Role of Quinone-Mediated Generation of Hydroxyl Radicals in the Induction of Glutathione *S*-Transferase Gene Expression†

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ABSTRACT: Induction of glutathione *S*-transferase (GST) Ya gene expression by a variety of chemical agents is mediated by a regulatory element composed of two adjacent AP-1-like binding sites and activated by the Fos/Jun heterodimeric complex (AP-1). We have previously shown that the induction of GST Ya gene expression and of AP-1 binding activity is regulated by intracellular glutathione (GSH) levels. To study the role of reactive oxygen species in the induction of AP-1 activity and GST Ya gene expression and their effect on intracellular GSH levels, we have exposed hepatoma cells to adriamycin and two synthetic quinones, Q_c^b and Q_n, with different capacities to generate oxygen radicals. The kinetics of quinone-mediated generation of hydroxyl radicals were monitored in intact cells by a spin trapping technique and EPR spectral measurements. We find that quinones which can chelate Fe(III) ions, adriamycin and Q_c^b, are more effective in hydroxyl radical production than the nonchelating quinone Q_n. Furthermore, we show that the induction of AP-1 binding activity and GST Ya gene expression by these quinones correlates with their oxygen radical production, adriamycin and Q_c^b being stronger inducers that Q_n. The present study indicates that the AP-1-mediated induction of GST Ya gene expression is part of the response to oxidative stress. A transient increase by 2.5-fold in the intracellular GSH level was observed 30 min after exposure of cells to quinone and was followed by a rapid depletion of GSH. This increase in the GSH level represents an induction of GSH synthesis since it was blocked by buthionine sulfoximine, an inhibitor of γ-glutamylcysteine synthetase. The oxygen radical-mediated induction of γ-glutamylcysteine synthetase, the first and rate-limiting step in GSH synthesis, appears therefore to be an early event in cells exposed to quinone metabolism. These findings therefore indicate a correlation between production of oxygen radicals, GSH level, and induction of AP-1-mediated GST Ya gene expression.

The capacity of mammalian cells to maintain cellular functions during oxidative stress resides in a rapid induction of protective enzymatic activities which by decreasing the level of compounds capable of generating reactive oxygen species decrease the level of oxidant stress. In this respect drug-metabolizing enzymes, e.g., glutathione transferases, glucoronosyl transferases, and NADP(H):quinone reductase that function as intracellular detoxication systems of toxic and carcinogenic compounds, are part of the inducible pathway for antioxidant defense (Sies, 1991). The activity of these enzymes is induced by a variety of chemical agents such as planar aromatic hydrocarbons, diphenols, PMA,[1] barbiturates, and electrophilic compounds (Talalay *et al.*, 1987). The recent study of a mouse GSY Ya gene has shown that all these chemicals operate through a single cis-regulatory element defined as EpRE to induce the expression of this gene (Friling *et al.*, 1990, 1992; Pinkus *et al.*, 1993; Bergelson *et al.*, 1994a). We have previously shown that the EpRE regulatory element of the mouse GST Ya gene is composed of two adjacent AP-1-like binding sites that bind and are transactivated by the Fos/Jun heterodimeric complex (AP-1) (Friling *et al.*, 1992; Daniel *et al.*, 1993; Bergelson & Daniel, 1994). A number of other chemical-inducible genes were observed to be regulated through enhancers similar in structure to the EpRE of mouse GST Ya (Sakai *et al.*, 1988; Rushmore & Pickett, 1990; Diamond *et al.*, 1990; Favreau & Pickett, 1991; Li & Jaiswall, 1992). Such regulatory elements termed ARE in rat GST Ya and rat quinone reductase genes (Rushmore & Pickett, 1990; Favreau & Pickett, 1991) were also found to be activated by the AP-1 complex (Bergelson *et al.*, 1994a). An Ets-binding site, located immediately upstream of the two AP-1-like sites in EpRE and ARE of GST Ya genes, was recently found to mediate a cooperative interaction between Ets and AP-1 transcription factors which contributes to increase the inducibility of these enhancers (Bergelson & Daniel, 1994). Treatment of hepatoma HepG2 or H4II cells with chemical agents such as β-naphthoflavone, 3-methylcholanthrene, *tert*-butyl hydroquinone, *trans*-4-phenyl-3-buten-2-one, PMA, phenobarbital, hydrogen peroxide, arsenite, arsenate, and heavy metals was found to induce an increase in AP-1 binding activity (Pinkus *et al.*, 1993; Daniel *et al.*, 1993; Bergelson *et al.*, 1994a). Dioxin, an inducer of the GST Ya gene (Friling et al., 1990), was also reported to induce an

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[1] Abbreviations: BSO, L-buthionine *S,R*-sulfoximine; DMPO, 5,5′-dimethyl-1-pyroline *N*-oxide; DMSO, dimethyl sulfoxide; EPR, electron paramagnetic resonance; GSH, glutathione; GST, glutathione *S*-transferase; NAC, *N*-acetylcysteine; PMA, phorbol 12-myristate 13-acetate; PTK, protein−tyrosine kinase; PTP, protein−tyrosine phosphatase; Q_c^b, 2-phenyl-4-(butylamino)naphtho[2,3-*h*]quinoline-7,12-dione; Q_n, 2-phenyl-5-nitronaphth[2,3-*g*]indole-6,11-dione.

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increase in AP-1 activity in Hepa 1c1c7 cells (Puga *et al.*, 1992).

The finding that chemical agents of diverse structure all activate GST Ya and quinone reductase gene expression through the induction of AP-1 transcription factor that interacts with the respective EpRE or ARE enhancers led us to assume that the various chemicals may produce a common transduction signal responsible for AP-1 induction. It was observed that all chemical inducers of GST Ya and quinone reductase gene expression have the potential to induce conditions of oxidative stress and depletion of reduced GSH (Daniel, 1993). In fact, these chemicals can generate by metabolism reactive oxygen species or can modify thiol compounds which in both cases may cause a depletion of GSH and a change of intracellular redox equilibrium toward a more oxidizing environment. Recent studies support the hypothesis that the cellular redox state plays an important role in the regulation of AP-1-mediated activation of GST Ya and quinone reductase gene expression. We have reported that the induction of AP-1 binding activity and EpRE-mediated GST Ya gene expression by chemical agents is inhibited by thiol compounds, *N*-acetylcysteine and GSH, and that both basal and inducible activities are enhanced by lowering the intracellular GSH level (Pinkus *et al.*, 1993; Bergelson *et al.*, 1994b). These findings indicate that the induction of AP-1 activity and GST Ya and quinone reductase gene expression is in fact associated with an increase in intracellular oxidant levels. Similarly, the induction of mammalian heme oxygenase gene by a variety of chemical treatments such as UV radiation, heavy metals, PMA, or sodium arsenite (Applegate *et al.*, 1991) was reported to correlate with a depletion of intracellular GSH levels (Lautier *et al.*, 1992).

