The Response of Renal Tubular Epithelial Cells to Physiologically and Chemically Induced Growth Arrest*

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Cells respond to a variety of stresses by activating the transcription of a battery of “acute phase” or “stress response” genes. The nature of this response is tailored to the nature of the stress. The extent to which physiologically and pathophysiologically induced growth arrest share common genomic responses is unclear. We therefore compared the effects of a physiologically induced (serum and nutrient deprivation) and a chemically induced (2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ) stress in renal tubular epithelial cells (LLC-PK1). The response to physiological stress, induced by serum depletion, involves growth arrest characterized by an inhibition of DNA synthesis that occurs in the absence of a decrease in histone mRNA or an increase in gadd153 mRNA, one of the growth arrest and DNA damage inducible genes. In contrast, the chemical-induced stress involves growth arrest accompanied by a decrease in histone mRNA, particularly core histone H2B and H2A mRNA, and the induction of gadd153. Chemical-induced changes in histone mRNA inversely correlate to changes in the expression of a stress gene, hsp70, whose expression is dependent upon the maintenance of appropriate nucleosomal structure.

Cells respond to a variety of stresses by activating the transcription of a battery of “acute phase” or “stress response” genes (1, 2). The nature of this response is tailored to the nature of the stress. For example, serum- and nutrient-deprived cells usually enter a quiescent state (G0) that requires both the up-regulation and down-regulation of genes involved in growth control. In contrast, the initial response to DNA-damaging agents includes arrest of cell cycle progression, presumably to facilitate the transcription of a battery of genes involved in the DNA repair process (3) prior to DNA replication (G1 arrest) and cell division (G2 arrest). The extent to which physiologically and pathophysiologically induced growth arrest share common genomic responses is unclear and is the focus of the present studies.

The conjugation of ortho-, or para-quinones with GSH results in the formation of conjugates that frequently exhibit more potent toxicological activity than the parent quinone (4). GSH conjugates of polyphenolics are also formed as metabolites of a variety of “non-genotoxic” carcinogens (5–7). As a model of quinone-thioether-mediated toxicity we have been investigating the cellular and molecular responses to 2-Br-bis-(glutathion-S-y1)hydroquinone (2-Br-bis-(GSyl)HQ) and 2-Br-6-(glutathion-S-y1)hydroquinone (2-Br-6-(GSyl)HQ). 2-Br-bis-(GSyl)HQ (30 μmol/kg) causes margination of heterochromatin and loss of chromatin staining when administered to male Fischer 344 rats (8), and in renal tubular epithelial cells (LLC-PK1) it causes the formation of single strand breaks in DNA (9), rapid inhibition of DNA synthesis (10), and the induction of the growth arrest and DNA damage-inducible gene, gadd153 (10). Quinone-thioethers therefore provide a useful model with which to investigate chemically induced growth arrest and the consequences of chemically induced chromatin disruption. We have therefore compared physiologically induced (serum and nutrient deprivation) and chemically induced (2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ) growth arrest in renal tubular epithelial cells (LLC-PK1) and determined some of the factors involved in regulating gene expression in response to the chemically induced stress. We report that the response to physiological stress (serum deprivation) involves growth arrest characterized by an inhibition of DNA synthesis in the absence of changes in histone or gadd153 mRNA. In contrast, the chemically induced stress involves growth arrest accompanied by a decrease in histone mRNA, particularly core histone H2A and H2B mRNA, and the induction of gadd153.

MATERIALS AND METHODS

Chemicals and Probes—Unless specified otherwise, chemicals were obtained from Sigma. 2-Br-6-(GSyl)HQ and 2-Br-6-(GSyl)HQ were synthesized according to established methodology (11). Ethylene glycol-bis-(β-aminoethyl ether) N,N,N′,N″-tetraacetic acid (EGTA-AM) was purchased from Molecular Probes, Inc. (Eugene, OR). A plasmid pBlue-script containing 600-bp fragment of cDNA of gadd153 was kindly provided by Dr. Nikki J. Holbrook (NIA, National Institutes of Health, Gerontology Research Center). The cDNA probe for histone H2B was purchased from Oncor (Gaithersburg, MD), and the probes for histone H2A and H3 were available from the American Type Culture Collection (Rockville, MD). Probes for histone H1 and H4 were kindly provided by Drs. Gary and Janet Stein (Dept. Cell Biology, University of Massachusetts), who constructed plasmids containing fragments of the histone genes. pFNC16A (H1) and pF0002 (H4) were transformed into a competent cell line (DH5α; Life Technologies, Inc.). Transformed cells were cultured in LB medium (Difco) containing ampicillin at 37 °C. Plasmid DNA was isolated following large scale preparation and digested with restriction enzymes (EcoRI/PvuII for H1 and BamHI/PstI for H4) to obtain the correct inserts. The probes were purified (1.45 kilobase of H1 and 2.3 kilobase of H4), and the inserts were used for Northern blot analysis. Dr. Sue Fischer (M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX) provided 7 S ribosomal RNA cDNA.

