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The Activation of c-Jun NH_2-terminal Kinase (JNK) by DNA-damaging Agents Serves to Promote Drug Resistance via Activating Transcription Factor 2 (ATF2)-dependent Enhanced DNA Repair*

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The activating transcription factor 2 (ATF2) is a member of the ATF/cAMP-response element-binding protein family of basic-leucine zipper proteins involved in cellular stress response. The transcription potential of ATF2 is enhanced markedly by NH_2-terminal phosphorylation by c-Jun NH_2-terminal kinase (JNK) and mediates stress responses including DNA-damaging events. We have observed that four DNA-damaging agents (cisplatin, actinomycin D, MMS, and etoposide), but not the cisplatin isomer, transplatin, which does not readily damage DNA, strongly activate JNK, p38, and extracellular signal-regulated kinase (ERK), and strongly increase phosphorylation and ATF2-dependent transcriptional activity. Selective inhibition studies with PD98059, SB202190, SP600125, and the dominant negative JNK indicate that activation of JNK but not p38 kinase or ERK kinase is required for the phosphorylation and transcriptional activation of ATF2. Stable expression of ATF2 in human breast carcinoma BT474 cells increases transcriptional activity and confers resistance to the four DNA-damaging agents, but not to transplatin. Conversely, stable expression of a dominant negative ATF2 (dnATF2) quantitatively blocks phosphorylation of endogenous ATF2 leading to a marked decrease in transcriptional activity by endogenous ATF2 and a markedly increased sensitivity to the four agents as judged by decreased cell viability. Similarly, application of SB202190 at 50 μM or SP600125 inhibited JNK activity, blocked transactivation, and sensitized parental cells to the four DNA-damaging drugs. Moreover, the wild type ATF2-expressing clones exhibited rapid DNA repair after treatment with the four DNA-damaging agents but not transplatin. Conversely, expression of dnATF2 quantitatively blocks DNA repair. These results indicate that JNK-dependent phosphorylation of ATF2 plays an important role in the drug resistance phenotype likely by mediating enhanced DNA repair by a p53-independent mechanism. JNK may be a rational target for sensitizing tumor cells to DNA-damaging chemotherapy agents.

Activating transcription factor 2 (ATF2)[1]/cyclic AMP-responsive element-binding protein-1 is a member of the leucine zipper protein family which regulates gene transcription by interacting with ATF/cAMP-response elements of genes. ATF2 commonly plays an important role in the cellular stress responses (1–4). Various forms of cellular stress including genotoxic agents, inflammatory cytokines, and UV irradiation stimulate the transcriptional activity of ATF2 (5–7). ATF2 target genes include TNF-α (8), topoisomerase I (9), DNA polymerase-β (10), nuclear factor-κB (11), and c-Jun (12), genes that are known to play important roles in the stress response. Although ATF2 target genes have been implicated in these effects, the physiological role of ATF2 in regulating responses to stress remains largely uncharacterized.

Cisplatin is a DNA-damaging anticancer drug that is used widely in the treatment of tumors such as ovarian, testicular, head and neck, bladder, and lung cancer. Although treatment with cisplatin for breast cancer had not been frequently used, interest in platinum compounds was reawakened because preclinical and clinical studies of breast cancer patients that overexpress HER2/neu have demonstrated synergistic responses after combined treatment of platinum salts with the monoclonal antibody trastuzumab (13–15).

A major limitation to the use of platinum salts is the occurrence of inherent cisplatin resistance or the acquisition of resistance to cisplatin by initially responsive tumors. Recent studies have revealed a number of resistance mechanisms such as enhanced drug efflux in a c-Jun NH_2-terminal kinase (JNK)/c-Jun-dependent mechanism (16) or by JNK/c-Jun-dependent enhanced DNA repair (17, 18). We have demonstrated previously that NH_2-terminal phosphorylation of c-Jun by JNK is required for DNA repair and cell survival after cisplatin treatment in various human cancer cell lines including glioblastoma, ovarian, breast, and prostate carcinoma cells (19–22). These studies demonstrate that the JNK/c-Jun pathway is activated by cisplatin-induced DNA damage. Thus, cells modified to express a nonphosphorylatable dominant negative in-

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[1] The abbreviations used are: ATF2, activating transcription factor-2; CBP, CREB-binding protein; cisplatin, *cis*-diaminodichloroplatinum; CMV, cytomegalovirus; CREB, cyclic AMP-responsive element-binding protein; dnATF2, dominant negative ATF2; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; Luc, luciferase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; Me_2SO, dimethyl sulfoxide; MMS, methionine-*S*-methylsulfonium chloride; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt; SAPK, SAPK, stress-activated protein kinase; tk, thymidine kinase; TNF-α, tumor necrosis factor-α; transplatin, *trans*-diaminodichloroplatinum; wtATF2, wild type ATF2.

hibitor of c-Jun stably fail to repair cisplatin-DNA adducts. These cells are sensitized to the cytotoxic effects of cisplatin at concentrations that have little or no effect on parental and control cell lines.

