elevation of glutathione levels and induction of phase 2 detoxication enzymes, including glutathione *S*-transferases (GSTs), NAD(P)H:(quinone-acceptor) oxidoreductase [quinone reductase (QR)], epoxide hydrolase, and UDP-glucuronosyltransferases (1–3). These widely distributed enzymes detoxify electrophiles, thereby protecting cells against the toxic and neoplastic effects of carcinogens. Understanding the chemistry and molecular mechanisms of these inductions is therefore of primary importance in devising strategies for chemoprotection against cancer. (For definitions of phase 1 and phase 2 enzymes and of bifunctional and monofunctional inducers, see ref. 4.)

The upstream enhancer elements of the mouse and rat liver glutathione transferase Ya genes that respond to these inducers are nearly identical 41-bp segments and have been termed the electrophile-responsive element (EpRE) (5) and the antioxidant-responsive element (ARE) (6), respectively†; the mouse and rat 41-bp segments differ at only two sites—bases 1 and 14 are G in the rat segment, leaving it with only one ARE instead of the two AREs in the mouse segment.

The critical DNA sequences of the EpRE that respond to monofunctional inducers (labeled ARE in Fig. 1*B*) have been presumed to resemble AP-1-binding sites; AP-1 constitutes a family of transcription-activating DNA-binding proteins that bind to phorbol 12-tetradecanoate 13-acetate (TPA)-responsive elements (TREs) (7). Similar sequences have also been identified in the upstream regions of the human and rat QR genes (8–11) and the heavy subunit of the human γ-glutamylcysteine synthetase gene (12).

Apparently, all monofunctional phase 2 enzyme inducers increase phase 2 gene transcription via their upstream EpRE/ARE elements (2, 3, 5, 6, 13, 14), but the identity of the EpRE/ARE enhancer-binding protein(s) is controversial. Daniel and coworkers (7) have demonstrated that TRE-binding proteins (c-Fos and c-Jun) can activate this enhancer in cotransfection studies and that *in vivo* treatment of cells with many inducers increases TRE binding activity in nuclear extracts, as demonstrated by gel mobility-shift assays (15–18). In contrast, Nguyen and Pickett (19) have shown that a unique protein(s) binds specifically to the EpRE/ARE sequence as determined by gel mobility-shift and UV-crosslinking studies. More recently, others (13, 20, 21) have demonstrated, by gel mobility-shift assays, that the ARE sequence of the human QR gene interacts with a unique nuclear binding protein(s) that is not AP-1. Moreover, Yoshioka *et al.* (20) demonstrated that TBHQ stimulated transcriptional activity from an EpRE/ARE enhancer but inhibited TPA-induced gene expression from a TRE. These authors observed induction of Fra-1, which forms a complex with c-Jun that binds to and inhibits the TRE. Thus, although increased binding to the TRE sequence occurs in gel mobility-shift assays with nuclear extracts from cells treated with TBHQ *in vivo*, this does not necessarily signify stimulation of transcription, contrary to suggestions of others

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Abbreviations: TPA, phorbol 12-tetradecanoate 13-acetate; TRE, TPA-responsive element; ARE, antioxidant-responsive element; EpRE, electrophile-responsive element; GH, growth hormone; QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; TBHQ, *tert*-butylhydroquinone; CV, coefficient of variation; GST, glutathione *S*-transferase.

*To whom reprint requests should be addressed.

†The EpRE was so named because it was activated by dimethyl fumarate, *trans*-4-phenylbut-3-en-2-one, and *tert*-butylhydroquinone (TBHQ) (5), all of which contain (or can generate) electrophilic (Michael acceptor) groups. The ARE (6) was so named as it was responsive to redox-active quinone precursors (TBHQ, catechol, hydroquinone) or 1,4-benzoquinone itself, but was unresponsive to redox inactive diphenols (resorcinols). In common with most other monofunctional phase 2 enzyme inducers, the redox-active quinones are also electrophilic Michael reaction acceptors (1). We prefer the term electrophile-responsive element, since the common chemical characteristic of monofunctional phase 2 enzyme inducers and EpRE activators is that they are electrophiles; most are not antioxidants (2, 3). However, we respect the original nomenclature and thus refer to the enhancer as EpRE/ARE in this report.