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Chemical-induced Growth Arrest

Cell Culture and Treatment Conditions—LLC-PK1 cells (American Type Culture Collection, Rockville, MD) are a renal proximal tubule epithelial cell line derived from the New Hampshire Mini-pig, and were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and high glucose without pyruvate (Life Technologies, Inc.) at 37°C in a humified incubator containing 5% CO₂. Cells were seeded at a density of 2 × 10⁵ cells/well in six-well plates and were used following overnight culture. Cells were washed twice with Earl’s balanced salt solution (EBSS; 0.8 mM MgSO₄·7H₂O, 116.3 mM NaCl, 10.0 mM NaHCO₃, 1.0 mM Na₂HPO₄·H₂O, 5.4 mM KCl, 5.5 mM d-glucose, 20 mM HEPES, and 1.8 mM CaCl₂·2H₂O) and treated with 2-Bis-(S-glutathionyl)hydroquinone and 2-Br-6-(glutathionyl)HQ (200 μM) for 2 h or pretreated with deferoxamine (10 mM) for 1 h, washed in DMEM-Hepes, and then exposed to 2-Bis-(S-glutathionyl)HQ (200 μM) for 2 h. Cultures were then washed twice with DMEM containing Hepes without fetal bovine serum. Each well was pulsed with 5 μCi/ml of [³H]thymidine (specific activity, 58 Ci/mmol). After incubation with [³H]thymidine for 4 h, cells were washed three times with phosphate-buffered saline, and 15% trichloroacetic acid was added. Cells were harvested by gentle scraping, and centrifuged for 5 min at 14,000 rpm. The supernatant was removed, and the pellet was washed by adding 5% trichloroacetic acid to remove unincorporated [³H]thymidine. The same procedure was repeated three times. A final washing with absolute ethanol was performed to remove residual trichloroacetic acid, and the pellet was dried. Dried pellets were dissolved in 1 ml of 0.1 N NaOH and used for the determination of protein concentration and radioactivity by liquid scintillation spectroscopy. Protein concentrations were determined by the Bio-Rad protein method using bovine serum as the standard.

Northern Blot Analysis—Total RNA was isolated using RNAzol (Cinna/Biotecx Laboratory International Inc., Friendswood, TX) and the procedure of Chomczynski and Sacchi (12). For Northern blot analysis, RNA was electrophoresed on 0.8% agarose gel containing formaldehyde and transferred to Zeta Probe membranes (Bio-Rad). cDNA probes were labeled with [³²P]dCTP (ICN, Costa Mesa, CA) by the random primer method (13) using a kit (Boehringer Mannheim, Mannheim, Germany). All blots were hybridized in 0.5 M Na₂HPO₄, pH 7.2, and 7% SDS overnight at 65°C. Blots were washed twice with 25 mM Na₂HPO₄, pH 7.2, and 5% SDS at 65°C for 30 min and twice with 25 mM Na₂HPO₄, pH 7.2, and 1% SDS at 65°C for 30 min. All blots were rehybridized with the cDNA probe for gadd153 mRNA to correct for potential variations in RNA loading by stripping the original probe with 0.1 × SSC and 0.5% SDS at 95°C for 0.5–1 h. Autoradiography was performed using Kodak XAR-5 film (Sigma) exposed for between 1 h to 5 days, depending on the probe, at ~70°C with intensifying screens.