ATF2 can form homodimers and more commonly heterodimers with c-Jun which bind to AP-1-like target sites characterized by an 8-bp response element with a consensus sequence of 5′-TGACGTCA-3′. This interaction together with coactivators such as p300/CBP leads to activation of transcription (12, 23–26). The interactions with p300/BP are dependent upon phosphorylation of serine 121 of ATF2, which is also required for interaction with the basal transcription complex (27). Like c-Jun, cellular stress stimulates the transcriptional activity of ATF2 by phosphorylation of the amino acid residues threonine 69 and threonine 71 (5–7). NH_2-terminal phosphorylation of ATF2 is mediated by stress-activated protein kinases (SAPKs) including JNK (5–7, 28) and p38 mitogen-activated protein kinase (MAPK) (29, 30). These observations suggest that ATF2 may be activated by JNK after DNA-damaging agent events. The effect of ATF2 has been studied extensively after UV irradiation (5–7, 28, 31, 32). Activated ATF2 confers resistance of human melanoma MeWo and WM3211 cells after irradiation (26, 31). It was shown to work by inhibiting the transcription of TNF-α thereby modulating Fas-mediated cell death (32). Regulation of apoptosis through phosphorylation of BAD has also been observed as a mechanism of drug resistance in human ovarian carcinoma Caov-3 cells (33).

We examined the role of ATF2 on chemosensitization and DNA repair in the breast cancer cell line BT474. These cells overexpress the Her2/ErbB2 receptor (34), a molecule up-regulated in almost 40% of human breast cancers (35, 36). Also, similar to many human breast cancers, BT474 cells express a mutated p53 gene product (37, 38), thereby providing a means of examining the DNA damage response pathway without the complicating effects of p53-induced apoptosis or cell cycle arrest. Here, we provide evidence that DNA-damaging agents such as cisplatin, MMS, etoposide, but not the cisplatin isomer, transplatin, which does not readily damage DNA, lead to the phosphorylation and enhanced transactivation potential of ATF2 in a JNK-dependent manner. Further, cells modified to express even low levels of wild type ATF2 exhibit accelerated DNA repair as judged by quantitative PCR and are resistant to the DNA-damaging agents. Conversely, cells modified to express stably a similar level of a nonphosphorylatable dominant negative inhibitor of ATF2 (dnATF2) or cells treated with a pyridinyl imidazole inhibitor, SB202190, or an anthrapyraxolone, SP600125, exhibit complete inhibition of phosphorylation of endogenous ATF2, blocked DNA repair, and a markedly decreased cell viability after treatment with the four different DNA-damaging agents. These results suggest that ATF2 plays an important role in the modulation of DNA repair and in determination of the drug-resistant phenotype.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfections—The human breast cancer BT474 cell line was obtained from American Type Culture Collection. The cells were cultured at 37 °C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in a water-saturated atmosphere of 95% O_2 and 5% CO_2. BT474 cells were transfected using LipofectAMINE Plus (Invitrogen). The total amount of DNA was kept constant at 2 μg by adding the empty vector plasmid DNA to the transfection mixtures. The experiments were repeated at least three times.

DNA Constructs—Wild type ATF2 (wtATF2), pLHCATF2, and a nonphosphorylatable dominant negative ATF2 (dnATF2) pLHCdnATF2(T69A,T71A) were constructed by insertion of the cDNA for wtATF2 and dnATF2 into the retroviral plasmid pLHCX, where L is the retroviral long terminal repeat, H is the hygromycin phosphotransferase gene for resistance to hygromycin B, C is an abbreviated human cytomeglia virus promoter, and X is a polylinker thereby creating pLHCwtATF2 and pLHCdnATF2, respectively. The vector pLHCX itself was constructed as described previously (22, 39). The cDNAs for wtATF2 and dnATF2 were excised from pECE-ATF2 or pECE-ATF2(T69A,T71A), kindly provided by Dr. Z. Ronai and Dr. M. Green. The resulting retroviral vectors were characterized by restriction enzyme digests and by the ability to impart hygromycin B resistance and by confirmation of plasmid protein expression (see below). The plasmids encoding the dominant negative SAPK/JNK (pcDL-SRα-SAPK-VPF) and the wild type SAPK/JNK (pcDL-SRα-wt-SAPK) (40) were kind gifts from Dr. E. Nishida. The reporter constructs p5xjun2tk-Luc and control vector ptk-Luc (7, 41) were kindly provided by Dr. P. Angel.

