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Nuclear Factor I/CCAAT Box Transcription Factor *trans*-Activating Domain Is a Negative Sensor of Cellular Stress

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ABSTRACT

The adaptive response to cellular stress requires the reprogramming of gene expression. So far, research has focused on induction mechanisms; several transcription factors activated by cellular stress have been shown to trigger the induction of repair and detoxification enzymes. Using the hepatoma cell line HepG2, we report that the *trans*-activating function of the nuclear factor I/CCAAT box transcription factor (NFI/CTF-1) is, on the contrary, repressed by various stress conditions, including inflammatory cytokine treatment, glutathione depletion, heat and osmotic shocks, and chemical stress. Under the same conditions, other transcription factors were not affected. We show that when Cys-427 within the *trans*-activating domain of NFI/CTF-1 is mutated into a serine, the repressive effect triggered by cellular stresses is no longer observed. In addition, this effect is abolished in cells transfected with a thioredoxin expression vector. Using the dichlorofluorescein fluorescent probe, we provide direct evidence that the stress conditions elicit an intracellular reactive oxygen species generation, which can, in turn, negatively regulate NFI/CTF-1. In agreement with these observations, we show that the CYP1A1 mRNA and the *CYP1A1* gene promoter, which is a target of NFI/CTF-1, are repressed by stress conditions. Thus, through the redox regulation of its *trans*-activating function, NFI/CTF-1 constitutes a novel biologically relevant negative sensor of several stress stimuli.

The response to environmental stress comprises the modulation of several cellular functions such as growth and differentiation, energetic metabolism, and gene expression. The latter point is an important feature of this response because the control of protein synthesis allows cells to adapt to environmental stress through the activation of enzymatic defenses (Brostrom and Brostrom, 1998). Biological macromolecules may be altered after cellular insults such as oxidative stress, xenobiotics afflux, heat shock, osmotic shock, laminar shear stress, or UV irradiation. Under such conditions, both repair and detoxification mechanisms can be activated. The repair process controls the integrity of the genome (Yu et al., 1999) and the correct structure of proteins (Cotto and Morimoto, 1999). In addition to repair mechanisms, detoxifying enzymes are activated upon cellular stress, particularly at the transcriptional level. The activation of the enzymatic defenses involves the modulation of the activity of critical transcription modulators that control the expression of stress-response genes [also called "immediate early" or "alert" genes (Morel and Barouki, 1999)].

A well-documented example of transcription factor activation is NF-κB (Legrand-Poels et al., 1997). This transcription factor is present in the cytosol but is inactive because of its interaction with a repressor I-κB. Several stimuli, including cellular stress, trigger the dissociation of I-κB from NF-κB, which then translocates into the nucleus and activates the transcription of target genes (Baeuerle, 1998). Other transcription factors, such as the p53 protein and heat shock factors, are also activated upon cellular stress (Sugano et al., 1995; Cotto and Morimoto, 1999). Moreover, some kinases are specifically involved in the stress response. The stress-activated protein kinases (Kyriakis et al., 1995) mediate a wide range of stress responses.

Several signaling pathways trigger the stimulation of stress-response genes. Physiological signals (such as cytokines) are released during infection or inflammation and can activate membrane receptors and/or downstream signaling pathways, leading to gene expression modulation (Kyriakis, 1999). Nuclear receptors that activate transcription when binding a xenobiotic ligand also play a major role in the

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ABBREVIATIONS: NF-κB, nuclear factor-κB; AP-1, activator protein-1; AhR, aryl hydrocarbon receptor; ROS, reactive oxygen species; NFI/CTF, nuclear factor 1/CCAAT box transcription factor; RSV, Rous sarcoma virus; TAD, *trans*-activating domain; AP-2, activator protein-2; CMV, cytomegalovirus; H_2DCF-DA, 2′,7′-dichlorofluorescein diacetate; DCF, dichlorofluorescein; GSH, glutathione; BSO, L-buthionine-(*S*,*R*)-sulfoximine; TCDD, tetrachlorodibenzo-*p*-dioxin; TNFα, tumor necrosis factor α.

induction of detoxifying enzymes. The aryl hydrocarbon receptor (AhR) activates several cytochrome P450 genes, as well as other xenobiotic-metabolizing enzymes and stress-response genes (Nebert et al., 1993). Other receptors bearing similar functions have been identified (Waxman, 1999), such as the peroxisome proliferator-activated receptor, the steroid and xenobiotic receptor.

Reactive oxygen species (ROS), such as H_2O_2, play an important role in the stress response. The transcription factors NF-κB, AP-1, and NF-E2 related factor 2 are activated by oxidative stress (reviewed in Sen and Packer, 1996; Morel and Barouki, 1999). ROS can activate specific kinases, but they can also directly modulate the activity of transcription factors through redox mechanisms (Morel and Barouki, 1999). Most studies have focused on the alteration of their DNA-binding activities by ROS (for a recent review, see Dalton et al., 1999). However, we have shown recently that intracellular oxidative stress could modulate the *trans*-activating domain of the NFI/CTF-1 transcription factor (Morel et al., 1999). This ubiquitous factor regulates the activity of a wide range of cellular and viral gene promoters, such as that of the collagen or the cytochrome P450 1A1 genes (Morel and Barouki, 1998, and references therein). This factor, originally identified as an activator of adenovirus DNA replication, is part of a family of transcription factors encoded by four different genes (*NFI-A*, *NFI-B*, *NFI-C/CTF-1*, and *NFI-X*) (Chaudhry et al., 1998). They form homo- or heterodimers that can bind the consensus $TGGCN_5GCCA$ sequence. In hepatoma cells, we previously showed that NFI/CTF-1 was the most abundant isoform and that its function was repressed by oxidative stress (Morel and Barouki, 1998). Recently, we have identified a cysteine residue within the *trans*-activating domain of NFI/CTF-1 as the regulatory target of H_2O_2 (Morel and Barouki, 2000). Because H_2O_2 is suspected to be a second messenger of some endogenous or environmental stimuli, we investigated whether the *trans*-activating function of NFI/CTF-1 was sensitive to various stress conditions.