RESULTS

Nutrient Depletion-mediated Growth Arrest: Effects on the Growth Arrest and DNA Damage-inducible Gene, gadd153, and Histone mRNA in Renal Tubular Epithelial Cells—Depriving cells of serum and nutrients was sufficient to induce gadd153 expression (Fig. 1). Supplementation of EBSS with glucose failed to overcome gadd153 induction, but DMEM, with or without added serum, prevented the increase in gadd153 (Fig. 1). Thus, the induction of gadd153 in physiologically stressed LLC-PK1 cells appears to be a response to the absence of specific nutrients rather than to an absence of glucose or serum factors. Although incubating cells in DMEM in the absence of serum did not induce gadd153 expression, there was a significant (~98%) reduction in [³H]thymidine incorporation into newly synthesized DNA (Fig. 2) indicative of a state of growth arrest. Because histone synthesis is tightly coupled to DNA synthesis, we examined the effects of this physiologically induced state of growth arrest on the expression of histone mRNA. Neither core (H2A, H2B, H3, H4) nor linker (H1) mRNA levels were decreased in cells grown in the absence of serum and/or nutrients (Fig. 3; for illustrative purposes, only data for H2B mRNA are presented). Thus, nutrient deprivation causes growth arrest and gadd153 expression, and although DNA synthesis is inhibited, histone mRNA levels are unaffected. To determine whether LLC-PK1 cells respond to chemical-induced growth arrest in a similar manner to the physiological (nutrient depletion) growth arrest, we examined the cellular response to chemical toxicants known to cause both DNA damage and growth arrest.

Linker Histone (H1) mRNA Down-regulation in Response to Quinone-thioether-mediated Cytotoxicity—Quinone-thioethers cause rapid disruption to chromatin in vivo (8) and single stranded breaks in DNA in renal epithelial cell culture (9). 2-Bis-(S-glutathionyl)hydroquinone and 2-Br-6-(glutathionyl-S-yl)
hydroquinone also cause a rapid inhibition of DNA synthesis and induce gadd153 expression in LLC-PK\(_1\) cells (10). We therefore utilized this model of chemically induced cell stress to compare the cellular response to physiologically and chemically induced growth arrest by examining the effects of quinone-thioethers on the expression of histone mRNA. 2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ caused a time- and concentration-dependent decrease in linker (H1) histone mRNA expression (Fig. 4). Suppression of H1 mRNA levels was maximal between 2 and 5 h after exposure of LLC-PK\(_1\) cells to a 200 \(\mu\)M concentration of either 2-Br-bis-(GSyl)HQ or 2-Br-6-(GSyl)HQ, and the latter conjugate was more potent than the former at decreasing the levels of H1 mRNA.

Core Histone (H2A, H2B, H3, H4) mRNA Down-regulation in Response to Quinone-thioether-mediated Cytotoxicity—Both 2-Br-bis-(GSyl)HQ (Fig. 5) and 2-Br-6-(GSyl)HQ (Fig. 6) caused a substantial reduction in H2A and H2B mRNA levels in LLC-PK\(_1\) cells, in a time- and concentration-dependant manner. Interestingly, H2A and H2B mRNA levels in LLC-PK\(_1\) cells exposed to the highest concentration of 2-Br-6-(GSyl)HQ (500 \(\mu\)M) were higher than in cultures exposed to 200 \(\mu\)M 2-Br-6-(GSyl)HQ. 2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ also decreased H3 and H4 mRNA levels (Figs. 5 and 6), but these effects were less pronounced than those seen with H2A and H2B, and exposure of LLC-PK\(_1\) cells to 200 \(\mu\)M 2-Br-bis-(GSyl)HQ or 2-Br-6-(GSyl)HQ for 5 h had little effect on H3 and H4 mRNA levels. Thus, quinone-thioethers, which cause single strand breaks in DNA (9, 10) and inhibit DNA synthesis (10), decrease histone gene expression in an apparently selective manner. Moreover, only toxicant-mediated growth arrest, and not nutrient deprivation-mediated growth arrest, affect histone gene expression in LLC-PK\(_1\) cells, indicating that the cellular response to the chemically induced stress differs from that of the physiologically induced stress.