In the present study we further investigated the role of reactive oxygen species in the induction of AP-1 activity and GST Ya gene expression. The generation of •OH radicals by quinone metabolism was monitored by a DMPO−ȮH spin trapping technique and EPR spectral measurements in intact cells exposed to antitumor quinone adriamycin and two synthetic quinones, Q_c^b and Q_n, with different capacities to generate oxygen radicals (Dikalov *et al.*, 1992; Weiner, 1994). This is the first report showing a correlation between production of •OH radicals by the different quinones and the induction of AP-1 binding activity and GST Ya gene expression.

MATERIALS AND METHODS

Materials. Adriamycin hydrochloride, 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO), *d*-α-tocopherol acetate (vitamin E), L-buthionine *S*,*R*-sulfoximine, glutathione, *N*-acetylcysteine, cycloheximide, and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO). Quinones 2-phenyl-4-(butylamino)naphtho[2,3-*h*]quinoline-7,12-dione (Q_c^b) and 2-phenyl-5-nitronaphth[2,3-*g*]indole-6,11-dione (Q_n) and ṘS−SṘ nitroxide biradical were synthesized and their structures analyzed by Dikalov *et al.* (1992) and Khramtsov *et al.* (1989), respectively.

Cell Cultures. Human HepG2 and rat H4II (a differentiated cell line that still expresses GST Ya gene) hepatoma cells were grown in F12 and Dulbecco's modified Eagle's medium (1:1) with 10% fetal calf serum. HepG2 and H4II cells have been previously found to yield similar results concerning AP-1 binding of nuclear extracts, transient expression of GST Ya gene constructs, and intracellular measurements of GSH levels (Bergelson *et al.*, 1994a,b). Cell viability was determined by trypan blue dye exclusion. Under the conditions used in this study exposure to quinones had little effect, if any, on cell viability. The number of viable cells after 3-h exposure to 100 μM adriamycin, Q_c^b, or Q_n did not vary by more than 5%.

Electrophoretic Mobility Shift Assays. HepG2 cells were grown on 100-mm plates, and after exposure to chemicals nuclear extracts were prepared (Schreiber *et al.*, 1989) and assayed by electrophoretic mobility shift for ^{32}P-labeled AP-1 oligonucleotide binding (Bergelson *et al.*, 1994a).

RNA Extraction and RNA Blot Analysis. Total cellular RNA was prepared by the guanidinium thiocyanate extraction method (Chomczynski & Sacchi, 1987) and was fractionated by electrophoresis on 1% agarose−formaldehyde gels, followed by transfer onto nitrocellulose filters. The RNA blot was hybridized with ^{32}P-labeled probes for GST Ya cDNA and rRNA. The hybridization levels were quantitated by the Image quant method in a Molecular Dynamics 300A computing densitometer.

Measurement of Cellular Sulfhydryl Groups. Total cellular −SH groups were measured in intact cells by the EPR spectroscopy method of Weiner *et al.* (1991). HepG2 cells grown to log phase were harvested by scraping, washed twice in phosphate-buffered saline (pH 7.2), and suspended in the same buffer at a density of 1×10^5 to 2.5×10^6 cells/mL. For measurement of −SH groups an aliquot of 200 μL of the cell suspension containing $(1-5) \times 10^3$ cells was mixed with ṘS−SṘ nitroxide biradical to a final concentration of 80 μM. The stable biradical ṘS−SṘ contains a disulfide bond that upon thiol/disulfide exchange with thiols yields a characteristic EPR spectrum (Khramtsov *et al.*, 1989; Weiner *et al.*, 1991). Reaction mixtures were incubated in air at room temperature for 5 min before the EPR spectrum was measured in a Bruker Electron Spin Resonance ER200D-SRC spectrometer. The instrumental parameters were as follows: microwave frequency, 9.7 GHz; incident microwave power, 10 mW; center of the field, 3480; scan range, 100 G; field modulation, 1 G; receiver gain, 6.3×10^4; and time constant, 640 ms. The calibration curve for GSH determination was obtained as described before (Weiner *et al.*, 1991).

Detection of Free Radical Intermediates. HepG2 cells were prepared as described above and •OH radical formation was followed by EPR of spin adduct DMPO−ȮH (Buettner & Mason, 1990). A typical 200-μL incubation mixture for trapping •OH radicals contained 2.5×10^5 cells, 100 mM DMPO, and 2 μM to 1.6 mM quinone. The DMPO in phosphate-buffered saline (pH 7.2) was purified before use by passing it twice through a syringe containing activated charcoal. The EPR spectrum of DMPO−ȮH consisted of a quartet (1:2:2:1) with hyperfine splitting constants of $a_N = a_H = 14.9$ G and was recorded by the EPR spectrometer under the same conditions described above for −SH measurements except for receiver gain, 6.3×10^5. The amplitude of the second peak in the quartet (Figure 1) was used for further calculations.