Materials and Methods

Chemicals. H_2O_2 was used from a 30% stock obtained from Merck (Darmstadt, Germany) and TNFα was from Tebu (Le Peray, France). Other chemicals were obtained from Sigma (Saint-Quentin Fallavier, France).

Cell Culture. The human hepatoma cell line HepG2 was maintained as described elsewhere (Morel et al., 1999). These cells were used because NFI-driven gene promoters (such as that of CYP1A1) are regulated by H_2O_2 and because of their good transfection efficiency (Morel et al., 1999).

Plasmids. The construction of several plasmids used here has been described previously. Briefly, the firefly luciferase expression plasmid pG5-FL contains five Gal-4-binding sites. The *Renilla reniformis* luciferase expression plasmid pαglob-RL, which is not sensitive to oxidative stress (Morel and Barouki, 1998), was used as an internal control for transfection efficiency. The pRSV.Gal.CTF, pRSV.Gal.AP-2, and pRSV.Gal.Oct vectors are derived from the pGal(399–499), pGalAP2, and pGalOct plasmids (described in Alevizopoulos et al., 1995) in which the simian virus 40 promoters have been replaced by RSV promoters. This swap has been performed because the simian virus 40 promoter is highly sensitive to even moderate oxidative stress, whereas that of RSV is not (Morel and Barouki, 1998). They express fusion proteins containing the Gal-4 DNA-binding domain fused to the *trans*-activating domain (TAD) of the human NFI/CTF-1, AP-2, and Oct2 transcription factors, respectively. The pRSV.Gal.CTFmutC427S, which expresses a mutated fusion protein (Cys-427 within the TAD of NFI/CTF-1 replaced by a serine) was described elsewhere (Morel and Barouki, 2000). The pCMV-Trx plasmid, a kind gift from Dr. Fradellizzi (INSERM, Paris), expresses the human thioredoxin under the control of the cytomegalovirus (CMV) promoter. pcDNA 1.1 AmpR (Invitrogen, San Diego, CA), an empty expression vector also named pCMV-MCS in this study, was used as a control for pCMV-Trx. The p1A1-FL and pmut1A1-FL plasmids have already been described (Morel and Barouki, 1998). The p1A1-FL vector expresses the firefly luciferase gene driven by 1.6 kb of the human *CYP1A1* gene promoter. The pmut1A1-FL is identical with p1A1-FL, except for a double mutation in the NFI-binding site located within the proximal promoter.

Transfection Experiments. Transfection experiments were performed in HepG2 cells as described previously (Morel et al., 1999). Briefly, 1 day before the transfection, cells (0.5×10^6 cells/6-cm dish) were seeded into the usual culture medium. The vectors expressing the fusion protein (2.5 μg), firefly and *Renilla* luciferase expression vectors (2 and 1 μg, respectively), were introduced into the cells by the calcium phosphate coprecipitation technique, followed 4 h later by a 2-min glycerol shock. Five hours later, cells were exposed to stress conditions by adding chemicals to the culture medium or, in the case of heat shock, by incubating them at 42°C for 45 min. After an overnight incubation, cells were homogenized for enzymatic assays. Dual luciferase assay (firefly and *Renilla*) was performed with a Promega kit (Promega, Madison, WI) according to the manufacturer's instructions. *Renilla* luciferase activity was used to normalize the transfection efficiency in all culture dishes. Blanks were obtained by assaying luciferase activity in mock-transfected cells. Results were expressed as (firefly luciferase − blank)/(*Renilla* luciferase − blank).

Intracellular H_2O_2 Generation Assay. The oxidation-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) is a nonpolar compound that readily diffuses into cells, where it is hydrolyzed by endogenous esterases (Royall and Ischiropoulos, 1993). The resulting compound is not fluorescent but yields the fluorescent DCF when oxidized. HepG2 cells were cultured in six-well plates (Costar, Corning, NY). Eighteen hours after chemical addition mimicking cellular stress, H_2DCF-DA (200 μM) was added directly to the culture medium, and cells were cultured in standard conditions for 1 h. In the case of heat shock, H_2DCF-DA was added directly after a 45-min incubation at 42°C. The fluorescence of DCF was then measured with a Bio-Tek FL-600 fluorimeter (Fisher, Elancourt, France) using 485 and 530 nm as excitation and emission wavelengths, respectively. In each well (diameter, 3.5 cm), 109 measurements were made with an optic of 3 mm in diameter to cover the whole well surface. The result given for each well was expressed as the addition of the 109 values obtained.

Northern Blots. Total RNA preparation was performed with the RNA Easy Midi Kit (Qiagen, Les Ulis, France). Northern blots were performed as described previously (Morel et al., 1999). Probes were synthesized from cDNAs with the Megaprime DNA-labeling kit (Amersham Pharmacia Biotech, Saclay, France) according to the manufacturer's instructions. Quantifications were performed with a PhosphorImager and ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Statistics. Student's two-tailed *t* tests were performed using a Statview software (Abacus Concepts, Inc., Berkeley, CA).