A Comparison of Consensus ARE and TRE Sequences

Consensus ARE GTGACNNNGC
A

Consensus TRE TGACTCA
G A

B Comparison of EpRE Enhancer and Mutants Used in this Study

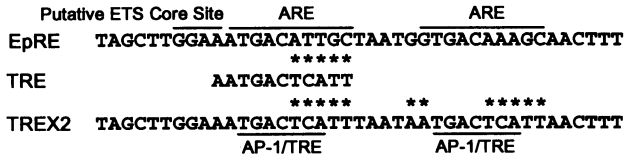

FIG. 1. (*A*) Comparison of consensus ARE (6, 21) and TRE (21–23) sequences. Although the TGAC sequence is common to both elements, the remaining sequences are different. Indeed, these two consensus sequences can share <50% homology. (*B*) Comparison of EpRE enhancer and mutants used in this study. The 41-bp EpRE from the 5′ upstream region of the mouse GST Ya gene is compared to the mutated EpRE sequences used in this study. The locations of the ETS core sequence site (2, 3) along with the direct ARE consensus repeats are shown by lines above the EpRE sequence; ETS constitutes a family of transcription factors, products of c-*ets* protooncogenes, known to cooperate with other regulatory proteins for activation of a variety of gene enhancers. The sequences present in pTRE-284YaGH and pTREX2-284YaGH are shown. The changes between the EpRE and the mutated EpRE sequences are indicated by stars above the sequence. Lines below the TREX2 sequence represent the location of the consensus TRE sites. Changes in the EpRE were made such that the consensus TRE site would be created and the 3′-GC crucial for ARE inducibility would be changed. Thus, ATTGC and AAAGC were changed to TCATT. The GG preceding the second ARE of the EpRE was changed to AA, thus making the sequences adjacent to the two TRE sequences identical and avoiding inadvertent ARE similarity on the complementary strand.

(15–18). Wang and Williamson (21) observed two gel mobility-shift bands when either the ARE or TRE consensus sequences of the human QR gene were incubated with nuclear extracts of several cell lines. The slower migrating band was competitively blocked by a consensus TRE sequence and was supershifted by c-Fos or c-Jun antibodies, thus identifying an AP-1-containing complex. The faster migrating band was competitively blocked only by a consensus ARE sequence, did not supershift with antibodies to AP-1, and corresponded to a 160-kDa protein or protein complex. These investigators identified a consensus sequence for the ARE that was nearly identical to that determined by Rushmore *et al.* (6). The ARE consensus sequence is (G or A)TGACNNNGC, while the TRE consensus sequence is TGA(C or G)T(C or A)A (Fig. 1*A*) (22, 23). Thus, there is mounting evidence that each of these sequences has unique binding specificity and responses.

We have previously demonstrated that at least eight distinct classes of inducers act via the mouse EpRE/ARE site. Recently, it has been shown that one compound (TBHQ) from these classes specifically stimulates rat ARE (13) and mouse EpRE (20) sequences but fails to activate a TRE site (20) or a rat ARE sequence that had been changed to TRE (13). Thus, we examined the responses of the mouse EpRE/ARE site and one or two TRE consensus sequences to all eight chemical classes of monofunctional phase 2 enzyme inducers in order to define more completely and distinguish the chemical inducibility of these enhancers.

MATERIALS AND METHODS

Cell Culture. Hepa 1c1c7 cells were from J.P. Whitlock, Jr., Stanford. Hep G2 and F9 cells were from American Type Culture Collection. F9 cells were discarded after eight passages. Cells were grown in Eagle's minimal essential medium containing Earle's balanced salt solution, nonessential amino acids, sodium pyruvate, and glutamine (Hep G2) or in Dulbecco's modified Eagle's medium (DMEM high glucose) (Hepa 1c1c7, F9) in an humidified atmosphere containing 5–7% CO_2 at 37°C; 10% (vol/vol) fetal calf serum, penicillin, and streptomycin were added to all media.

Compounds. Most inducers were obtained commercially. Sulforaphane and 1,2-dithiole-3-thione were gifts from G. H. Posner and T. W. Kensler, respectively.