RESULTS

•OH Radical Formation in Adriamycin-Treated Cells. In order to quantitate the adriamycin-mediated •OH radical

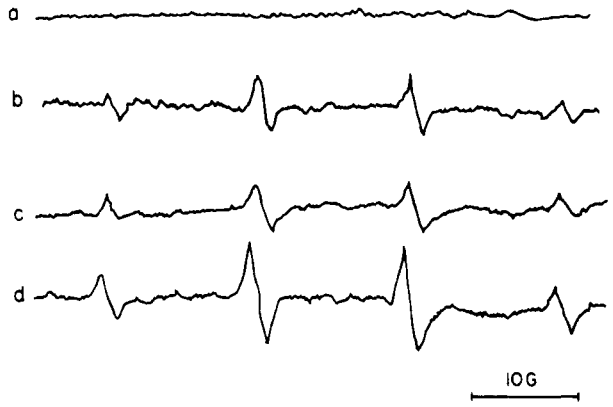

FIGURE 1: EPR spectra of DMPO−ȮH radical spin adduct obtained from intact HepG2 cells in the (a) absence or presence of (b) 100 μM adriamycin, (c) 3 mM sodium azide, or (d) 100 μM adriamycin and 3 mM sodium azide. The 200-μL reaction mixtures containing 2.5×10^5 cells in phosphate-buffered saline and 100 mM DMPO were incubated for 15 min at 22 °C before EPR spectra measurements. The instrumental parameters were as described in Materials and Methods.

formation in cultured cells, we have used the spin trapping of •OH by DMPO and measured the EPR spectrum of the resulting DMPO−ȮH adduct. Figure 1 shows that incubation of intact HepG2 cells with 100 μM adriamycin in the presence of 100 mM DMPO for 15 min before the EPR spectrum was measured resulted in the formation of an EPR spectrum consisting of a quartet (1:2:2:1) with hyperfine splitting of 14.9 G, which is characteristic for the DMPO−ȮH spin adduct (Buettner, 1987). A DMPO−ȮH signal with a similar amplitude was observed when cells were treated with the catalase inhibitor sodium azide, indicating the involvement of H_2O_2 in •OH formation. Treatment of cells with both adriamycin and sodium azide resulted in an additive amount of DMPO−ȮH adduct (Figure 1). The kinetics of •OH formation in adriamycin-treated cells were studied by measuring the amplitude of the EPR signal of the DMPO−ȮH spin adduct produced by exposure for different time periods to adriamycin concentrations from 2 μM to 1.6 mM. In repeated experiments using HepG2 or H4II cells a similar dependence of •OH radical production on time and concentration of adriamycin was observed. Figure 2 presents the results of a typical experiment. It is seen that both the kinetics of •OH radical formation and the maximum amplitude of the spin adduct EPR signal augment with the increase in adriamycin concentration.

Effect of Adriamycin on Intracellular GSH Level. The intracellular GSH levels were measured in intact HepG2 cells exposed to adriamycin by the EPR spectroscopy method of Weiner *et al.* (1991). It was observed that the exposure to adriamycin caused an initial increase in GSH level up to 250%, followed by a rapid decrease to about 50% of GSH level in untreated cells (Figure 3). The increase in GSH level, which has a lag of 7−10 min behind the •OH radical production, reaches a peak in about 30 min after exposure to adriamycin. BSO, a specific inhibitor of γ-glutamylcysteine synthetase which is responsible for the first and rate-limiting step in GSH synthesis (Meister, 1991), when added together with adriamycin blocks completely the increase in GSH levels (Figure 3). In the presence of BSO the exposure to adriamycin actually causes a steady depletion of intracellular GSH which in 90 min reaches about 50% of the GSH level in untreated cells. These findings suggest that

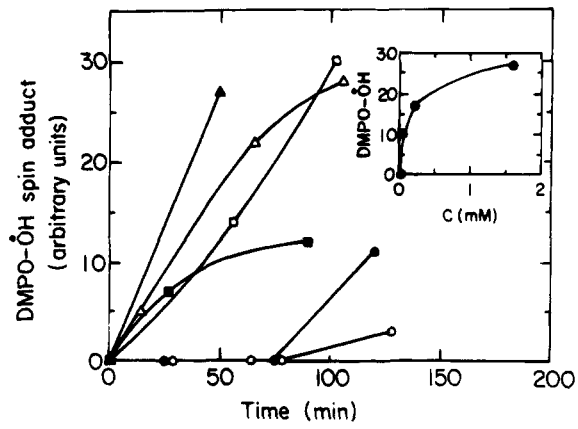

FIGURE 2: Formation of DMPO−ȮH radical spin adduct in adriamycin-treated cells as a function of time and dose dependence. Aliquots of 200 μL of HepG2 cells containing 2.5×10^5 cells and 100 mM DMPO were treated with increasing concentration of adriamycin, (○) 2.0 μM, (●) 10 μM, (■) 50 μM, (□) 100 μM, (△) 200 μM, or (▲) 1.6 mM, for different periods of time. The DMPO−ȮH radical spin adduct was measured from the amplitude of the second component of the quartet in the EPR spectrum (Figure 1). The insert presents the amount of DMPO−ȮH spin adduct produced in 50 min as a function of adriamycin concentration.

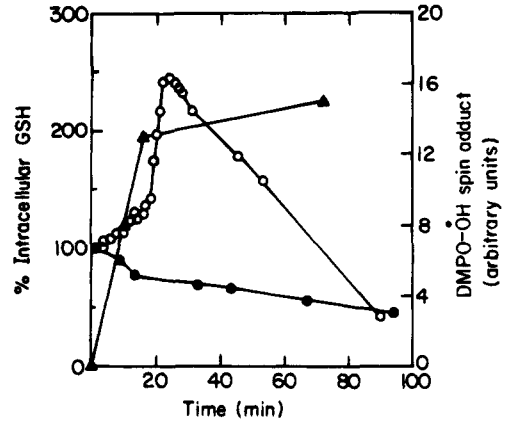

FIGURE 3: Effect of adriamycin on intracellular GSH level. ·ȮH radical production (▲) was measured in a 200-μL reaction mixture containing 2.5×10^5 HepG2 cells, 100 μM adriamycin, and 100 mM DMPO as described in Figure 2. Total cellular −SH levels were measured in 200-μL reaction mixtures containing 1.5×10^3 HepG2 cells and 80 μM R̈S−SR nitroxide biradical by incubation with 100 μM adriamycin in the (○) absence or (●) presence of 50 μM BSO.

the short-term increase in GSH levels in adriamycin-treated cells represents an induction of GSH synthesis which is prevented by BSO.