Results

The *trans*-Activating Function of NFI/CTF-1 Is Specifically Repressed by Various Cellular Stresses. In a previous study, we have shown that both the DNA-binding and *trans*-activating functions of the transcription factor NFI/CTF-1 were sensitive to oxidative stress (Morel and Ba-

rouki, 1998). However, these functions are not regulated by oxidative stress in a similar manner. In human hepatoma cells (HepG2), DNA binding is repressed by millimolar H_2O_2 concentrations, whereas the *trans*-activating function is repressed by micromolar concentrations. Because the TAD of NFI/CTF-1 was particularly sensitive to H_2O_2, we tested the effect of other cellular stresses on the transcriptional activity of this transcription factor. For this purpose, we cotransfected HepG2 cells with vectors expressing Gal-4 fusion proteins containing the DNA-binding domain of the yeast Gal-4 transcription factor fused to the TAD of mammalian transcription factors and the pG5-FL vector containing the firefly luciferase reporter gene driven by a Gal-4-sensitive promoter. Therefore, the firefly luciferase reporter gene expression reflects the *trans*-activating efficiency of the TAD.

In the following experiments, cell cultures underwent various stress conditions for 16 h before reporter gene assay. Inflammation was mimicked by the treatment of cell cultures with the inflammatory cytokine TNFα. The role of intracellular glutathione (GSH) content was also investigated. GSH level is a sensor of cellular stress (Wilhelm et al., 1997): its intracellular concentration drops upon oxidative stress or xenobiotic afflux. Here, GSH depletion was achieved using buthionine-(*S*,*R*)-sulfoximine (BSO) as an inhibitor of γ-glutamylcysteine synthase. This enzyme is rate limiting in the synthesis of GSH, and its inhibition causes a strong depletion of the intracellular GSH pool (in HepG2 cells, the use of 50 μM BSO caused a 75% decrease of the GSH pool, data not shown). In addition, physical stresses (heat and osmotic shocks) were also tested. The heat shock consisted of incubating culture dishes at 42°C for 45 min, followed by further incubation at the normal temperature of 37°C. Osmotic shock was achieved by addition of 100 mM sorbitol to the culture medium. The effect of chemical challenge was tested by treatments with either rifampicine or benzo(*a*)pyrene. The metabolism of benzo(*a*)pyrene leads to DNA alteration, and several studies have shown that it is a potent carcinogen (Guengerich and Shimada, 1998). Rifampicine is a common macrolide antibiotic. In our cellular model, both xenobiotic compounds induce and interfere with the metabolism driven by detoxification enzymes such as cytochromes P450 (Morel et al., 1999; Sumida et al., 1999). All of these conditions did not affect the growth nor the aspect of cells. We previously showed, using cytotoxicity assays, that the HepG2 cell line was resistant to several stress conditions, including treatment with H_2O_2, TNFα, BSO, or benzo(*a*)pyrene without any significant loss of viability (Morel and Barouki, 1998; Morel et al., 1999).

As shown in Fig. 1, all the stress conditions that we tested repressed the activity of the TAD of NFI/CTF-1 by up to 65% (compare bars 2–7 with bar 1). The most potent repression was observed with osmotic shock. These regulations did not result from a global repression of transcriptional mechanisms. Indeed, in control experiments, the TADs of two other transcription factors, AP-2 and Oct, were used. The basal activity of the AP-2 TAD was similar to that of NFI/CTF-1 (compare bars 8 and 1), whereas that of the Oct TAD was 50% lower (compare bars 15 and 1). Using the same stress conditions, no significant variation of the AP-2-*trans*-activating function was observed (compare bars 9–14 with bar 8). In the case of Oct, only limited positive or negative variations were observed (compare bars 16–21 with bar 15).

ROS Are Produced After Cellular Stress. Several stimuli have been suggested to trigger the production of ROS as second messenger (reviewed in Morel and Barouki, 1999). In our experimental model, we have assessed the intracellular