Clone Selection—BT474 cells were transfected for 12 h in six-well tissue culture plates with 2 μg of pLHCdnATF2(T69A,T71A), pLHCATF2, pLHCcJun(S63A,S73A), or the empty vector pLHCX with LipofectAMINE Plus. The preparation of pLHCX and pLHCcJun(S63A,S73A) has been described (51, 52). The vector pLHCcJun(S63A,S73A) has been used previously for the stable expression of a nonphosphorylatable form of c-Jun in various human tumor lines (19, 42). Clone selection was performed by adding hygromycin B to the medium to a 400 μg/ml final concentration 2 days after the transfection. After 3 weeks several clones were isolated using cloning rings. Selected clones were then maintained in medium supplemented with 400 μg/ml hygromycin B, and only low passage cells ($p < 10$) were used for the experiments described here.

Western Analysis—Cells were lysed in a solution containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, and protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin. Equal amounts of lysates (50 μg) were size fractionated in 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences) after incubation of the polyvinylidene difluoride membranes with specific antibodies.

In Vitro Kinase Assay—JNK assay to c-Jun and p38 MAPK kinase assays to ATF2 were performed with assay kits for the respective kinases (Cell Signaling Technology Inc.) following the company's instructions. Briefly, treated cells were lysed in buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM α-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin) for 15 min on ice. The cell lysates (250 μg) were incubated overnight at 4 °C with immobilized c-Jun (Cell Signaling Technology Inc.) or anti-phospho-p38 MAPK (Cell Signaling Technology Inc.) for JNK and p38 MAPK, respectively. In preliminary experiments, it was confirmed that anti-phospho-p38 reliably and uniformly precipitated p38, and this was confirmed for all conditions (*lanes*) of Fig. 1*C*. The immunoprecipitated products were washed twice with the cell lysis buffer and twice with kinase buffer with 25 mM Tris, pH 7.5, 5 mM glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM $MgCl_2$. The pellets were suspended in the kinase buffer containing 100 μM ATP for the JNK assay, 200 μM ATP, and 2 μg of ATF2 for the p38 MAPK assay. Each reaction was then incubated for 30 min at 30 °C. Western blotting was performed with the phospho-c-Jun and phospho-ATF2 antibodies, respectively. JNK assay for the phosphorylation of ATF2 was performed using an ATF2 fusion protein (Cell Signaling Technology Inc.) as a substrate. Briefly, The cell lysates (250 μg) were incubated with JNK antibody (Cell Signaling Technology Inc.) overnight at 4 °C. The immunoprecipitated products were washed twice with the cell lysis buffer and twice with kinase buffer. In preliminary studies, it was confirmed that anti-JNK reliably precipitated JNK, and this was confirmed for all conditions (*lanes*) examined here (*i.e.* Fig. 1*C*). The pellets were suspended in the kinase buffer containing 200 μM ATP and 2 μg of ATF2. Each reaction was then incubated for 30 min at 30 °C. Western blotting was performed with the phospho-ATF2 antibodies. In some of the experiments, BT474 cells cultured in 100-mm dishes were transfected with hemagglutinin-tagged wild type SAPK/JNK expression plasmid (1 μg of pcDL-SRα-wt-SAPK) or hemagglutinin-tagged dominant negative SAPK/JNK expression plasmid (1 μg of pcDL-SRα-SAPK-VPF) using LipofectAMINE Plus. At 72 h after transfection, treated cells were lysed, and 250 μg of cell lysates were immunoprecipitated with anti-hemagglutinin antibody (Santa Cruz Biotechnology), and the JNK activity was measured as described above.

Cytotoxicity—To assess viability, cells were seeded at a density of 1,000 cells/well in 96-well tissue culture plates, and on the following day, they were treated in the same medium with various cytotoxic agents for 1 h. After all treatments, the cells were washed with phosphate-buffered saline and supplemented with fresh complete medium.