Role of Quinone−Iron(III) Interaction in •OH Radical Generation and GSH Depletion. Recent studies on the quinone-dependent generation of •OH radicals in cell-free extracts have demonstrated that quinones capable of forming complexes with Fe(III) ions stimulate the generation of oxygen radicals more efficiently than their nonchelating analogues (Weiner, 1994). This effect was proposed to be due to intramolecular electron transfer from the semiquinone into the Fe(III) ion (Dikalov *et al.*, 1992). To study the effect of chelation of iron ions by quinones on the generation of •OH radicals in the intact cell, we have compared the activity of adriamycin, which can form a complex with Fe(III) (Powis, 1989), with that of two synthetic quinones, Q_c^b and Q_n (Dikalov *et al.*, 1992). The Q_c^b can form complexes with Fe(III) ions while the Q_n quinone has similar redox

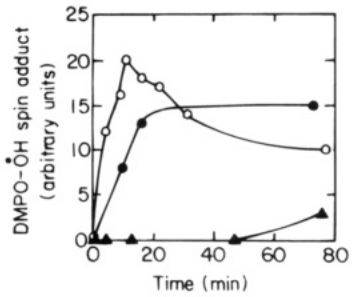

FIGURE 4: Kinetics of DMPO−ȮH radical spin adduct formation in cells exposed to different quinones. Reaction mixtures of 200 μL containing 2.5 × 10^5 HepG2 cells and 100 mM DMPO were incubated with 100 μM (●) adriamycin, (○) quinone Q_c^b, or (▲) quinone Q_n, and the formation of DMPO−ȮH adducts was measured by EPR spectroscopy.

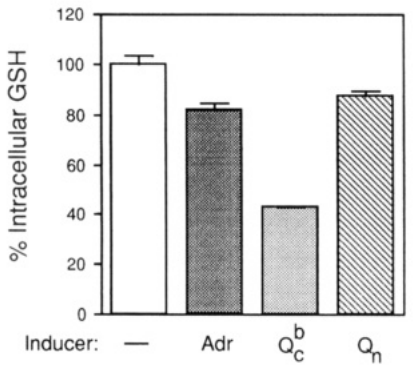

FIGURE 5: Effect of quinones on the intracellular GSH levels. HepG2 cells were untreated (−) or exposed for 3 h to 10 μM adriamycin (Adr), quinone Q_c^b, or quinone Q_n. Total −SH was determined by EPR (Materials and Methods) and by Ellman reagent as described by Tieze (1969).

properties without the chelating group. HepG2 cells were exposed to 100 μM adriamycin, Q_c^b, or Q_n in the presence of 100 mM DMPO. The kinetics of the EPR signal of the DMPO−ȮH spin adduct produced by the quinone-treated cells show that both quinones that can form complexes with Fe(III), adriamycin and Q_c^b, present a faster rate of ·OH radical generation as compared with then nonchelating quinone Q_n (Figure 4). Moreover, as previously observed with an *in vitro* system (Dikalov *et al.*, 1992; Weiner, 1994), Q_c^b stimulated the formation of ·OH radicals in intact cells with a significantly higher rate and amount of spin adduct production than adriamycin.

A decrease in intracellular GSH levels in cells exposed to the different quinones was observed (Figure 5). Treatment of cells for 3 h with 10 μM adriamycin or Q_c^b reduces the GSH levels to 80% and 40%, respectively, of that in untreated cells. Exposure to Q_n under similar conditions also causes a depletion of intracellular GSH levels to 80−90%. The relatively small effect of 10 μM adriamycin in reducing the GSH level may be due to the lag in ·OH radical production at this concentration (see Figure 2) and the transient increase in GSH synthesis previously observed (Figure 3).

Induction of GST Ya Gene Expression by Quinones. To test a possible involvement of ·OH radical production in the induction of GST Ya gene expression, H4II hepatoma cells were exposed to different concentrations of adriamycin, Q_c^b, and Q_n. Total RNA was extracted and analyzed by RNA blot hybridization with a GST Ya cDNA probe. The

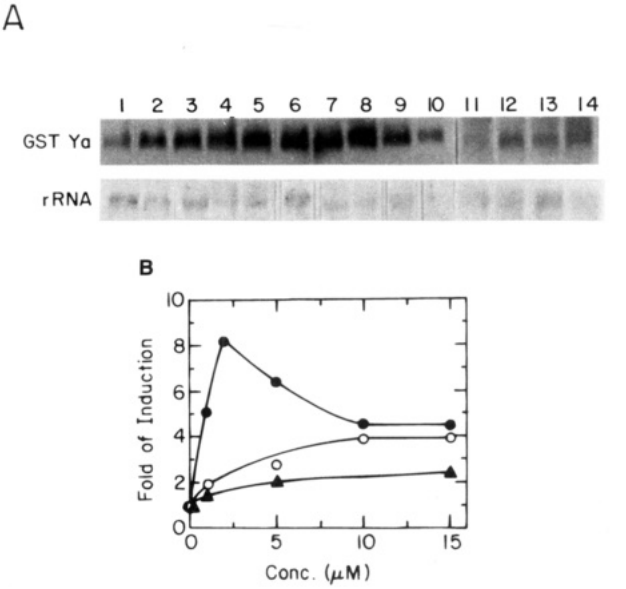

FIGURE 6: Induction of GST Ya gene expression by quinones. (A) RNA blot analysis of total cellular RNA (20 μg) isolated from H4II cells untreated (lanes 1 and 11) or exposed for 3 h to 1, 5, 10, or 15 μM adriamycin (lanes 2−5), 1, 2, 5, 10, or 15 μM quinone Q_c^b (lanes 6−10), or 1, 5, or 15 μM quinone Q_n (lanes 12−14). The blot was hybridized to GST Ya cDNA and rRNA ^{32}P-labeled probes. (B) Autoradiograms were quantitated by densitometry, the induction of GST Ya mRNA was calculated from the GST Ya mRNA/rRNA ratios, and the fold of induction was related to the uninduced basal level arbitrarily set as 1. Curves: (○) adriamycin; (●) quinone Q_c^b; (▲) quinone Q_n.