The Effects of Combined Nutrient Depletion and Chemical Stress on gadd153 and Histone H2B mRNA—It seemed possible that differences in the cellular response to nutrient deprivation and chemical challenge might simply reflect different points on a continuum of a single stress response. In other words, the chemical stress, being more intense than the physiological stress, elicits a more intense stress response. LLC-PK\(_1\) cells were therefore treated with increasing concentrations of

2-Br-6-(GSyl)HQ for 2 h in either EBSS (nutrient-deprived cells) or DMEM. Interestingly, 2-Br-6-(GSyl)HQ-mediated decreases in histone H2B mRNA were less severe under conditions of nutrient depletion (Fig. 7). For example, at 50 \(\mu\)M 2-Br-6-(GSyl)HQ, H2B expression decreased 55% in DMEM but only 31% in EBSS. Moreover, nutrient depletion alone appears sufficient to induce maximal gadd153 expression in LLC-PK\(_1\) cells. Thus, after culturing cells for 2 h in EBSS, gadd153 mRNA levels are 6-fold higher than in cells cultured in DMEM (Fig. 8). The addition of 2-Br-6-(GSyl)HQ to EBSS actually decreases the gadd153 response to 86% (50 \(\mu\)M) and 79% (100 \(\mu\)M) of EBSS alone. In contrast, the chemical stress (2-Br-6-(GSyl)HQ) induces gadd153 expression only 3-fold in DMEM (Fig. 8). Finally, the ability of 2-Br-6-(GSyl)HQ to attenuate the gadd153 response is not merely a consequence of delaying the stress response. We examined gadd153 expression
out to 10 h. Nutrient depletion (EBSS) caused a continual increase in *gadd153* mRNA throughout the experiment (27-fold increase), and 2-Br-6-(GSyl)HQ blunted the response to nutrient depletion (11-fold increase), which reached a plateau by 10 h (Fig. 9). Clearly, the effects of nutrient depletion and chemical stress in LLC-PK1 cells are not additive, indicating that the growth arrest response to chemical stress (DNA damage) and nutrient deprivation are different.

Correlation between H2B Histone and hsp70 Expression—Because changes in chromatin structure are coupled to changes in histone function (gene expression) we examined the effects of 2-Br-6-(GSyl)HQ on *hsp70* expression, a stress gene whose expression is dependent upon the maintenance of appropriate nucleosomal structure (14, 15). H2B histone mRNA levels in LLC-PK1 cells exposed to increasing concentrations of 2-Br-6-(GSyl)HQ were inversely correlated with *hsp70* mRNA (Fig. 10). Thus, *hsp70* expression is elevated at 200 μM 2-Br-6-(GSyl)HQ, when H2B expression is at a nadir. At 500 μM 2-Br-6-(GSyl)HQ, H2B mRNA levels rebound, with a concomitant decline in *hsp70* mRNA.

The Role of Transcription and Translation in Histone Gene Expression in Response to Quinone-thioethers—To initially determine whether the decrease in histone mRNA by 2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ is regulated at the transcriptional and/or post-transcriptional level, we investigated the effects of inhibiting RNA synthesis on gene expression. LLC-PK1 cells were preincubated for 1 h with actinomycin D (10...
LLC-PK1 cells were exposed for 2 h to renaltubularepithelialcells.

Fe²⁺ requires protein synthesis (transcription factors) LLC-PK1 cells were incubated in DMEM (control) with cycloheximide (50 μg/ml) for 1 h prior to treatment for 2 h with 200 μM 2-Br-6-(glutathion- Syl)HQ. Total RNA was extracted, separated, and examined by Northern blot analysis. The level of 7 S rRNA was used as the internal control. Experiments were repeated a minimum of three times and produced similar results.

The Role of Iron, Hydrogen Peroxide, and Calcium in Quinone-thioether-mediated Decreases in Histone Gene Expression—Growth arrest induced by quinone-thioethers, and not by nutrient deprivation, causes a corresponding decrease in histone gene expression. The next series of experiments was therefore carried out to ascertain factors involved in down-regulating histone gene expression. The cytotoxicity of 2-Br-bis-(GSyl)HQ and 2-Br-6-(GSyl)HQ in LLC-PK₁ cells involves the generation of reactive oxygen species (9) most likely via the iron-catalyzed Haber-Weiss reaction, in which O₂⁻ undergoes dismutation to form hydrogen peroxide, and reduces Fe³⁺ to Fe²⁺. The hydrogen peroxide then reacts with Fe²⁺ to generate the highly reactive hydroxyl radical, which may be the species responsible for the DNA damage. To determine whether iron-dependant events are related to the decrease in histone mRNA, we investigated the effects of deferoxamine and catalase on histone gene expression. The chelation of iron with deferoxamine decreased H2B mRNA in the absence of added conjugate (Fig. 12), and no further decreases in H2B mRNA were observed when 2-Br-bis-(GSyl)HQ (data not shown) and 2-Br-bis-(GSyl)HQ were added after pretreatment of cells with deferoxamine (Fig. 12). Similar effects were seen with H1, H2A, H3, and H4 mRNA (data not shown). Concomitant with the decrease in H2B mRNA, DNA synthesis was significantly inhibited in deferoxamine-pretreated cells (Fig. 13). Scaevenging hydrogen peroxide with catalase totally prevented 2-Br-bis-(GSyl)HQ-mediated decreases in H2B mRNA (Fig. 12). Chelation of intracellular calcium with the cell-permeant EGTA-AM decreased levels of H2B mRNA in the presence and absence of 2-Br-bis-(GSyl)HQ (Fig. 14). Similar effects were seen with H1, H2A, H3, and H4 mRNA (data not shown). Therefore, 2-Br-bis-(GSyl)HQ activates a genomic stress response, involving decreases in histone gene expression, via a signaling pathway that may include iron, calcium, and reactive oxygen species.