Plasmids and Their Constructions. Growth hormone (GH) reporter gene constructs p284YaGH, containing the mouse GST Ya gene minimal promoter region, and p41-284YaGH, containing the mouse GST Ya promoter and the mouse 41-bp EpRE (Fig. 1*B*), have been described (2). pTRE-284YaGH was made by directly ligating the oligonucleotide 5′-agc TAA TGA CTC ATT g-3′ and its complement 5′-tcg acA ATG AGT CAT T-3′ (TRE, Fig. 1*B*; lowercase letters signify cloning sites) into the *Hin*dIII and *Sal* I sites, located just 5′ of the minimal promoter sequence, of p284YaGH. pTREX2-284YaGH was made by directly ligating the oligonucleotide 5′-agc TTA GCT TGG AAA TGA CTC ATT TAA TAA TGA CTC ATT AAC TTT g-3′ and its complement 5′-tcg acA AAG TTA ATG AGT CAT TAT TAA ATG AGT CAT TTC CAA GCT A-3′ (TREX2, Fig. 1*B*) into the *Hin*dIII and *Sal* I sites of p284YaGH. All constructs were sequenced. pGL3-Control was from Promega.

Transfections and Transient Gene Expression Assays. Transfections were performed by the calcium phosphate method (24). Briefly, cells were plated at a density of 1.5×10^6 (Hepa 1c1c7), 7×10^6 (Hep G2), or 1.5×10^6 (F9) in 10-cm plates, and medium was replaced after 14–16 hr. After 3 hr, the transfection mixture containing 20 μg of the specific GH gene construct and 5 μg of the control luciferase gene construct (pGL3-Control) was added. Cells were either incubated with the precipitate overnight (Hepa 1c1c7, F9) or were incubated for 5 hr followed by a 2-min 15% (wt/vol) glycerol shock (Hep G2). After a further 24 hr, cells from each 10-cm plate were trypsinized, pooled, and evenly distributed among four 24-well plates containing 1.0 ml (Hepa 1c1c7, Hep G2) or 1.5 ml (F9) of medium per well. Before treatment with compounds, the cells were left to recover overnight (Hepa 1c1c7, F9) or for 6 hr (Hep G2). Inducers dissolved in either dimethyl sulfoxide or water (arsenicals and metal salts) were then added (0.5% final dimethyl sulfoxide concentration). After 48 hr, 100 μl of medium was removed from each duplicate well and assayed for GH (2). For correction of interexperimental transfection efficiency, luciferase was assayed with a kit (Promega) with use of a scintillation counter in which the coincidence was inactivated. Viability was determined by staining with crystal violet (25). Results were validated by at least three independent transfections.

Standardization of GH Gene Expression Assay. We have demonstrated the accuracy and reproducibility of the GH transient gene expression assay used herein (2). In this study basal GH secretion (uncorrected) in three independent transfections with p41-284YaGH in Hep G2 cells was 6.13 ± 8.9%, 5.96 ± 3.0%, and 2.84 ± 6.2% ng of GH secreted per ml of medium in 48 hr [means of six intraexperimental replicates ± coefficient of variation (CV)]. This assay is ideal for comparing effects of many compounds because splitting cells after transfection but prior to inducer treatment obviates the need to control transfection efficiency. Before transfection, neither Hep G2, Hepa 1c1c7, nor F9 cells expressed detectable GH; GH added to the assay systems (0–10 ng/ml; $n = 6$) was recovered quantitatively from the medium. GH addition did not alter expression of GH by Hep G2 cells transfected with p41-284YaGH. No compounds caused induction of the enhancerless p284YaGH.

Measurement of Potency for Induction of Quinone Reductase. F9 cells were plated at 70,000 per well in 24-well plates

24 hr before addition of inducers in dimethyl sulfoxide. After a further 24 hr, cells were gently washed with phosphate-buffered saline and lysed in 150 μl of digitonin solution, and 55 μl of this lysate was assayed for QR activity (25). Protein was assayed on 20 μl of the lysate (26) with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Induction of Quinone Reductase in F9 Cells. Since undifferentiated murine F9 embryonal carcinoma cells do not express endogenous TRE binding activity (27–29), these cells are valuable in elucidating the mechanism of phase 2 enzyme induction. Extensive experiments with Hepa 1c1c7 cells (25, 30–33) showed that QR induction is a useful indicator of overall phase 2 enzyme induction. The basal specific activity of QR in undifferentiated F9 cells [697 nmol/min per mg; CV, 10.5%; means of two separate experiments, $n = 4$ per experiment] was significantly higher than that of Hepa 1c1c7 cells (208 nmol/min per mg) (25). Fig. 2 shows that exposure of F9 cells to compounds **1, 3, 5**, and **6** (Fig. 3) gave significant, concentration-dependent inductions of QR. These results strongly suggest that induction of QR does not involve TRE function, unless the inducers cause the accumulation of AP-1 proteins in these cells (see below).