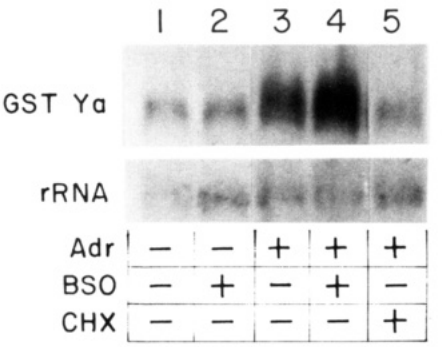

FIGURE 7: Effect of BSO and cycloheximide on the induction of GST Ya gene expression by adriamycin. RNA blot analysis of total RNA (30 μg) isolated from H4II cells untreated (lanes 1 and 2) or incubated for 3 h with 2 μM adriamycin (lanes 3−5) in the absence (−) or presence (+) of 60 μg/mL cycloheximide (CHX) or 50 μM BSO as indicated. The RNA blot was hybridized with ^{32}P-labeled probes for GST Ya and rRNA and autoradiographed.

autoradiogram shown in Figure 6 indicates that a 3-h exposure to these quinones causes a 2−8-fold induction of GST Ya mRNA expression. While Q_c^b appears to be the most effective, an 8-fold induction at a concentration of 2 μM, Q_n has the lowest ability to activate GST Ya gene expression. Depletion of intracellular GSH levels by exposure of cells for 24 h to 50 μM BSO was found to enhance GST Ya mRNA induction by adriamycin, and the presence of the protein synthesis inhibitor cycloheximide was found to abolish this induction (Figure 7). An inhibition of GST Ya mRNA induction in H4II cells by cycloheximide was previously observed for inducers such as zinc chloride (Bergelson *et al.*, 1994a), *tert*-butyl hydroquinone (Daniel, 1993), phenobarbital, and hydrogen peroxide (data not shown).

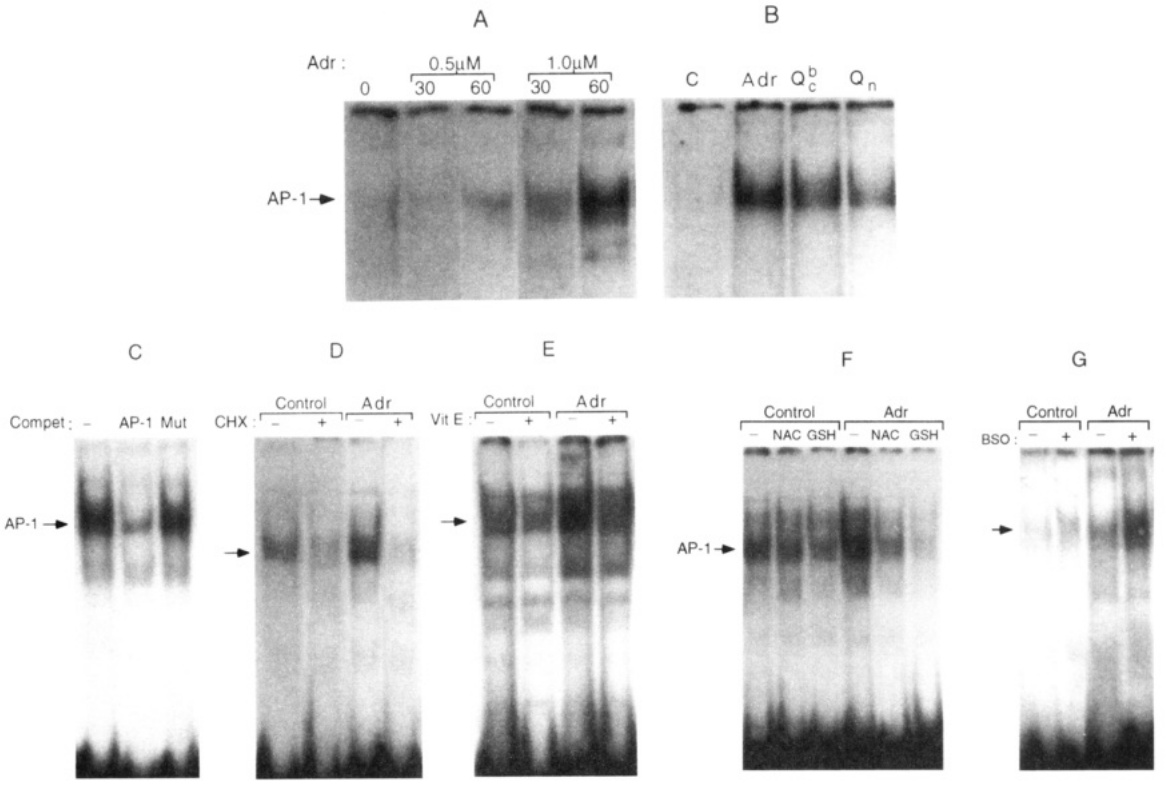

FIGURE 8: Induction of AP-1 binding activity by quinones. (A) HepG2 cells were exposed for 30 and 60 min to 0.5 or 1.0 μM adriamycin; nuclear extracts were prepared and analyzed by electrophoretic mobility shift assay for ^{32}P-labeled AP-1 oligonucleotide binding as described in Materials and Methods. (B) AP-1 binding activity in nuclear extracts from HepG2 cells untreated (C) or exposed for 3 h to 2 μM adriamycin (Adr), quinone Q_c^b, or quinone Q_n. (C) Competition experiment of AP-1 binding activity induced by 3-h exposure to 2 μM adriamycin with a 100-fold molar excess of unlabeled AP-1 or unlabeled AP-1 mutant (Mut) oligonucleotides (Bergelson *et al.*, 1994a) which were included in the reaction mixtures. (D) Effect of protein synthesis inhibition on AP-1 induction by adriamycin. Nuclear extracts were prepared from HepG2 cells incubated for 60 min in the absence or presence 60 μg/mL cycloheximide (CHX) followed by 3-h exposure to 2 μM adriamycin. AP-1 binding activity was measured as described in Materials and Methods. (E) Effect of vitamin E on AP-1 induction by adriamycin. Nuclear extracts were prepared from cells exposed for 60 min to 5 mM vitamin E as indicated, followed by 3-h incubation with 2 μM adriamycin (Adr). Control cells were incubated for 3 h in the absence of adriamycin. (F) Effect of NAC and GSH on AP-1 induction by adriamycin. Nuclear extracts were prepared from cells exposed for 60 min to 30 mM NAC or GSH as indicated, followed by 3-h incubation with 2 μM adriamycin (Adr). Control cells were incubated for 3 h in the absence of adriamycin (G) Effect of BSO on AP-1 induction by adriamycin. Nuclear extracts were prepared from cells exposed for 24 h to 50 μM BSO, followed by 3-h incubation with 2 μM adriamycin (Adr) as indicated.