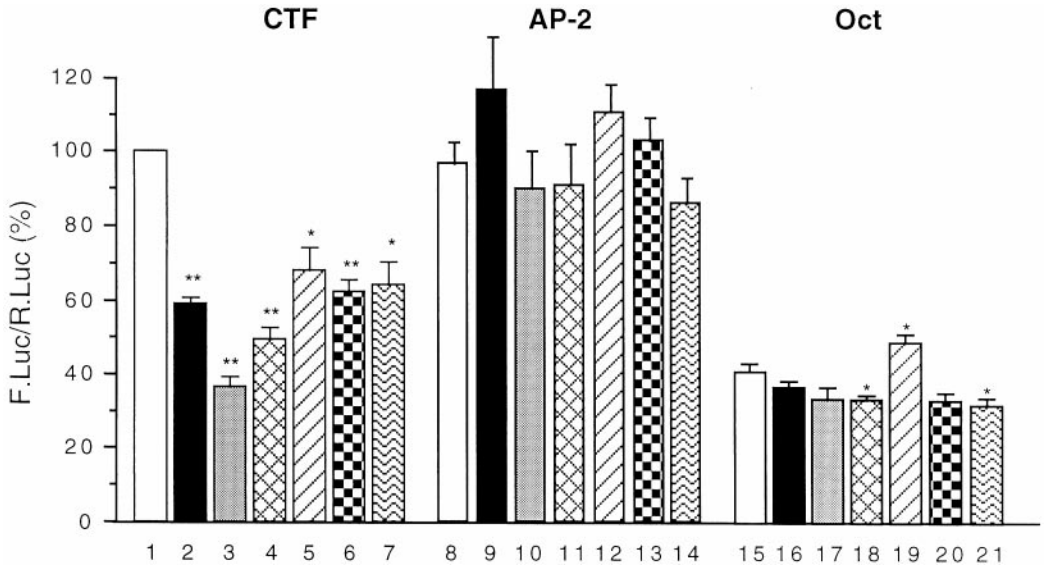

Fig. 1. Effect of cellular stress on the activity of various TADs. Cells were transfected with pG5-FL as a reporter vector and pαglob-RL as an internal control. They were cotransfected with pRSV.Gal.CTF (lanes 1–7), pRSV.Gal.AP-2 (lanes 8–14), or pRSV.Gal.Oct (lanes 15–21) expression vectors. Cell cultures were left untreated (bars 1, 8, and 15) or underwent various stress conditions and were harvested 16 h later. The chemicals were used at the following concentrations: TNFα, 5 ng/ml (bars 2, 9, and 16); sorbitol, 100 mM (osmotic shock; bars 3, 10, and 17); BSO, 50 μM (glutathione depletion; bars 5, 12, and 19); rifampicine, 50 μM (bars 6, 13, and 20); and benzo(*a*)pyrene, 2.5 μM (bars 7, 14, and 21). The dimethyl sulfoxide solvent vehicle (0.1%, v/v) had no effect. Heat shock was achieved by a 45-min incubation at 42°C, 16 h before cell harvest (bars 4, 11, and 18). Firefly and *Renilla* luciferases and were assayed as described under *Materials and Methods.* Results are expressed as [firefly luciferase activity (F. Luc)/*Renilla* luciferase (R. Luc) activity] (mean ± S.E.M., $n \geq 8$). One hundred percent corresponds to the Firefly luciferase/*Renilla* luciferase ratio in untreated control cells transfected with pRSV.Gal.CTF. For each Gal-4 fusion, statistically significant differences between stressed cells and the corresponding untreated controls (i.e., lanes 1, 8, and 15, respectively) are indicated: $^*P < .05$ and $^{**}P < .005$. □, untreated control; ■, TNFα; ▨, osmotic shock; ▩, heat shock; ◪, glutathione depletion; ⊠, rifampicine; ▧, benzo(*a*)pyrene.

production of ROS after stress challenges. This production in cultured HepG2 cells was assayed as described under *Materials and Methods* using H_2DCF-DA as a probe. When oxidized within the cell by ROS, especially H_2O_2, H_2DCF-DA yields DFC, a fluorescent compound (Royall and Ischiropoulos, 1993). We have previously shown in this system that BP elicits an H_2O_2 production and the subsequent fluorescence of DCF, which was abolished by catalase addition (Morel et al., 1999). Here we show that all the cellular stress that we tested induced an intracellular production of ROS (Fig. 2). The increase in intracellular ROS content was more potent in the case of TNFα, BP treatment, or osmotic shock (88% increase).

Cys-427 Mediates the Repression of NFI/CTF-1 by Cellular Stress. The TAD of NFI/CTF-1 contains two cysteine residues (Cys-405 and Cys-427). We have previously shown that Cys-427 was critical for the repression of the transcriptional activity of this factor by H_2O_2, whereas Cys-405 was not involved in this regulation (Morel and Barouki, 2000). Moreover, the same study suggested that the H_2O_2-mediated repressive effect was unlikely to involve a kinase pathway. In the present study, we have evaluated the role of Cys-427 in the regulation of the TAD of NFI/CTF-1 by the stress conditions described above. As shown in Fig. 3, the Cys-427-Ser mutation did not affect the basal activity of the TAD (compare lanes 8 and 1). However, this mutation totally abolished the repressive effect of all the stress conditions implemented in our experiments. Indeed, although the activity of the wild-type TAD was strongly repressed by these stress conditions (compare bars 2 to 7 with the untreated control bar 1), the activity of the mutated TAD did not display significant variations (compare bars 9–14 with untreated control bar 8). Thus, it seems that Cys-427 is required to mediate the repression of NFI/CTF-1 transcriptional activity by several cellular stresses: inflammatory cytokine treatment, heat and osmotic shock, glutathione depletion, and xenobiotic afflux.

Thioredoxin Prevents the Repression of NFI/CTF-1-*trans*-Activating Function by Cellular Stress. Because we observed that the stress conditions used here caused an intracellular ROS production and that Cys-427 was critical for the repressive effect, we hypothesized that the TAD of NFI/CTF-1 could undergo a direct oxidation and that natural antioxidant could reverse this effect. Thioredoxin is a 12-kDa protein able to migrate into the nucleus, where it can functionally interact with several nuclear proteins such as redox factor 1 (Hirota et al., 1997) and hormone nuclear receptors (Makino et al., 1999). The thioredoxin active site contains two close cysteine residues in a conserved Cys-Gly-Pro-Cys motif that can switch from dithiol to disulfide and thus reduce oxidized cysteine residues (Qin et al., 1994). In our experiments, the transfection of a thioredoxin expression vector did not significantly modify the basal transcriptional activity of NFI/CTF-1 (Fig. 4). However, it totally prevented the strong repressive effect of H_2O_2 on this activity. In addition, thioredoxin expression also abolished the repression of the TAD of NFI/CTF-1 caused by glutathione depletion and rifampicine or TNFα treatments. It also clearly limited the effect of heat and osmotic shocks and that of benzo(α)pyrene treatment. The protective efficiency of thioredoxin seems to be different for the various stress conditions tested. These differences could be related to kinetic parameters, but we do not have evidence for this. Thus, all these stress conditions trigger the repression of the transcriptional activity of NFI/CTF-1 by a signaling pathway that is sensitive to thioredoxin and is therefore likely to involve the oxidation of a cysteine. Data from Fig. 3 suggest that Cys-427 within the TAD of NFI/CTF-1 is a likely target of this redox mechanism. However, these data do not allow us to assess whether a direct thioredoxin-Cys-427 interaction occurs. The possibility that