Induction of the EpRE Enhancer Transfected into F9 Cells. When F9 cells were transfected with the p41-284YaGH plasmid (containing the 41-bp EpRE sequence from the murine GST Ya gene and its homologous promoter), significant and dose-dependent GH production was induced by several compounds (compounds **1–9** in Fig. 3) known to be inducers of QR in Hepa 1c1c7 cells. No GH response was observed with two related compounds (**10, 11**) inactive in the Hepa 1c1c7 system (Fig. 4). As expected, TPA was inactive in these transfected F9 cells (20). Basal GH secretion was 0.345 ng/ml (CV, 12%; $n = 5$). In a repeat experiment, basal activity was 0.535 ng/ml (CV, 11%; $n = 5$). For comparison, basal GH secretion by F9 cells containing the enhancerless plasmid p284YaGH was 0.13 ng/ml (CV, 22%; $n = 5$), which was barely detectable and comparable to the secretion (0.1 ng/ml, detection limit) of untransfected F9 cells.

It was possible that activation of the EpRE in F9 cells was due to accumulation of TRE-binding proteins. Friling *et al.* (7) observed 3-fold induction of chloramphenicol acetyltransferase activity by TBHQ driven by the EpRE enhancer in F9 cells and suggested that this might be due to induction of c-Fos

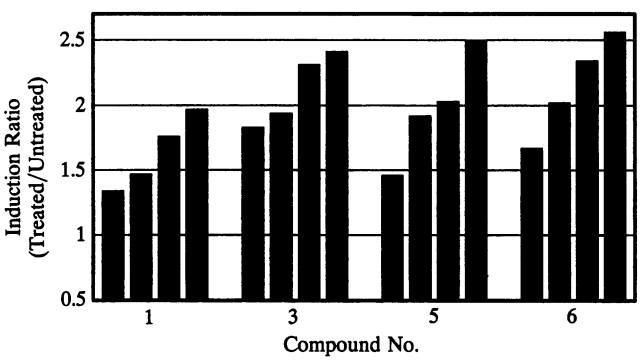

FIG. 2. Elevation of QR enzyme activity in F9 cells by representative inducers. F9 cells were treated with various micromolar concentrations (left to right for each compound) of compounds **1** (2.5, 5, 10, 20), **3** (0.63, 1.3, 2.5, 5), **5** (5, 10, 20, 40), and **6** (5, 10, 20, 40). Induction ratios are the ratios of specific activities of QR of treated/untreated cells and represent averages of two independent experiments. The average coefficient of variation was 9.7%. Compounds **1** and **3** are slightly less potent in these cells than previously observed for Hepa 1c1c7 cells (2, 3), but the overall potency order (**3** >> **1, 5, 6**) is similar in the two cell types.

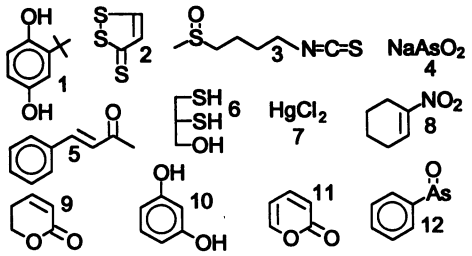

FIG. 3. Structures: **1**, TBHQ; **2**, 1,2-dithiole-3-thione; **3**, sulforaphane; **4**, sodium arsenite; **5**, *trans*-4-phenylbut-3-en-2-one; **6**, 2,3-dimercapto-1-propanol; **7**, mercury(II) chloride; **8**, 1-nitro-1-cyclohexene; **9**, 5,6-dihydro-2*H*-pyran-2-one; **10**, resorcinol; **11**, 2*H*-pyran-2-one; **12**, phenylarsine oxide.