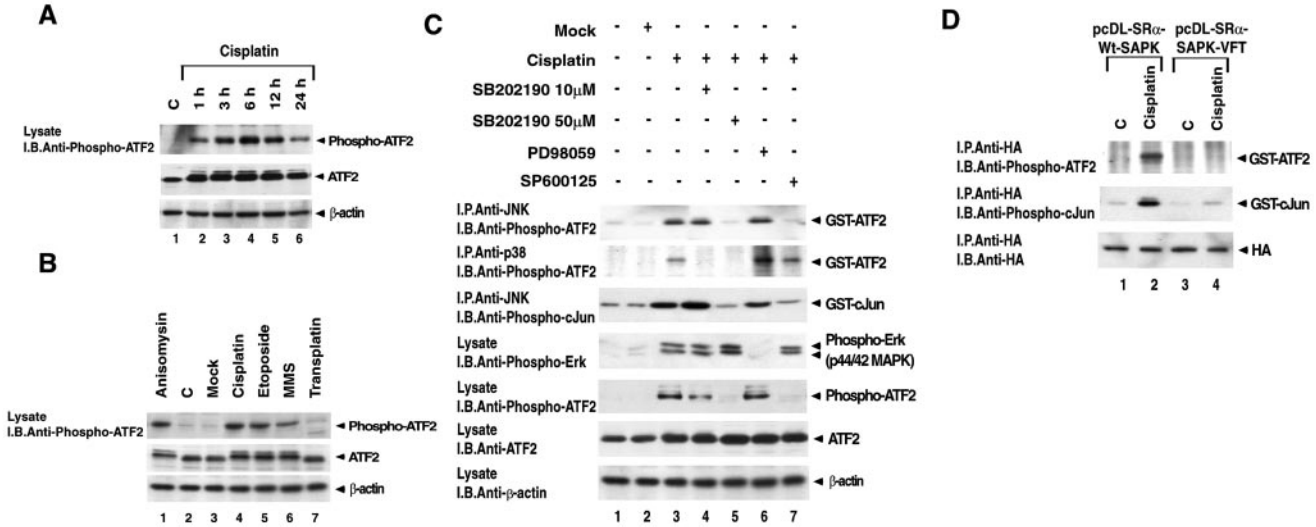

FIG. 1. **DNA-damaging agents induce the phosphorylation of ATF2 via the JNK pathway.** *A* and *B*, DNA-damaging agents phosphorylate ATF2. BT474 cells were treated with 100 μM cisplatin for the indicated times (*A*, *lanes 2–6*), 3 h (*B*, *lane 4*), 10 μg/ml anisomycin for 45 min (*B*, *lane 1*), 100 μM etoposide for 3 h (*B*, *lane 5*), 1 mM MMS for 3 h (*B*, *lane 6*), and 100 μM transplatin for 3 h (*B*, *lane 7*) and analyzed by Western blotting using anti-phospho-ATF2 and ATF2 antibody, as described under "Experimental Procedures." *C*, the phosphorylation of ATF2 depends on the JNK pathway. *Upper panel*, JNK and p38 MAPK kinase activity to ATF2, JNK kinase activity to c-Jun as a positive control and Western blot analysis with anti-phospho-ERK, anti-phospho-ATF2, and anti-ATF2 antibody of BT474 cells pretreated with 10 or 50 μM SB202190 (*lanes 4* and *5*), 50 μM PD98059 (*lane 6*), 30 μM SP600125 (*lane 7*) and Me_2SO (*Mock*) (*lane 2*) for 30 min after treatment with 100 μM cisplatin for 3 h were carried out as described under "Experimental Procedures." *D*, cells were transfected with pcDL-SRα-wt-SAPK (*lanes 1* and *2*) or pcDL-SRα-SAPK-VFT (*lanes 3* and *4*) and, after 72 h, were stimulated with 100 μM cisplatin for 3 h (*lanes 2* and *4*). Cell lysates were immunoprecipitated with anti-hemagglutinin antibody, and the JNK activity was measured as described under "Experimental Procedures." The experiments were repeated three times with essentially identical results. *I.B.*, imunoblot; *I.P.*, immunoprecipitation.

In some of the experiments, the cells were treated with SB202190, SP600125, or Me_2SO (mock) for 30 min before the addition of cytotoxic agents. The measurements of viable cell mass were performed 5 days later using a colorimetric based reaction after the addition of the dye MTS in accordance with the manufacturer's protocol (Promega), which is reduced in proportion to the amount of intact mitochondria, *i.e.* viable cell mass. All determinations were carried out with eight samples for each condition. Cell viability was expressed as viable cell mass after a given treatment by normalizing the averaged value to that of parallel cultures of untreated cells × 100 (viability, %) (21, 22). The chemicals and inhibitors used were purchased as follows: LatinolR®AQ cisplatin was used as aqueous Platinol® (Bristol-Myers Squibb Laboratories); etoposide was from Sigma; MMS was from Aldrich; and actinomycin D, SB202190, SP600125, and PD98059 were from Calbiochem.

Analysis of Transcription—BT474 cells cultured for 1 day in 24-well tissue culture plates were transfected with reporter constructs (p5xjun2tk-Luc or vector ptk-Luc) (7, 41) and pCMV-β-galactosidase plasmid (to normalize for cell viability and transfection efficiency) in combination with the indicated plasmids using LipofectAMINE Plus. At 24 h after transfection, the serum-deprived cells were incubated with various cytotoxic agents or in buffer alone (phosphate-buffered saline) for 1 h. In some of the experiments, the cells were treated with 10 or 50 μM SB202190 or 30 μM SP600125 for 30 min before the addition of 100 μM cisplatin. After 16 h, the cells were lysed by exposure to three sequential freeze-thaw cycles in 100 mM potassium phosphate, pH 7.8, and 10 mM dithiothreitol. The frozen/thawed cells were vortexed vigorously to enhance cell lysis. The lysates were clarified by centrifugation (microfuge) at 10,000 rpm for 10 min at 4 °C. The aliquots of the supernatants were used in the subsequent luciferase and β-galactosidase assays. Luciferase activity was assayed using the luciferase assay mixture contained 20 mM NaOH, pH 7.8, 1 mM dithiothreitol, 3.7 mM $MgSO_4$, 270 μM coenzyme A, 530 μM ATP, and 470 μM luciferin. A 100-μl portion of the luciferase assay mixture was added to a 20-μl aliquot of cell extract just before recording the intensity of phosfluorescent light emission, which was measured in duplicate during the first 20 s of the reaction at 25 °C in a luminometer (EG & G Berthhold, LB96V luminometer, Bundoora, Australia).