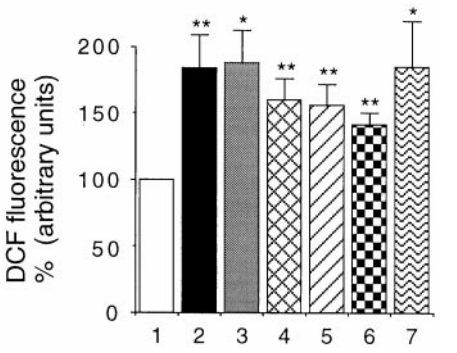

Fig. 2. Stress conditions cause intracellular ROS generation. H_2O_2 levels within HepG2 cells were assayed as described under *Materials and Methods.* Cell cultures were left untreated (bar 1) or underwent various stress conditions as described in Fig. 1: TNFα treatment (bar 2), osmotic shock (bar 3), heat shock (bar 4), glutathione depletion (bar 5), rifampicine (bar 6) and benzo(α)pyrene treatment (bar 7). Eighteen hours after the addition of chemicals mimicking cellular stress, plates were read in a fluorimeter, and fluorescence is expressed in arbitrary units. Results are expressed as means ± S.E.M. (n = 6), normalized to 100% for control cells. Statistical differences compared with the untreated control are indicated: *P < .05 and **P < .01.□, untreated control; ■, TNFα; ▨, osmotic shock; ▩, heat shock; ▨, glutathione depletion; ▩, rifampicine; ▨, benzo(α)pyrene.

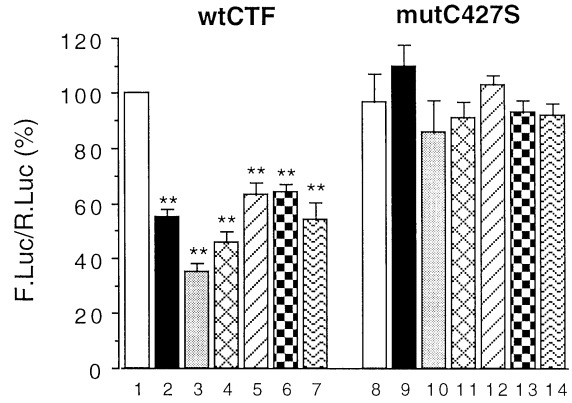

Fig. 3. Cys-427 within the TAD of NFI/CTF-1 mediates the repression by cellular stress. Cells were transfected with pG5-FL as a reporter vector and pαglob-RL as an internal control. They were cotransfected with either pRSV.Gal.CTF (lanes 1–7) or pRSV.Gal.CTF mutC427S (lanes 8–14) expression vectors. Cell cultures were left untreated (bars 1 and 8) or underwent various stress conditions as described in Fig. 1: TNFα treatment (bars 2 and 9), osmotic shock (bars 3 and 10), heat shock (bars 4 and 11), glutathione depletion (bars 5 and 12), rifampicine (bars 6 and 13) and benzo(α)pyrene treatment (bars 7 and 14). Cells were harvested 16 h later. Firefly and *Renilla* luciferases were assayed as described under *Materials and Methods*. Results are expressed as [firefly luciferase (F. Luc) activity/*Renilla* luciferase (R. Luc) activity] (mean ± S.E.M., $n \geq 8$). One hundred percent corresponds to the firefly luciferase/*Renilla* luciferase ratio in untreated control cells transfected with pRSV.Gal.CTF. For the wild-type (wt) and mutated (mut) TADs, statistically significant differences between stressed cells and the corresponding untreated controls (i.e., lanes 1 and 8) are indicated: **P < .001. □, untreated control; ■, TNFα; ▨, osmotic shock; ▩, heat shock; ▨, glutathione depletion; ▩, rifampicine; ▨, benzo(α)pyrene.

the protective role of thioredoxin stems from a mere ROS-sink effect cannot be excluded.

The *CYP1A1* Gene Is Repressed by Cellular Stress Conditions. The cytochrome P450 1A1 gene (*CYP1A1*) is a member of the multigenic cytochrome P450 family. It is highly inducible at the transcriptional level by polycyclic aromatic compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 3-methylcholanthrene, and benzo(*a*)pyrene (Morel et al., 1999, and references therein), which are ligands of the AhR. The promoter of this gene contains two main functional regions: the so-called xenobiotic responsive elements, which are recognized by the activated AhR, and the proximal promoter, which contains binding sites for the Sp1 and NFI transcription factors. We have previously reported that the NFI transcription factor played an important role in the *trans*-activation of the *CYP1A1* gene promoter (Morel and Barouki, 1998). Its *trans*-activating domain displays a functional synergy with the AhR-signaling pathway (Morel et al., 1999). Thus, we investigated whether the stress conditions that inhibit NFI-*trans*-activating function altered the activity of the *CYP1A1* gene promoter. As shown in Fig. 5A, all the cellular stresses tested in this experiment decreased the mRNA levels of the *CYP1A1* gene in TCDD-treated cells. These results are in agreement with previous studies of the repression of the *CYP1A1* gene by oxidative stress and inflammatory cytokines (Morel and Barouki, 1999, and references therein). Some conditions (such as osmotic shock) are more effective, which is consistent with other data presented in this study (ROS generation, NFI/CTF-1 repression).