and Jun-B proteins in response to TPA treatment, as observed by Chiu *et al.* (34). Furthermore, when c-Fos and c-Jun were overexpressed in F9 cells by transfection, cotransfected EpRE sequences were activated (7). Various chemical treatments can induce differentiation of F9 cells (27, 35, 36) and the expression of various enhancer binding proteins. Hence we transfected F9 cells with the construct pTRE-284YaGH in which the 41-bp EpRE is replaced by a consensus TRE site (Fig. 1*B*). Cells were then treated with compounds **1–12** and with TPA over a range of concentrations. In no case was any induction observed (data not shown), although we detected low basal GH secretion (0.19 ng/ml; CV, 17%; $n = 5$). Transfection was confirmed by detection of luciferase activity when cells were also transfected with pGL3-Control. When pTRE-284YaGH was transfected into Hep G2 cells, however, TPA treatment led to a large induction of GH reporter gene expression (discussed below), showing that this construct is functional and can respond to TPA in the correct environment. Therefore, it seems unlikely that these cells are expressing TRE-activating proteins under the conditions of this study. These results are consistent with those of Yoshioka *et al.* (20), who observed significant stimulation by TBHQ of only the EpRE/ARE but not the TRE.

Comparison of Efficiencies of Inducer Responses of Plasmids pTRE-284YaGH, pTREX2-284YaGH, and p41-284YaGH in Hep G2 and Hepa 1c1c7 Cells. Friling *et al.* (7) showed that a single "so-called" TRE sequence was inducible by TBHQ and also demonstrated that AP-1 proteins bind to

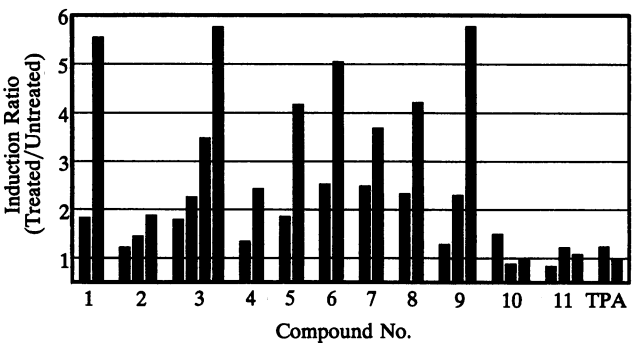

FIG. 4. Effect of different concentrations of inducers on GH production in F9 cells transfected with p41-284YaGH. The compounds are numbered as in Fig. 3, and their micromolar concentrations were as follows (left to right for each compound): **1**, 6.25 and 12.5; **2**, 13, 25, and 50; **3**, 0.63, 1.3, 2.5, and 5; **4**, 0.25 and 0.50; **5**, 6.25 and 12.5; **6**, 5 and 10; **7**, 0.5 and 1.0; **8**, 0.63 and 1.3; **9**, 6.3, 13, and 25; **10**, 6.3, 13, and 25; **11**, 6.3, 13, and 25; and TPA, 100 and 200 ng/ml. The ratio of GH production in 48 hr in treated/untreated cells is shown. Values were corrected for cell number by staining with crystal violet. Rank order of potencies is similar to that observed in similar transfections in Hepa 1c1c7 cells (2, 3) except that compound **2** is much less potent in F9 cells.

the EpRE. Thus, they concluded that the two "AP-1-like" sites in the EpRE were simply binding sites for AP-1 binding proteins and further concluded that TBHQ and other inducers act via AP-1 proteins. However, they used the sequence ATGACTCAGCA, which includes the 3′-GC bases crucial for ARE inducibility (8, 13), and hence they were actually testing a combined EpRE/ARE and TRE sequence. These crucial 3′-GC bases are not included in the classical consensus TRE (22, 23) and thus were changed in our study. We compared the responses of the EpRE sequence and two sequences containing either one or two consensus TRE sites to the eight chemical classes of inducers (Fig. 1*B*). The three plasmids containing these sequences, pTRE-284YaGH, pTREX2-284YaGH, and p41-284YaGH, were transfected into Hep G2 cells and treated with a range of concentrations of inducers **1–8, 12**, and TPA (Fig. 5). Basal GH secretions, expressed as ng of GH secreted per ml of medium in 48 hr, were 3.28 ± 39% for pTRE-284YaGH, 8.56 ± 41% for pTREX2-284YaGH, and 8.77 ± 15% for p41-284YaGH (average ± CV of three independent transfections, normalized for cell number by crystal violet staining, each with six intraexperimental replicates). Thus, basal activity is comparable for all three enhancers.