**DISCUSSION**

DNA synthesis is rapidly inhibited in serum-deprived subconfluent cultures of renal proximal tubular epithelial cells (LLC-PK₁) (Fig. 1), but this physiological mode of growth arrest is insufficient to induce gadd153 expression (Fig. 2). However, removal of serum and nutrients, including glucose, from the culture medium is sufficient to induce the expression of gadd153 (Fig. 2). Chen et al. (16) also noted that culturing LLC-PK₁ cells in EBSS induces the expression of gadd153. The addition of glucose to serum- and nutrient-deprived LLC-PK₁
cells did not overcome the induction of gadd153 (Fig. 1). Glucose is not essential for energy production in cultured cells (17), and glutamine, not glucose, is the major energy source in HeLa cells (18). The mitochondrial glutamine to α-ketoglutarate pathway is also the major regulatory site of ammoniagenesis in LLC-PK₁ cells (19). Increased expression of gadd153 occurs in H4-II-E rat hepatoma cells grown in a limited amino acid medium (20). Glutamine can also serve as a precursor for pyrimidine nucleotides, via the glutamate-aspartate transamination reaction, and cells grow readily in sugar-free medium supplemented with uridine or cytidine (17). The absence of glutamine, rather than glucose, probably initiates gadd153 expression in LLC-PK₁ cells, and changes in the nucleotide pool may be a signal for gadd153 expression. Gadd153 is induced by growth arrest and DNA damage (3), and the nucleotide pool will also decline as DNA repair is initiated in cells experiencing DNA damage, again signaling gadd153 expression. The p53-dependent G₀–G₁ cell cycle arrest is induced by ribonucleotide depletion (21), suggesting that p53 may serve as a ribonucleotide sensor. However, although p53 lies upstream of gadd45 (22), it does not appear to control gadd153 expression (23).

The expression of histone genes is tightly coupled to DNA replication, but the levels of histone mRNA were unchanged in both serum- and nutrient-deprived LLC-PK₁ cells (Fig. 3), despite significant reductions in DNA synthesis (Fig. 2). Although changes in the level of histone mRNA occur between G₁ and S phase cells, the signal for rapid histone mRNA degradation appears to be confined to the S-G₂ border (24, 25), and histone mRNA levels only decline rapidly as cells exit the S phase of the cell cycle. Moreover, the half-life of histone mRNA is comparatively long during S phase, ranging from 110 min (25) to 4–5 h (26).

In contrast to the lack of effects of serum and nutrient deprivation on gadd153 and histone mRNA, chemical-induced growth arrest of LLC-PK₁ cells causes a decrease in both core and linker histone mRNAs (Figs. 4–6) and an increase in gadd153 mRNA (Figs. 8 and 9). Differences in the cellular response to nutrient deprivation and chemical challenge might simply reflect different points on a continuum of a single stress response. We therefore compared the effects of a chemical stress (2-Br-6-(GSyl)HQ exposure in DMEM) and the combination of chemical stress under conditions of nutrient depletion (2-Br-6-(GSyl)HQ exposure in EBSS). However, not only are the two stresses not additive, but decreases in H2B expression are attenuated when the chemical and nutrient stress are combined (Fig. 7). In addition, nutrient deprivation for 2 h causes a 6-fold increase in gadd153 mRNA, and the combination of nutrient deprivation and 2-Br-6-(GSyl)HQ actually decreases the gadd153 response (Fig. 8). The ability of 2-Br-6-(GSyl)HQ to attenuate the gadd153 response is not simply a consequence of a delay in the stress response. Nutrient depletion causes a continual increase in gadd153 mRNA, whereas the combination of nutrient depletion and 2-Br-6-(GSyl)HQ blunts the gadd153 response by 60% (Fig. 9). The chemical challenge, therefore, appears to interfere with pathways utilized by the cell to signal the physiological stress to the nucleus. Whether the same, albeit compromised, pathways are utilized to signal the chemical stress response is unclear, but likely involves the recruitment of alternative signaling pathways. Nonetheless, the genomic response to nutrient deprivation and chemically induced growth arrest in LLC-PK₁ cells is clearly not additive.