Using the p1A1-FL vector, which expresses the firefly luciferase reporter gene driven by the *CYP1A1* gene promoter, we tested whether the repression of the *CYP1A1* gene oc-

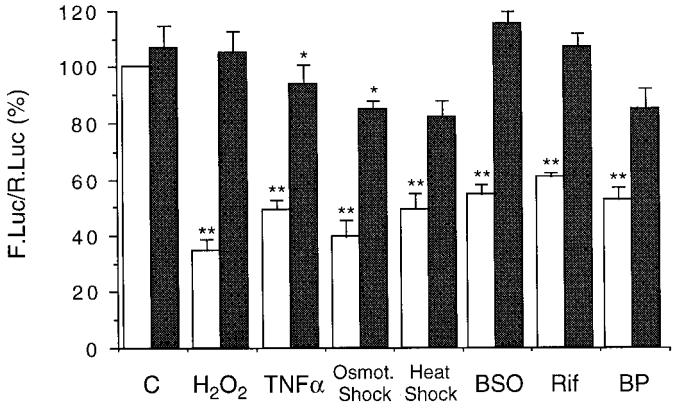

Fig. 4. Thioredoxin prevents the inhibition of NFI/CTF-1 TAD by cellular stress. Cells were transfected with pG5-FL as a reporter vector, pαglob-RL as an internal control, and the pRSV.Gal.CTF expression plasmid. In order to express the thioredoxin protein, cells were cotransfected with 250 ng of the pCMV-Trx expression vector (filled bars). In control cells (open bars), a plasmid lacking the thioredoxin cDNA (pCMV-MCS) was transfected to have an equivalent amount of total transfected DNA. Cell cultures underwent various stress conditions as described in Fig. 1. In addition, the effect of H_2O_2 (50 μM) was investigated. Cells were harvested 16 h after treatments. Firefly and *Renilla* luciferases were assayed as described under *Materials and Methods*. Results are expressed as [firefly luciferase (F. Luc) activity/*Renilla* luciferase (R. Luc) activity] (mean ± S.E.M., $n \geq 8$). One hundred percent corresponds to the firefly luciferase/*Renilla* luciferase ratio in untreated control cells transfected with pCMV-MCS. Statistically significant differences with this control are indicated: $^*P < .05$ and $^{**}P < .001$. □, CMV-MCS; ■, CMV-Trx.

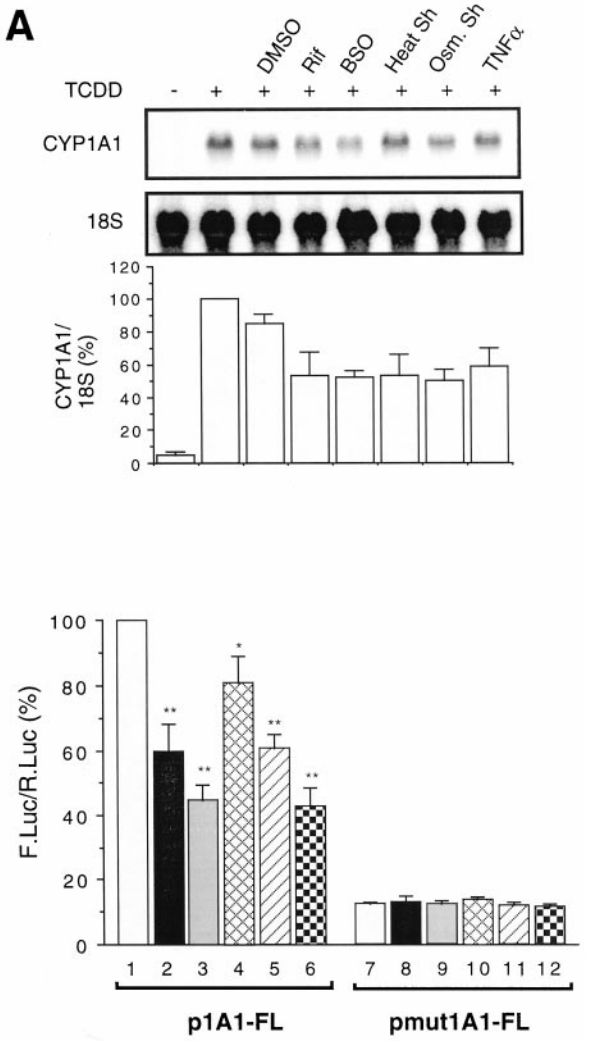

Fig. 5. The CYP1A1 gene is inhibited by various cellular stresses. A, CYP1A1 mRNA levels are decreased by cellular stress. Cells were treated with 3 nM TCDD except for the control lane. Cell cultures were left untreated or underwent various stress conditions as mentioned in the same conditions as described in Fig. 1 and below. The level of CYP1A1 mRNA was normalized to that of ribosomal 18S RNA. The mean ± S.E.M. of three experiments is shown on the diagram. One hundred percent corresponds to cells treated with TCDD but that underwent no cellular stress. DMSO, dimethyl sulfoxide; Rif, rifampicine. B, the *CYP1A1* gene promoter activity is repressed by cellular stress. Cells were transfected with either p1A1-FL or pmut1A1-FL as a reporter vector and pαglob-RL as an internal control. In order to stimulate the *CYP1A1* gene promoter, cells were treated with 3 nM TCDD. Cell cultures were left untreated (bars 1 and 7) or underwent various stress conditions as described in Fig. 1: TNFα treatment (bars 2 and 8), osmotic shock (bars 3 and 9), heat shock (bars 4 and 10), glutathione depletion (bars 5 and 11), rifampicine treatment (bars 6 and 12). The dimethyl sulfoxide solvent vehicle used for rifampicine (0.1%, v/v) did not influence promoter activities (97 ± 10 and 109 ± 12% for p1A1-FL and pmut1-FL, respectively, not shown). Cells were harvested 16 h after treatments. Firefly and *Renilla* luciferases were assayed as described in the Methods section. Results are expressed as [firefly luciferase (F. Luc) activity/*Renilla* luciferase (R. Luc) activity] (mean ± S.E.M., $n \geq 8$). One hundred percent corresponds to the firefly luciferase/*Renilla* luciferase ratio in cells transfected with p1A1-FL and undergoing no stress condition. For p1A1-FL-transfected cells, statistically significant differences with this control are indicated: $^*P < .05$ and $^{**}P < .005$. □, untreated control; ■, TNFα; ▨, osmotic shock; ▩, heat shock; ◪, glutathione depletion; ⊠, rifampicine.