β-Galactosidase was assayed using the β-galactosidase buffer containing 60 mM sodium phosphate, pH 7.5, 1 mM $MgCl_2$, 0.80 mg/ml *O*-nitrophenyl-β-D-galactopyranoside, and 40 mM β-mercaptoethanol. A standard curve for reactions containing 100–2 microunits of β-galactosidase was made with each assay. A 30-μl aliquot of cell extract prepared as described above was incubated with assay buffer until color developed (30–120 min), and the reaction was then stopped by adding 150 μl of 1 M sodium bicarbonate. The absorbance at 420 nm was determined using a spectrophotometer (Molecular Devices, SPECTRA Max Plus 384).

Luciferase-catalyzed light emission measured in arbitrary units was normalized to the activity of β-galactosidase observed for control samples. The resulting normalized values were averaged and expressed as average -fold stimulation relative to the control values ± S.E.

Analysis of DNA Damage and Repair—Cisplatin adduct formation and repair were analyzed by a PCR-based DNA damage assay (PCR stop assay) as described previously (19, 20, 43, 44). Briefly, the assay is based on the observation that the efficiency of amplification of cisplatin-treated DNA is inversely proportional to the degree of the platination (43). Genomic DNA was isolated immediately or at the indicated times after treatment of cells for 1 h with varying amounts of cisplatin using the DNeasy tissue kit (Qiagen, Valencia, CA) and PCR-amplified using primers complementary to the hypoxanthine phosphoribosyltransferase gene giving rise to a 2.7-kb product. The polymerase progress and therefore the amount of PCR product formed is reduced if cisplatin adduct formation takes place within this 2.7-kb span. A nested primer giving rise to a 0.15-kb fragment of the hypoxanthine phosphoribosyltransferase gene is also included. The small 0.15-kb segment of genomic DNA sustains undetectable levels of DNA damage under our conditions and serves as an internal control, and the PCR values are used for normalization of the values observed for the amplification of the 2.7-kb fragment. The 5′- and 3′-primer sequences were TGGGATTACACGTGTGAACCAACC and TGTGACACAGGCAGACTGTGGATC, respectively, with a 5′-nested primer sequence of CCTAGAAAGCACATGGAGAGCTAG. For quantitative analysis, primers were end labeled with [α-^{32}P]ATP (ICN Pharmaceuticals Inc.), and the PCR products were separated on 4% or 12% acrylamide gels for 0.15- and 2.7-kb products, respectively. The gels were dried and exposed to films for 12–24 h (see Fig. 7, *A* and *B*, *insets*). The resulting autoradiographed band intensity was quantified by using an AlphaImager™ (Alpha Innotech Co.). The intensity of the integrated 2.7-kb band is normalized to that of the integrated 0.15-kb band, and the efficiency of amplification was determined (see Figs. 7 and 8, *ordinate*).

Statistics and Criteria of Significance—Statistical analysis was performed using an unpaired Student's *t* test as implemented by JSTAT, and $p < 0.01$ was considered significant. Data are expressed as the mean ± S.E.

RESULTS

DNA-damaging Agents Promote the Activation of ATF2 via the JNK Pathway—To test whether ATF2 is phosphorylated upon DNA damage, cultured BT474 cells were exposed to the intrastrand cross-linking agent, cisplatin, and to transplatin, an isomer that does not cross-link DNA under similar conditions of exposure (Fig. 1*A*). Cell lysates were prepared after different lengths of exposure and subjected to Western analysis using an anti-phospho-ATF2 antibody. Phospho-ATF2 is undetectable in resting cells; however, phosphorylation of ATF2 after treatment of the cells with cisplatin was readily detectable at 1 h, reached a broad plateau from 3 through 6 h, and was still readily detected at 24 h (Fig. 1*A*), whereas total ATF2 was not changed detectably. Cisplatin induced the phosphorylation of ATF2 in a dose-dependent manner (data not shown). Treatment by transplatin at the same concentrations had no apparent effect on ATF2 phosphorylation (Fig. 1*B*). Because cisplatin but not transplatin forms covalent cross-links between the N7 position of adjacent guanine or adenine-guanine residues (18, 45), these results indicate that the phosphorylation of ATF2 was dependent on drug-induced DNA damage.