None of the phase 2 enzyme inducers strongly stimulated the consensus TRE constructs (Fig. 5). No compound led to any significant induction of the construct containing one consensus TRE except for phenylarsine oxide (2.1-fold induction at 25 nM). When two consensus TRE sites were present, slight induction was seen with most compounds, with questionable dose dependency. However, in the same experiment, cells transfected with the EpRE-containing construct gave large concentration-dependent inductions, consistent with our previous observations (2). Thus, the EpRE is significantly more inducible than the TRE in almost all cases. Even $HgCl_2$ (7), which is a poor inducer of the EpRE sequence, still induced this sequence much more efficiently than the TREX2 sequence. Only sodium arsenite (3) showed no significant difference in induction between the EpRE and TREX2 sequence. However, TPA induced all three constructs to a similar extent. TPA induction is also an internal control for these experiments, providing strong evidence that all plasmids are functional. Identical results were obtained in Hepa 1c1c7 cells, except that these cells do not respond (for unclear reasons) to TPA. These results agree with those found for TBHQ and TPA induction by Yoshioka *et al.* (20) for mouse EpRE, and for a single rat GST Ya ARE by Nguyen *et al.* (13). Clearly, while the sequences can all respond in a similar manner to TPA, the response to most phase 2 enzyme monofunctional inducers is restricted to the EpRE sequence. It has been suggested (7, 37) that ARE sites are simply low-affinity AP-1-binding sites. If this were correct, however, replacing the two ARE sites of the EpRE sequence with TRE consensus sequences should either increase or have no effect on the responses of these sequences to inducers. Although this occurs for TPA, antioxidants and electrophilic inducers did not greatly induce constructs in which the EpRE/ARE sequence was changed to a consensus TRE sequence. This suggests that the EpRE/ARE sequence interacts specifically with a protein that is unable to bind efficiently to the TRE.

Simultaneous Treatment of Transfected Cells with TPA and Inducers. We compared the responses of the three plasmids to combined treatment with TPA and compounds **1–7** (Fig. 6), using concentrations of inducers that provide significant induction of the EpRE reporter construct. For the TRE-containing plasmids, treatment with compounds **1–7** together with TPA had no effect on the inductions produced by TPA alone (Fig. 6 *Top*). In contrast, the same experiment with the EpRE/ARE enhancer element produced additive or even synergistic inductions of this enhancer element (Fig. 6 *Bottom*),

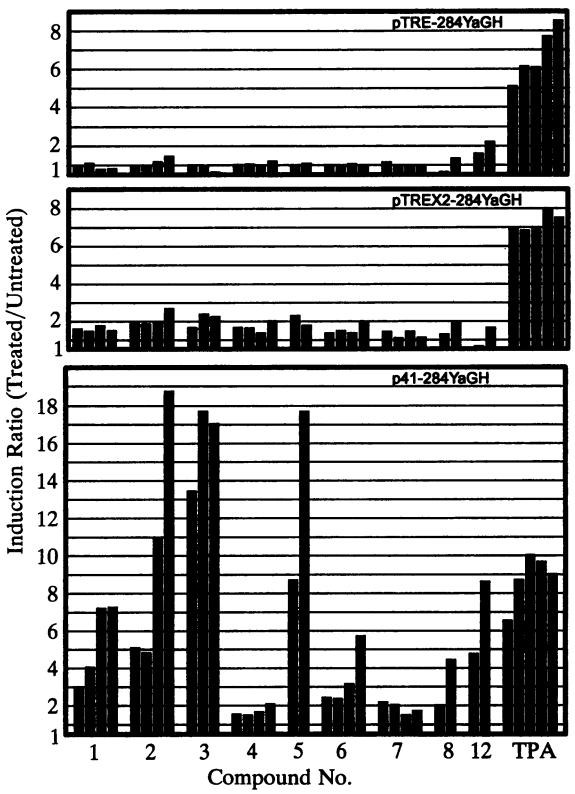

FIG. 5. Effect of different concentrations of inducers on GH production in Hep G2 cells transfected with pTRE-284YaGH (*Top*), pTREX2-284YaGH (*Middle*), or p41-284YaGH (*Bottom*). The compounds are numbered as in Fig. 3, and their micromolar concentrations were as follows (left to right for each compound): **1**, 6.3, 13, 25, and 50; **2**, 19, 38, 63, and 130; **3**, 1.3, 2.5, and 5.0; **4**, 0.5, 1, 2, and 4; **5**, 13 and 25; **6**, 5.0, 10, 20, and 40; **7**, 0.5, 1.0, 2.0, and 4.0; **8**, 0.63 and 1.3; **12**, 0.025 and 0.05; and TPA, 4, 10, 20, 50, and 100 ng/ml. Values were corrected for cell number by staining with crystal violet.