curred at the transcriptional level. As shown in Fig. 5B, all the cellular stress tested in the experiment significantly decreased the promoter activity (compare bars 2–6 with unstressed control bar 1). In these experiments, BP was not tested because it is an inducer of both *CYP1A1* gene promoter activity and intracellular ROS generation. This question was specifically addressed previously and the metabolism of BP, which generates H_2O_2, was shown to limit *CYP1A1* gene expression (Morel et al., 1999).

When the NFI site located within the proximal promoter of the *CYP1A1* gene was mutated, the activity of the promoter was strongly decreased (compare bars 1 and 7). However, the mutated promoter can still be induced by TCDD (although less than that of the wild-type promoter) and, in the presence of this compound, displays an activity that is higher than that of the basal wild-type promoter (see Table 1). These data are in agreement with previous results underlying the role of NFI in the activity of the *CYP1A1* gene promoter (Morel and Barouki, 1998). Interestingly, under the same stress conditions as above, the mutated promoter was not affected by cellular stress (compare bars 8 to 12 with bar 7). It should be added that, in the same experiments, the basal (i.e., non induced with TCDD) activity of the wild-type was repressed by stress conditions (data not shown).

These data suggest that the NFI transcription factor is involved in the mediation of the repressive effect caused by cellular stresses on the activity of the *CYP1A1* gene promoter. These results are consistent with a previous study in our laboratory of the regulation of this promoter by oxidative stress (Morel and Barouki, 1998).

Discussion

In this article, we have studied a signaling pathway leading to transcriptional repression by several cellular stresses. This pathway involves ROS as second messengers of the stress stimuli. Various mechanisms may lead to the production of ROS (reviewed in Morel and Barouki, 1999), such as mitochondrial metabolism dysfunction or stimulation (Schulze-Osthoff et al., 1993; Esposito et al., 1999) and the activation of NADPH oxidases or myeloperoxidase [in the case of phagocytes (Hampton et al., 1998)]. In addition, the catalytic activity of several oxidases and mono-oxygenases may lead to ROS release (Puntarulo and Cederbaum, 1998). A wide range of either endogenous or environmental stimuli could be associated with intracellular ROS production. For example, it has been shown that infection and inflammation activate the oxidases of phagocytes as a defense mechanism. The inflammatory cytokine TNFα causes a disruption of the mitochondrial electron transport chain, resulting in intracellular ROS release (Schulze-Osthoff et al., 1993). Moreover, the afflux of xenobiotic compounds (i.e., chemical stress), such as redox-cycling agents and cytochromes P450 uncoupled substrates (Morel et al., 1999), may cause intracellular ROS generation. Within the cellular context, several cytochrome P450 isoforms are typical H_2O_2-generating enzymes (Puntarulo and Cederbaum, 1998). In the hepatoma cell model used in this study, we have previously shown that CYP1A1 and CYP2E1 activity generates H_2O_2 (Morel et al., 1999, 2000). UV irradiation was also shown to induce H_2O_2 generation within the cell (Hockberger et al., 1999). Other stress conditions were suggested to be associated with H_2O_2 production (reviewed in Morel and Barouki, 1999). For example, osmotic shock activates p38 (Nadkarni et al., 1999), a kinase typically activated by H_2O_2 (Clerk et al., 1998). Moreover, heat shock and oxidative stress elicit a similar activation of heat shock factors and the expression of heat shock proteins (Morano and Thiele, 1999). Physical stresses that activate NF-κB were also suspected to have ROS as second messengers: for example, laminar shear flow (Hsieh et al., 1998) and endoplasmic reticulum overload (Pahl and Baeuerle, 1997). In the present study, we have shown that, in an hepatocyte-derived cell line, several stress conditions induce an increase in intracellular ROS, including TNFα treatment, osmotic and heat shocks, glutathione depletion, and xenobiotic afflux [benzo(α)pyrene and rifampicine]. Thus, among other intracellular mediators such as calcium, nitric oxide, or ceramide, ROS—presumably H_2O_2—seems to be an important second messenger triggering the stress response.

A large number of studies have reported that cellular stress modulates the expression of gene expression (Brostrom and Brostrom, 1998). Owing to the historical focus on gene inductions, most studies have addressed positive modulations. In contrast, we report here that the *trans*-activating function of NFI/CTF-1 is repressed by various stress conditions. Our experiments suggest that the mechanism involves the direct oxidation of the TAD because 1) all the stress conditions tested caused an intracellular ROS generation, 2) Cys-427 within the TAD is required to mediate the repressive effect, and 3) thioredoxin, an endogenous thiol reducer, prevents this repression. However, further biochemical studies are required to ascertain the role of Cys-427 and its oxidation within the native NFI protein. In addition, such studies could allow the detection of a direct interaction between NFI and thioredoxin.