Induction of AP-1 Binding Activity by Quinones. In previous studies we have shown that the induction of AP-1 binding activity mediates the activation of GST Ya gene expression by chemical agents (Daniel *et al.*, 1993; Pinkus *et al.*, 1993; Bergelson *et al.*, 1994a; Bergelson & Daniel, 1994). To test whether induction of GST Ya gene expression by quinones is mediated by an increase in AP-1 binding activity, hepatoma HepG2 cells were exposed to 0.5 and 1.0 μM adriamycin for 30 and 60 min; nuclear extracts were prepared and analyzed by electrophoresis mobility shift for AP-1 binding activity using an AP-1 binding site oligonucleotide probe. Figure 8A shows that, compared with nuclear extract from uninduced cells, exposure to adriamycin resulted in a time- and dosage-dependent increase in AP-1 binding. Exposure of cells for 3 h to a 2 μM quantity of the different quinones used in this study shows that the Fe(III)-chelating quinones, adriamycin and Q_c^b, which are more efficient producers of •OH radicals and GST Ya mRNA induction, are also better inducers of AP-1 binding activity than Q_n (Figure 8B). The competition experiment shown in Figure 8C indicates that the adriamycin-induced AP-1 binding activity is specific for the AP-1 site. Thus the AP-1 complex binding to the ^{32}P-labeled AP-1 oligonucleotide probe is competed by a 100-fold molecular concentration of unlabeled AP-1 oligonucleotide and not by a mutant of the AP-1 oligonucleotide. Inhibition of protein synthesis by treatment of HepG2 cells for 60 min with cycloheximide before exposure to adriamycin is found to inhibit the induction of the AP-1 binding activity (Figure 8D). Exposure of HepG2 cells to different antioxidants such as vitamin E, NAC, or GSH, for 60 min before the addition of adriamycin, was observed to inhibit the adriamycin-induced increase in AP-1 binding activity (Figure 8E,F). Treatment of cells for 24 h with 50 μM BSO, which was previously shown to reduce by 50−60% the endogenous GSH levels (Bergelson *et al.*, 1994b), is found to enhance the adriamycin-induced AP-1 activity (Figure 8G).

DISCUSSION

In order to define the role of reactive oxygen species in the regulation of glutathione *S*-transferase gene expression, we have compared the quinone-mediated hydroxyl radical formation in HepG2 cells by adriamycin and synthetic quinones Q_c^b and Q_n. Because the highly reactive •OH radical is short-lived and has no detectable EPR spectrum at room temperature, the spin trapping EPR technique was utilized to detect the quinone-induced •OH formation. In the presence of the spin trapping agent DMPO the quinone-stimulated generation of •OH in intact cells was detected by EPR spectra of DMPO−ȮH spin adducts. The •OH radical

formation was quantitated from the amplitude of the EPR spectrum of the DMPO−ȮH adduct, which consisted of a characteristic quartet (1:2:2:1) with hyperfine splitting constants of $a_N = a_H = 14.9$ G (Figure 1). The formation of the •OH radical was confirmed by the addition of 0.14 M DMSO, which reacted with free •OH to form the •CH_3 radical and abolished the DMPO−ȮH EPR spectrum. The addition of sodium azide markedly enhanced the adriamycin-stimulated •OH formation in hepatoma cells (Figure 1), while a 5 mM concentration of antioxidant vitamin E was found to inhibit by 50% the formation of the EPR spectrum quartet (data not shown).

The sequence of reactions which takes place in quinone-mediated electron-transfer chains leading to •OH radical formation is transition metal ion dependent (Powis, 1989). In this process the ions Fe(III), Cu(II), etc. or their complexes with organic ligands undergo cycles of reversible oxidation−reduction. Recent studies bring additional evidence for the key role played by Fe(III) ions in the quinone−mediated •OH radical generation. It was shown that a synthetic quinone, Q_c^b, capable of chelating Fe(III) stimulated the formation of •OH radicals in a cell-free system consisting of NADPH and NADPH−cytochrome P-450 reductase more efficiently than the nonchelator quinone Q_n (Dikalov *et al.*, 1992; Weiner, 1994). In the presence of Fe(III) and using the DMPO spin trap, these experiments have indicated the absence of a lag period in the spin adduct appearance for Q_c^b and a higher rate of DMPO−ȮH spin adduct formation for Q_c^b as compared with Q_n. Also, under these conditions a larger concentration of spin adduct was attained for Q_c^b. In the present study we have measured the generation of •OH radicals by the spin trapping technique in intact cells exposed to the different quinones. Taking in consideration that the cell growth medium contains significant amounts of Fe(III), the present results support the previous observations of Dikalov *et al.* (1992). The Q_c^b − Fe(III) complex stimulated the generation of •OH radicals more efficiently, without a lag period and at a higher rate of DMPO−ȮH spin adduct production, than the nonchelator quinone Q_n (Figure 4). Adriamycin, which forms complexes with Fe(III) that are active in •OH radical formation (Powis, 1989), was slightly less effective than Q_c^b in the rate and amount of DMPO−ȮH spin adduct formation during th first 20 min of exposure.

In mammalian cells the metabolism of quinones is associated with the consumption of intracellular GSH as a result of alkylation by electrophilic metabolites or oxidation by reactive oxygen species (Bellomo *et al.*, 1990). GSH, the most abundant intracellular thiol compound, is an essential cofactor for GSH transferases and the GSH peroxidase activities. It plays a major role in maintaining the intracellular redox potential by regulating the levels of reactive oxygen species via the GSH peroxidase/GSSG reductase system. The GSH consumption induced by the redox metabolism of quinones appears to be mostly the result of enhanced detoxification of hydrogen peroxide by GSH peroxidase and oxidation of GSH to GSSG (Bellomo *et al.*, 1990). Our present data indicate that in adriamycin-treated hepatoma cells the formation of •OH radicals is actually accompanied by a transient increase of 250% in intracellular GSH levels (Figure 3). The kinetics of increase in GSH levels indicate that it has a short lag period of about 10 min behind the •OH radical production, reaches a peak in 30 min, and declines in 90 min to 50% of the GSH level in uninduced cells. The adriamycin-induced increase in GSH levels was completely abolished when cells were concomitantly exposed to the γ-glutamyl synthetase inhibitor BSO. These findings indicate that the short-term increase in GSH levels in adriamycin-treated cells is due to an induction of GSH synthesis and represents an early cellular response to •OH radical production. The enhanced synthesis of GSH as an early effect of treatment with oxidizing agents or with thiol-reactive compounds to produce higher than normal intracellular levels of GSH was observed in a number of biological systems (Deneke & Fanburg, 1989; Plummer *et al.*, 1981). The magnitude and duration of increase in GSH levels vary in the different types of cells studied. Thus in hepatoma cells the induction of GSH synthesis by •OH radicals is transient, and after 3-h exposure to the different quinones used in the present study, the GSH levels drop by 20% to 60% of the initial level in untreated cells (Figure 5). In a recent study, it was observed that, in exposure of bovine pulmonary artery endothelial or rat lung epithelial (L-2) cells to naphthoquinones that generate hydrogen peroxide but cannot conjugate with GSH, there is an increase in GSH levels to sustained elevated concentrations (Forman *et al.*, 1993). The same study has suggested that the increased GSH levels may constitute an adaptive response to oxidative stress which is mediated by an increased expression of γ-glutamylcysteine synthetase and *de novo* GSH synthesis. It should be observed that cystine transport, an important regulator of GSH levels, is also induced by conditions of oxidant stress (Deneke *et al.*, 1993; Bannai *et al.*, 1991).