To determine whether these results were general, the phosphorylation of ATF2 was examined after treatment with various known DNA-damaging agents (Fig. 1*B*). Both the topoisomerase inhibitor etoposide and the multifunctional alkylating agent MMS markedly induced the phosphorylation of ATF2 compared with untreated, mock-treated, or transplatin-treated cells to an extent nearly equal to that after cisplatin treatment (Fig. 1*B*). Moreover, phosphorylation remained detectable for up to 24 h (data not shown) after a single treatment. These observations suggest that phosphorylation of ATF2 for prolonged periods commonly follows from treatment with DNA-damaging agents.

ATF2 has been shown to be phosphorylated *in vivo* and *in vitro* by distinct members of the MAPK family. To determine which MAPK pathways were involved, kinase inhibitors were utilized. A pyridinyl imidazole inhibitor, SB202190, has been identified as a specific p38 MAPK inhibitor (46) at low concentration (10 μM) but has been also reported to inhibit JNK activity at high concentration (50 μM) as efficiently as it inhibits p38 MAPK (47, 48). Moreover, in specificity studies of 28 kinase inhibitors against a panel of 34 kinases, SB202190 was among those compounds that exhibited the most impressive selectivity profiles (49). We therefore tested the effect of various concentration of SB202190 upon JNK and p38 MAPK activity (Fig. 1*C*). Pretreatment of the cells with a low concentration (10 μM) of SB202190 nearly eliminated cisplatin-induced p38 MAPK activity, whereas JNK activity and the phosphorylation of ATF2 remained readily detectable. However, pretreatment of the cells with a high concentration (50 μM) efficiently inhibited both p38 MAPK and JNK activity and completely abolished the cisplatin-induced ATF2 phosphorylation.

To test this result further, we examined a second JNK inhibitor, the anthrapyraxolone SP600125, characterized previously as a specific inhibitor for the JNK group of MAP kinases (50, 51). Pretreatment of the cells with a 30 μM concentration of this agent also considerably reduced JNK activity compared with the levels observed with low concentrations of SB202190 and eliminated phosphorylation of ATF2 similar to the effects of high concentrations of SB202190 (Fig. 1*C*). However, pretreatment with SP600125 did not significantly alter either p38 MAPK activity or the phosphorylation of ERK. The MEK inhibitor PD98059 had no effect on ATF2 phosphorylation, although ERK activation was effectively inhibited (Fig. 1*C*). Moreover, An expression plasmid that encodes a dominant negative SAPK/JNK (pcDL-SRα-SAPK-VPF) was used to inhibit the JNK cascade (40) (Fig. 1*D*). Cisplatin-induced JNK activation in cells transfected with pcDL-SRα-SAPK-VPF was clearly attenuated compared with that in cells transfected with pcDL-SRα-wt-SAPK (Fig. 1*D*) and eliminated phosphorylation of ATF2. The combined results strongly indicate that the phosphorylation of ATF2 induced by cisplatin is predominately dependent on the JNK signaling pathway. Consistent with this conclusion, treatment of the cells with anisomycin, a strong stimulator of JNK, leads to a marked increase in phospho-ATF2 (Fig. 1*B*), further supporting the role of JNK in the activation of ATF2.

DNA-damaging Agents Promote Transcriptional Activity of ATF2—Amino-terminal phosphorylation of ATF2 at threonine residues 69 and 71 has been shown to increase transcriptional activity and effect gene regulation (5–7). To determine whether the phosphorylation events observed here might influence gene regulation, the transactivation potential was tested (Fig. 2). To monitor ATF2-mediated transactivation, a reporter construct containing five repeats of the octomeric ATF2/AP-1 DNA sequence from the promoter of the c-*jun* gene termed TRE-Jun2 was utilized (7, 41). These sequences preferentially bind ATF2/c-Jun heterodimers (7, 41). When BT474 cells were transfected with an expression vector of this reporter, little activation was observed compared with mock-transfected cells. However, a reproducible and significant increase (2–3-fold) in ATF2 transcriptional activities was observed after cisplatin treatment (Fig. 2*A*). Treatment with transplatin had no effect on the ATF2 transcriptional activities. The results were observed with all three DNA-damaging agents, and treatment with all three led to significantly increased activation of transcription ($p < 0.01$). Treatment with other DNA-damaging agents such as etoposide and MMS also led to activation of the reporter (Fig. 2*A*). These results suggest that DNA damage promotes transcriptional activity of ATF2. To examine this effect further, wild type ATF2 (wtATF2) was expressed in the BT474 cells. Only a modest activation of 2-fold over that observed with an empty expression vector was observed. However, when the cells were stimulated with cisplatin, a substantial increased in activation was observed which was significant compared with the effects of cisplatin on the reporter construct in the presence of the empty vector control (Fig. 2*B*). In contrast, cells cotransfected with a nonphosphorylatable dnATF2 expression vector significantly inhibited cisplatin-induced ATF2 transactivation (Fig. 2*B*). Thus, cisplatin-stimulated transactivation of the ATF2/AP-1-sensitive reporter is strictly proportional to the amount of wtATF2, and the transactivation is strongly antagonized by expression of dnATF2.