Chromatin structure plays an important role in regulating gene expression (27–30). For example, histones play an important role in maintaining chromatin structure and are intimately involved in the regulation of gene transcription (31, 32). The molecular mechanisms regulating gene expression in eukaryotes, therefore, include specific roles for the histone proteins. For example, when core histone H2A or H2B synthesis is repressed, the promoter elements of several inducible genes, which are normally repressed because of their incorporation into nucleosomes, become accessible to the basal transcriptional machinery (33, 34). Histones are therefore essential for nucleosomal structure and function. Interestingly, core histone H2A and H2B mRNAs are more sensitive to quinone-thioether-induced DNA damage than core histone H3 and H4 mRNA (Figs. 5 and 6). Because the H3/H4 histone tetramer forms the central core of the nucleosome, with H2A/H2B heterodimers capping each end of the core, H2A/H2B dimers are more readily displaced from the nucleosomal core than H3 and H4 (35). Histone mRNAs are rapidly degraded via the activation of a specific mRNA destabilization process (36) that is autoregulated by histone proteins and initiated by increases in cytoplasmic histones (37). Both core and linker histones induce the destabilization of histone H4 mRNA (38). Release of histones from nucleosomes during quinone-thioether-mediated nuclear disruption may therefore contribute to decreases in histone mRNA levels. Alternatively, quinones have been shown to inhibit ribonucleotide reductase (39), the first step in DNA synthesis. Such an effect may lead to a down-regulation in histone gene expression. However, this mechanism would not explain the differential effects of 2-Br-6-(GSyl)HQ on H2A/H2B and H3/H4 mRNA. Moreover, the activity of ribonucleotide reductase is reduced in serum-starved hamster kidney 21/C13 cells (40), and serum deprivation does not decrease histone mRNA in LLC-PK₁ cells (Fig. 3). Chemically induced changes to chromatin structure might therefore provide an important mechanism by which so-called “non-genotoxic” carcinogens influence gene expression and cell growth. Indeed, a variety of chemicals, including sodium butyrate, trichostatin A, and staurosporine, are known to alter the post-translational modification of histones, with corresponding changes in nucleosomal structure and function (41–53).

The compaction of nucleosomal templates containing histones H2A and H2B is more extensive than templates containing only H3/H4 tetramers, and removal of H2A and H2B from nucleosomal arrays enhances gene activity (54). Moreover, under conditions of efficient chromatin assembly, the hsp70 promoter is transcriptionally silent in Xenopus oocytes, and a role for chromatin structure in regulating the hsp70 promoter has been proposed (14, 15). Consistent with this scenario, levels of histone H2B mRNA in LLC-PK₁ cells exposed to increasing concentrations of 2-Br-6-(GSyl)HQ were inversely correlated with hsp70 mRNA (Fig. 10). Perhaps the increase in cytotoxicity at higher concentrations of 2-Br-6-(GSyl)HQ leads to an...
inhibition of protein synthesis with a complementary increase in histone mRNA stability by mechanisms described above. Alternatively, disruption to chromatin structure at higher concentrations of 2-Br-6-(GSyl)HQ may be sufficient to suppress the expression of hsp70, which appears to require precise assembly of the promoter template into chromatin for active transcription (14). This would imply that the structural requirements for gene transcription are less stringent for some genes than others.