In previous studies we have observed that the induction of the Fos/Jun (AP-1) complex and the activation of GST Ya gene expression are regulated by intracellular GSH levels. Thus a depletion of intracellular GSH by BSO inhibition of γ-glutamylcysteine synthetase or by direct oxidation of GSH by diamide was found to stimulate both basal and chemical-inducible expression of GST Ya and the AP-1 binding activity (Bergelson *et al.*, 1994b). We hypothesized that the diverse chemicals that induce the AP-1 complex leading to the AP-1-mediated transcriptional activation of GST Ya gene expression may act through a common mechanism involving the production of reactive oxygen species and depletion of reduced glutathione. The present results indicate that the induction of GST Ya gene expression in hepatoma H4II cells exposed to the different quinones used in this study reflects the ability of these compounds to generate •OH radicals. Thus the Fe(III)-chelating quinones, adriamycin and Q_c^b, are more efficient inducers of GST Ya mRNA than the non-chelating quinone Q_n (Figure 6). As observed previously for a number of chemical inducers, BSO treatment (50 μM, 24 h) of hepatoma cells which resulted in a 50−60% decrease in GSH level (Bergelson *et al.*, 1994b) is found now to enhance GST Ya mRNA induction by adriamycin (Figure 7).

In view of the role of the AP-1 complex in the regulation of induction of GST Ya gene expression by chemical agents (Friling *et al.*, Pinkus *et al.*, 1993; Bergelson *et al.*, 1994; Bergelson & Daniel, 1994), it was of interest to study the effect of the •OH radical-generating quinones as inducers of AP-1 binding activity. Our results indicate that exposure of hepatoma cells to adriamycin causes a dose- and time-dependent induction of AP-1 activity (Figure 8A). The

inhibitory effect of antioxidants such as vitamin E, NAC, and GSH (Figure 8 E–G) on this induction is significant and may indicate an involvement of •OH radicals in the increase in AP-1 binding activity. Furthermore, a decrease of intracellular GSH level by BSO treatment (50 μM, 24 h) that should stimulate the adriamycin-induced •OH generation (Dusre *et al.*, 1989) is found to enhance the induction of AP-1 binding activity (Figure 8G).

The activity of AP-1 is rapidly increased in response to a number of environmental stimuli (Angel & Karin, 1991) and chemical agents (Pinkus *et al.*, 1993; Puga *et al.*, 1992; Bergelson *et al.*, 1994a). This induction is due to the activation of *fos* and *jun* gene transcription (Treisman, 1994; Devary *et al.*, 1991) as well as to posttranslational modification by phosphorylation of both preexisting and newly synthesized Jun and Fos proteins (Smeal *et al.*, 1991, 1992; Pulverer *et al.*, 1991; Boyle *et al.*, 1991). The mechanism by which •OH radicals could induce an increase in AP-1 binding activity is not clear. The signal transduction pathway of •OH radicals may involve the oxidation of an –SH group that is essential for the function of redox-sensitive enzymes. The coupling of enzyme activity with cellular redox was proposed to involve a thiol/disulfide exchange mechanism by which the sulfhydryl oxidation state of proteins equilibrates with the thiol/disulfide status of the cell (Gilbert, 1984). Protein–tyrosine kinases (PTKs) and protein–tyrosine phosphatases (PTPs) which play an important role in signal transduction and cell proliferation are among the enzymes that seem to be regulated by the cellular redox status. It is well established that all PTPs have a cysteine residue in their active site which must be in the reduced form for catalytic activity (Zhang & Dixon, 1994; Guan & Dixon, 1991). Due to this feature the PTPs are highly sensitive to thiol oxidation, and a decrease in intracellular reducing environment could promote their inactivation. Since the steady-state level of tyrosine-phosphorylated proteins is determined by the relative activities of tyrosine kinases and phosphatases, the inactivation of the PTPs would result in a net increase in protein–tyrosine phosphorylations. An alteration of cellular redox equilibrium toward an oxidizing environment was reported to stimulate protein–tyrosine phosphorylation and tyrosine kinase activation (Heffetz *et al.*, 1990; Staal *et al.*, 1994). In view of the role of protein phosphorylation/dephosphorylation in the regulation of gene expression (Hunter & Karin, 1992) the redox-regulated enzymes may function as redox sensors in the signal transduction pathways by which oxidant stress causes induction of the AP-1 transcription factor.

The induction of GST Ya gene expression is presently shown to be part of a general response to oxidant stress which includes induction of enzymes such as heme oxygenase (Applegate *et al.*, 1991; Lautier *et al.*, 1992), γ-glutamylcysteine synthetase (Forman *et al.*, 1993), and cystine transport (Deneke *et al.*, 1993; Bannai *et al.*, 1991). In this respect the regulatory role played by the AP-1 transcription factor in the inducible expression of GST Ya and other drug-metabolizing enzymes by oxidants and sulfhydryl reagents (Bergelson *et al.*, 1994a,b) may be part of a general mechanism of regulation of gene expression by oxidative stress. It is noteworthy that the expression of heme oxygenase gene, which is induced by conditions of oxidative stress created by UVA radiation and hydrogen peroxide or GSH depletion (Lautier *et al.*, 1992), is also induced by PMA *via* multiple AP-1 sites activated by the Fos/Jun complex (Alam & Zhining, 1992). In view of these observations we hypothesize that the AP-1 complex may also be involved in the induction of heme oxygenase and γ-glutamylcysteine synthetase gene expression, cystine transport, and enhanced GSH synthesis during oxidative stress.