The modulation of NFI/CTF-1-*trans*-activating function could have a biological relevance. Indeed, this transcription factor plays a major role in the regulation of the expression of a wide range of genes. For example, as shown in this study, this transcription factor seems to be a critical mediator in the modulation of the *CYP1A1* gene transcription by various cellular stress conditions. In addition, the TAD of NFI/CTF-1 displays functional synergies with other transcriptional signaling pathways, such as those involving the estrogen receptor (Martinez et al., 1991) and the Ah receptor (Morel et al., 1999). It was also shown to interact directly with the TATA box binding protein (Xiao et al., 1994) and the cAMP response element-binding protein/p300 coactivator (Leahy et al., 1999). Moreover, NFI/

TABLE 1

Effect of TCDD on wild-type and mutated *CYP1A1* gene promoter

HepG2 cells were transfected with either the p1A1-FL or pmut1A1-FL vector. Cells were treated or not with 3 nM TCDD. The relative levels of expression of the firefly luciferase reporter gene driven by either the wild-type or the NFI-mutated *CYP1A1* gene promoter are shown.

Reporter Vector	p1A1-FL		pmut1A1-FL	
	TCDD−	TCDD+	TCDD−	TCDD+
Relative reporter activity[a]	1.0	25.8 ± 7.6	0.3 ± 0.1	3.1 ± 0.4

[a] HepG2 cells were transfected and reporter gene activity assessed as described in Fig. 5B. The activity of the wild-type *CYP1A1* gene promoter was arbitrarily set at 1.

CTF-1 also interacts with histones H1 and H3 (Alevizopoulos et al., 1995) REM, and its TAD contains a peptide sequence homologous with the RNA polymerase II carboxyl-terminal domain (Xiao et al., 1994), which might interact with other nuclear proteins. Apart from the *CYP1A1* gene regulation, it thus seems that the stress-induced repression of the activity of NFI/CTF-1 TAD is likely to interfere with the expression of several genes.

Previous data showing that NFI/CTF-1-*trans*-activating function is particularly sensitive to H_2O_2 compared with other transcription factors (Morel and Barouki, 2000), and data from this study suggest that NFI/CTF-1 could be an integrator of cellular stress via its oxidative repression. It seems to be a negative sensor of stress stimuli (in contrast to NF-κB). In this respect, NFI/CTF-1 could play an important role in the stress response that allows cells to adapt to physiological or environmental conditions through a reshaping of the transcriptome. As summarized in Fig. 6, ROS produced as second messengers can both activate and repress specific transcriptional modulators. On one hand, ROS are known activators of several transcription factors [AP-1, NF-kB, and NF-E2 related factor 2 (reviewed in Sen and Packer, 1996; Dalton et al., 1999; Morel and Barouki, 1999)] and thus trigger the expression of immediate early or alert genes, such as Egr-1, gadd153, p53, or p21. These positive modulations allow the activation of repair and detoxifying enzymes that are necessary to adapt. On the other hand, in addition to these inductions, it is also important to repress genes that can cause further cellular stress [such as *CYP1A1* or *CYP2E1*, which cause an intracellular oxidative stress when overexpressed (Morel et al., 1999, 2000)]. We show here that NFI/CTF-1 mediates the repression of the *CYP1A1* gene promoter by stress conditions that induce an intracellular ROS production. Moreover, under stress conditions, the cellular metabolism is reorganized to achieve energy savings (Hand and Hardewig, 1996). The global synthesis of proteins is slowed down (apart from the above-mentioned specific induction of stress-response enzymes). Thus, a global repression of nonessential gene transcription could be an important feature of the stress response. The functional repression of ubiquitous transcription factors, such as NFI/CTF-1 [and also Sp1 (Wu et al., 1996)], by H_2O_2 could thus be involved in this global mechanism.

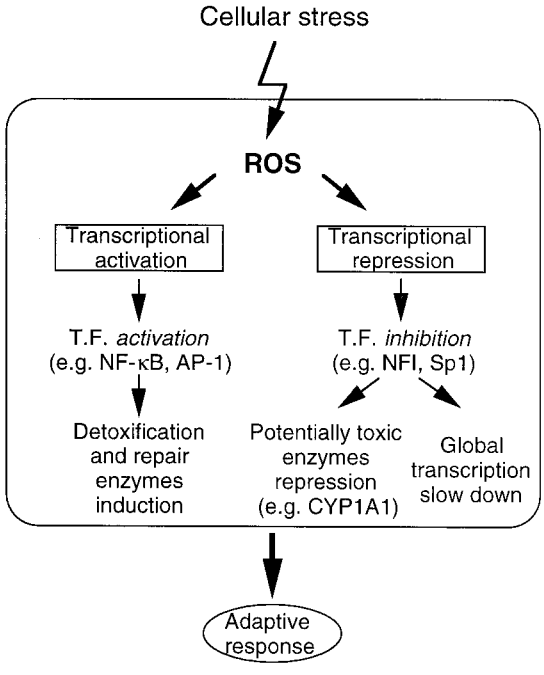

Fig. 6. The cellular stress response comprises both induction and repression of gene expression. This scheme summarizes one aspect of the cellular stress response regarding gene expression regulation. Several stress conditions elicit an intracellular H_2O_2 production. This second messenger triggers either kinase pathway stimulation or direct oxidation of transcriptional modulators. Several transcription factors (T.F.) are activated, which leads to the induction of repair and detoxification enzymes. However, other transcription factors are repressed, which leads to the inhibition of potentially toxic genes and may also contribute to the global slow-down of transcription to save energy during the stress response.

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