To determine whether these results reflected the same activation pathway as for the phosphorylation of ATF2, the effects of the MAPK inhibitors were examined (Fig. 2*C*). The addition of either PD98059 or SB202190 at low concentration did not alter the basal activities of the reporter constructs (Fig. 2*C*). Similarly, mock treatment (Me_2SO) or pretreatment with mock (Me_2SO), 10 μM SB202190, or PD98059 had no effect on cisplatin-induced ATF2 transactivation, whereas pretreatment with 50 μM SB202190 or pretreatment with the JNK-specific inhibitor SP600125 significantly attenuated the cisplatin-induced activation of the ATF2/AP-1 reporter activity, suggesting that, as for activation of ATF2, the cisplatin-induced transactivation was specifically dependent on the JNK signaling pathway. This conclusion is supported further by observations made after cotransfection with a dominant negative SAPK/JNK (pcDL-SRα-SAPK-VPF) (Fig. 2*D*). Cotransfection with pcDL-SRα-SAPK-VPF significantly attenuated cisplatin-induced ATF2 transactivation, whereas pcDL-SRα had no effect, indicating

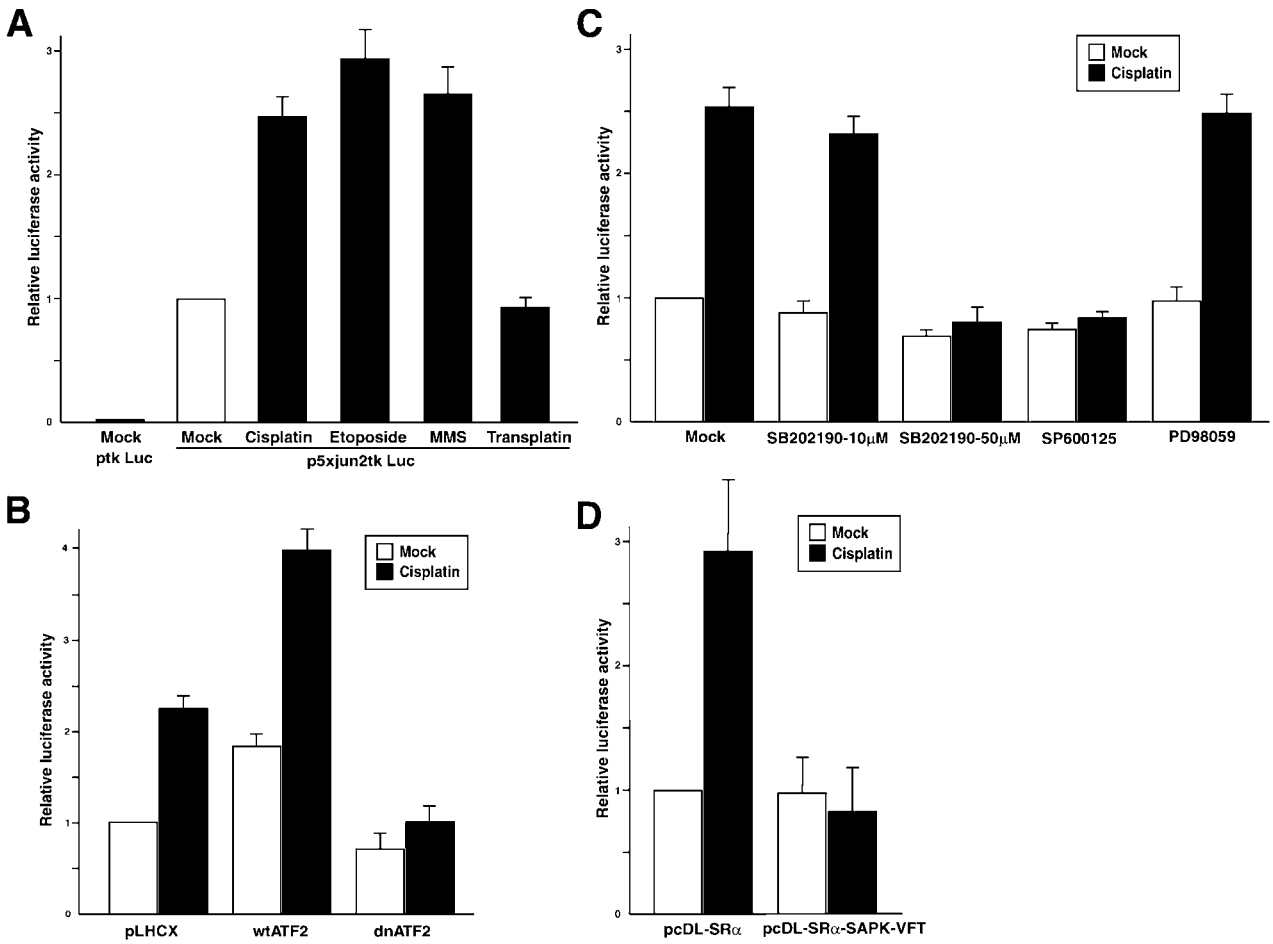

FIG. 2. **DNA-damaging agents promote transcriptional activity of ATF2 via the JNK pathway.** *A*, BT474 cells were transiently cotransfected with the reporter construct p5xjun2tk-Luc or ptk-Luc and an internal control, pCMVβgal. After transfection, cells were treated with 100 μM cisplatin, 100 μM etoposide, 1 mM MMS, 100 μM transplatin, and the solvent (phosphate-buffered saline) for 1 h followed by incubation in drug-free medium for 16 h and subjected to luciferase assay, as described under "Experimental Procedures." *B*, BT474 cells were transiently cotransfected with the reporter construct p5xjun2tk-Luc and an internal control, pCMVβgal with pLHCX or pLHCwtATF2 (wtATF2) or pLHCdnATF2 (dnATF2), as indicated. After transfection, cells were treated with 100 μM cisplatin followed by incubation in drug-free medium for 16 h and subjected to luciferase assay, as described under "Experimental Procedures." *C*, BT474 cells were transiently cotransfected with the reporter construct p5xjun2tk-Luc or ptk-Luc and an internal control, pCMVβgal. After transfection, cells were incubated with 10 or 50 μM SB202190, 30 μM SP600125, 50 μM PD98059, or the solvent (Me_2SO) for 30 min as indicated and then treated with 100 μM cisplatin for 1 h followed by incubation in drug-free medium for 16 h and subjected to luciferase assay, as described under "Experimental Procedures." *D*, BT474 cells were transiently cotransfected with the reporter construct p5xjun2tk-Luc and an internal control, pCMVβgal with pcDL-SRα or pcDL-SRα-SAPK-VFT as indicated. After transfection, cells were treated with 100 μM cisplatin followed by incubation in drug-free medium for 16 h and subjected to luciferase assay, as described under "Experimental Procedures." Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity of p5xjun2tk-Luc was set at 1.0. Data are expressed as the mean -fold activation ± S.E. of six transfections.