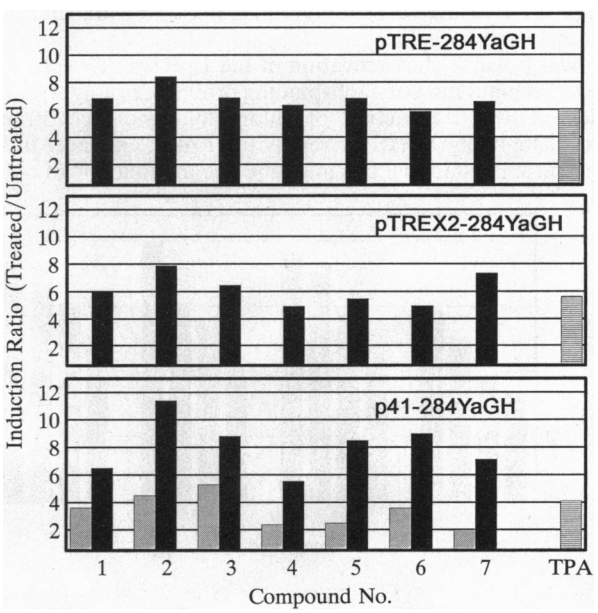

FIG. 6. Effect of combined treatment with TPA at 100 ng/ml and various inducers of GH production in Hep G2 cells transfected with pTRE-284YaGH (*Top*), pTREX2-284YaGH (*Middle*), or p41-284YaGH (*Bottom*). Micromolar concentrations of compounds: **1**, 25; **2**, 50; **3**, 1.3; **4**, 4.0; **5**, 13; **6**, 40; **7**, 4.0. The shaded bars on the right in all panels represent TPA treatment alone. Shaded bars associated with compounds **1–7** (*Bottom*) represent treatment with compounds **1–7** alone. Black bars indicate combined treatments with TPA.

further demonstrating dramatic differences in inducibility of consensus TRE and EpRE/ARE.

CONCLUSIONS

We have shown that: (*i*) an enzyme known to be induced via an EpRE/ARE sequence (QR) was induced in F9 cells; (*ii*) EpRE/ARE but not TRE enhancer constructs was stimulated in F9 cells by the same inducers; (*iii*) inducers stimulate reporter gene transcription from EpRE/ARE-containing constructs in Hep G2 and Hepa 1c1c7 cells but largely fail to stimulate transcription of reporter gene constructs containing either one or two TRE(s); (*iv*) TPA induces both EpRE/ARE and the TRE to a similar degree in Hep G2 cells, but synergistic or additive induction occurs only with the EpRE/ARE enhancer element; and (*v*) the basal activity of the EpRE/ARE enhancer and the TRE is very similar.

Apparently, the EpRE/ARE site can be induced both by TRE binding proteins [it can be activated by c-Fos and c-Jun in cotransfection studies according to Friling *et al.* (7)] and by an as-yet-unidentified EpRE/ARE-specific protein(s) that mediates induction by phase 2 monofunctional enzyme inducers. This might explain why the basal activity of the elements is similar and why both can be induced by TPA, as basal activation and TPA induction may be mediated through proteins capable of activating both sequences. However, the EpRE/ARE-activating protein does not activate the TRE consensus element. Overall, the conclusion that the response to monofunctional phase 2 enzyme inducers is mediated by AP-1 proteins (5, 15, 16, 18, 37) is clearly untenable in light of these and other results (13, 20, 21). Thus, it is crucial to identify the proteins that specifically interact with the EpRE/ARE sequences and mediate the action of these inducers.

Note Added in Proof. Recently, Xie *et al.* (38) compared the requirements for inducibility of "ARE" and "TRE" sequences in an effort to define the "response to xenobiotics and antioxidants." These studies involved the use of TPA and of β-naphthoflavone as inducers. It is unfortunately very difficult to compare our results with those of Xie *et al.* (38) because these authors used somewhat different definitions of ARE and TRE and not those proposed by the authors who discovered these entities. Furthermore, the only results published for "ARE" inducibility were obtained with a single compound—β-naphthoflavone—at extremely high concentrations (50 μM). β-Naphthoflavone is not an antioxidant and is a bifunctional inducer—i.e., it must undergo metabolism to induce phase 2 enzymes.

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