REFERENCES

Alam, J., & Zhining, D. (1992) *J. Biol. Chem. 267*, 21894–21900.

Angel, P., & Karin, M. (1991) *Biochim. Biophys. Acta 1072*, 129–157.

Applegate, L. A., Luscher, P., & Tyrrell, R. M. (1991) *Cancer Res. 51*, 974–978.

Bannai, S., Sato, H., Ishii, T., & Taketani, S. (1991) *Biochim. Biophys. Acta 1092*, 175–179.

Bellomo, G., Thor, H., & Orrenius, S. (1990) *Methods Enzymol. 186*, 627–635.

Bergelson, S., & Daniel, V. (1994) *Biochem. Biophys. Res. Commun. 200*, 290–297.

Bergelson, S., Pinkus, R., & Daniel, V. (1994a) *Oncogene 9*, 565–571.

Bergelson, S., Pinkus, R., & Daniel, V. (1994b) *Cancer Res. 54*, 36–40.

Boyle, W. J., Smeal, T., Defize, L. H., Angel, P., Woodgett, J. R., Karin, M., & Hunter, T. (1991) *Cell 64*, 573–584.

Buettner, G. R. (1987) *Free Radical Biol. Med. 3*, 259–303.

Buettner, G. R., & Mason, R. P. (1990) *Methods Enzymol. 186*, 127–133.

Chomczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Daniel, V. (1993) *CRC Crit. Rev. Biochem. Mol. Biol. 28*, 173–207.

Daniel, V., Bergelson, S., & Pinkus, R. (1993) in *Structure and Function of Glutathione S-Transferases* (Tew, K. D., Pickett, C. B., Mantle, T. J., Mannervik, B., & Hayes, J., Eds.) pp 129–136, CRC Press, Boca Raton, FL.

Deneke, S. M., & Fanburg, B. L. (1989) *Am. J. Physiol. 257*, L163–L173.

Deneke, S. M., Bukowski, D. M., & Deneke, C. F. (1993) *Free Radical Biol. Med. 15*, 506.

Devary, Y., Gottlieb, R. A., Lau, L. F., & Karin, M. (1991) *Mol. Cell. Biol. 11*, 2804–2811.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K., & Yamamoto, K. R. (1990) *Science 249*, 1266–1271.

Dikalov, S. I., Rumyantseva, G. V., Piskunov, A. V., & Weiner, L. M. (1992) *Biochemistry 31*, 8947–8953.

Dusre, L., Mimnaugh, E. G., Myers, C. E., & Sinha, B. K. (1989) *Cancer Res. 49*, 511–515.

Favreau, L. V., & Pickett, C. B. (1991) *J. Biol. Chem. 266*, 4556–4561.

Forman, H. J., Shi, M., Kugelman, A., & Choy, H. A. (1993) *Free Radical Biol. Med. 15*, 543.

Friling, R. S., Bensimon, A., Tichauer, Y., & Daniel, V. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6258–6262.

Friling, R. S., Bergelson, S., & Daniel, V. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 668–672.

Gilbert, H. (1984) *Methods Enzymol. 107*, 330–351.

Guan, K. L., & Dixon, J. E. (1991) *J. Biol. Chem. 266*, 17026–17030.

Heffetz, D., Bushkin, I., Dror, R., & Zick, Y. (1990) *J. Biol. Chem. 265*, 2896–2902.

Hunter, T., & Karin, M. (1992) *Cell 70*, 375–387.

Khramtsov, V. V., Yelinova, V. I., Weiner, L. M., Berezina, T. A., Martin, V. V., & Volodarsky, L. B. (1989) *Anal. Biochem. 182*, 58–63.

Lautier, D., Luscher, P., & Tyrrell, R. M. (1992) *Carcinogenesis 13*, 227−232.
Li, Y., & Jaiswall, A. K. (1992) *J. Biol. Chem 267*, 15097−15104.
Meister, A. (1991) *Pharmacol. Ther. 51*, 155−194.
Pinkus, R., Bergelson, S., & Daniel, V. (1993) *Biochem. J. 290*, 637−640.
Plummer, J. L., Smith, B. R., Sies, H. Y., & Bend, J. R. (1981) *Methods Enzymol. 77*, 50−59.
Powis, G. (1989) *Free Radical Biol. Med. 6*, 63−101.
Puga, A., Nebert, D. N., & Carrier, F. (1992) *DNA Cell Biol. 11*, 269−281.
Pulverer, B. J., Kyriakis, J. M., Avruch, J., Nikolakaki, E., & Woodgett, J. R. (1991) *Nature 363*, 670−674.
Rushmore, T. H., & Pickett, C. B. (1990) *J. Biol. Chem. 265*, 14648−14653.
Sakai, M., Okuda, A., & Muramatsu, M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9456−9460.
Schreiber, E., Matthias, P., Mueller, M. M., & Schaffner, W. (1989) *Nucleic Acids Res. 17*, 6419.
Sies, H. (1991) *Am. J. Med. 91*, 315−385.
Smeal, T., Binetruy, B., Mercola, D. A., Birrer, M., & Karin, M. (1991) *Nature 354*, 494−496.
Smeal, T., Binetruy, B., Mercola, D., Glover, B. A., Heidecker, G., Rapp, U. R., & Karin, M. (1992) *Mol. Cell. Biol. 12*, 3507−3513.
Staal, F. J. T., Anderson, M. T., Staal, G. E. J., Herzenberg, L. A., Gitler, C., & Herzenberg, L. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3619−3622.
Talalay, P., De Long, M. J., & Prochaska, H. (1987) in *Cancer Biology and Therapeutics* (Cory, J. G., & Szentivani, A., Eds.) pp 197−216, Plenum Publishing Corp., New York.
Tieze, F. (1969) *Anal. Biochem. 27*, 502−522.
Treisman, R. (1994) *Curr. Opin. Genet. Dev. 4*, 96−101.
Weiner, L. M. (1994) *Methods Enzymol. 233*, 92−103.
Weiner, L. M., Hu, H., & Swartz, H. M. (1991) *FEBS Lett. 290*, 243−246.
Zhang, Z.-Y., & Dixon, J. E. (1994) *Adv. Enzymol.*, 1−36.

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