Inhibiting protein synthesis with cycloheximide attenuated decreases in histone H2B mRNA (Fig. 11), consistent with the finding that the down-regulation of histone H1 caused by 1-β-d-arabinofuranosylcytosine is also prevented by cycloheximide (55). Inhibition of protein synthesis has been reported to prevent rapid degradation of histone mRNA (37), possibly by decreasing the interaction of a 45-kDa stem-loop-binding protein with the 3′-end of histone mRNA, which may be necessary for histone mRNA degradation (56). Thus, the regulation of histone mRNA following chemical stress in LLC-PK1 cells is in part mediated at the post-transcriptional level. Inhibition of transcription with actinomycin D virtually abolished histone H2B expression (Fig. 11), indicating that active transcription is required to maintain histone mRNA levels in these cells.

Quinone-thioether-mediated DNA damage and cytotoxicity requires the availability of Fe^{2+} (9). Treatment of LLC-PK1 cells with deferoxamine to chelate intracellular Fe^{2+} decreases H2B histone mRNA (Fig. 12) and DNA synthesis (Fig. 13). Iron is essential to maintain ribonucleotide reductase activity (57), the rate-limiting step in DNA synthesis (58), and deferoxamine has been shown to inhibit this enzyme (59). Iron is also required to maintain synthesis of the cell cycle-regulated protein p34^{cdk2} (60). Thus, intracellular iron concentrations, either directly or by modulating p34^{cdk2} synthesis and ribonucleotide reductase activity, influence cell cycle progression and histone gene expression. H2B histone mRNA levels in deferoxamine-treated cells were unaffected by treatment with 2-Br-6-(GSyl)HQ (Fig. 12), which is consistent with the ability of deferoxamine to prevent 2-Br-6-(GSyl)HQ-mediated DNA damage (10) and cytotoxicity (9, 10). Calcium also plays an important role in controlling cell proliferation and regulates progression through several cell cycle checkpoints, including the G_{1}/S phase transition, the G_{2}/M phase transition, and exit from mitosis (61). Calcium is also required for p34^{cdk2} histone H1 kinase activity (62). Therefore, decreasing intracellular calcium concentrations will delay cell cycle progression, decreasing the requirement for histone protein synthesis as DNA synthesis is suppressed. Chelation of intracellular calcium in LLC-PK1 cells with EGTA-AM decreases the constitutive expression of histone H2B mRNA and potentiates 2-Br-6-(GSyl)HQ-mediated decreases in H2B mRNA (Fig. 14). Although the relationship between intracellular calcium concentrations and histone gene expression is likely to be complex, our findings establish a link between the two.

In summary, renal tubular epithelial cells respond differently to physiologically and chemically induced stress. The response to physiological stress, induced by a combination of serum and nutrient depletion, involves growth arrest characterized by an inhibition of DNA synthesis that occurs in the absence of a decrease in histone mRNA or an increase in gadd153 mRNA. In contrast, a chemical-induced stress mediated by exposure of cells to quinone-thioethers involves growth arrest accompanied by a decrease in histone mRNA, particularly core histone H2B and H2A mRNA, and the induction of gadd153. Chemical-induced expression of hsp70 mRNA appears to be linked to decreases in histone mRNA.
52. Crissman, H. A., Gadbois, D. M., Tobey, R. A., and Bradbury, E. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88, 7580–7584
53. Abe, K., Yoshida, M., Usui, T., Horinouchi, S., and Beppu, T. (1991) *Exp. Cell Res.* 192, 122–127
54. Datta, R., Kharbanda, S., and Kufe, D. W. (1992) *Mol. Pharmacol.* 41, 64–68
55. Williams, A. S., Ingledue, T. C., Kay, B. K., and Marzluff, W. F. (1994) *Nucleic Acids Res.* 22, 4660–4666
56. Hansen, J. C., and Wolffe, A. P. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 2339–2343
57. Testa, U., Pelosi, E., and Peschle, C. (1993) *Crit. Rev. Oncol.* 4, 241–276
58. Elledge, S. J., Zhou, Z., and Allen, J. B. (1992) *Trends Biochem. Sci.* 17, 119–123
59. Fox, R. M. (1985) *Pharmacol. & Ther.* 30, 31–42
60. Terada, N., Or., R., Szepesi, A., Lucas, J. J., and Gelfand, E. W. (1993) *Exp. Cell Res.* 204, 260–287
61. van Dolah, F. M., and Ramsdell, J. S. (1996) *J. Cell Physiol.* 166, 49–56
62. Takuwa, N., Zhou, W., Kumada, M., and Takuwa, Y. (1993) *J. Biol. Chem.* 268, 138–145