that cisplatin-stimulated transactivation of the octomeric reporter construct is specifically mediated by JNK.

Dominant Negative ATF2 Quantitatively Inhibits Activation of Endogenous ATF2 and Blocks ATF2-dependent Transactivation—To determine whether DNA-damaging treatments that led to activation of ATF2 altered the phenotype of the cells, cell viability was examined. For these studies, clonal lines of BT474 were prepared which stably express increased levels of wtATF2 or dnATF2 (Fig. 3*A*). Thus, BT474 cells were transfected with either the retroviral vector pLHCwtATF2 or pLHCdnATF2, which led to the production of a number of hygromycin B-resistant clones. The clones were characterized further by examination of immunoreactive ATF2 levels, which confirmed elevated expression (Fig. 3*A*). Western analysis confirmed stable expression of more than 6-fold increased steady-state ATF2 protein in multiple isolated clones compared with the parental BT474 cells (Fig. 3*A*). Representative pairs of clonal lines with approximately equal expression of either wtATF2 or dnATF2 were selected for further analysis.

The functional properties of the expressed exogenous wtATF2 and dnATF2 were characterized further by activation of JNK by addition of anisomycin (52, 53). Treatment of parental or empty vector control clones leads to a shift in the electrophoretic migration of endogenous ATF2, leading to a slower migrating form. This retardation is consistent with the phosphorylation of ATF2 by JNK (6). Moreover, the results show that this effect is quantitative because *no* ATF2 is detectable at the position of migration of unphosphorylated ATF2 (Fig. 3*B*, *lanes 1*, *4–6*). This indicates, therefore, efficient JNK-dependent phosphorylation of endogenous ATF2 in anisomycin-treated cells (Fig. 3*B*). In the case of exogenous wtATF2-expressing clones, Western analysis revealed greatly increased amounts of retarded ATF2 (Fig. 3*C*, compare *lanes 5–8* with *1–4*). This suggests that all expressed exogenous ATF2 is accessible to and is a substrate of JNK. Again, no ATF2 was observed to migrate to the position of unphosphorylated ATF2. However, when anisomycin was used to activate dnATF2-expressing clones in a parallel experiment, no such retardation was observed, and all ATF2 migrated as for the unphosphorylated form (Fig. 3*C*, *lanes 9–12*). This observation indicates that

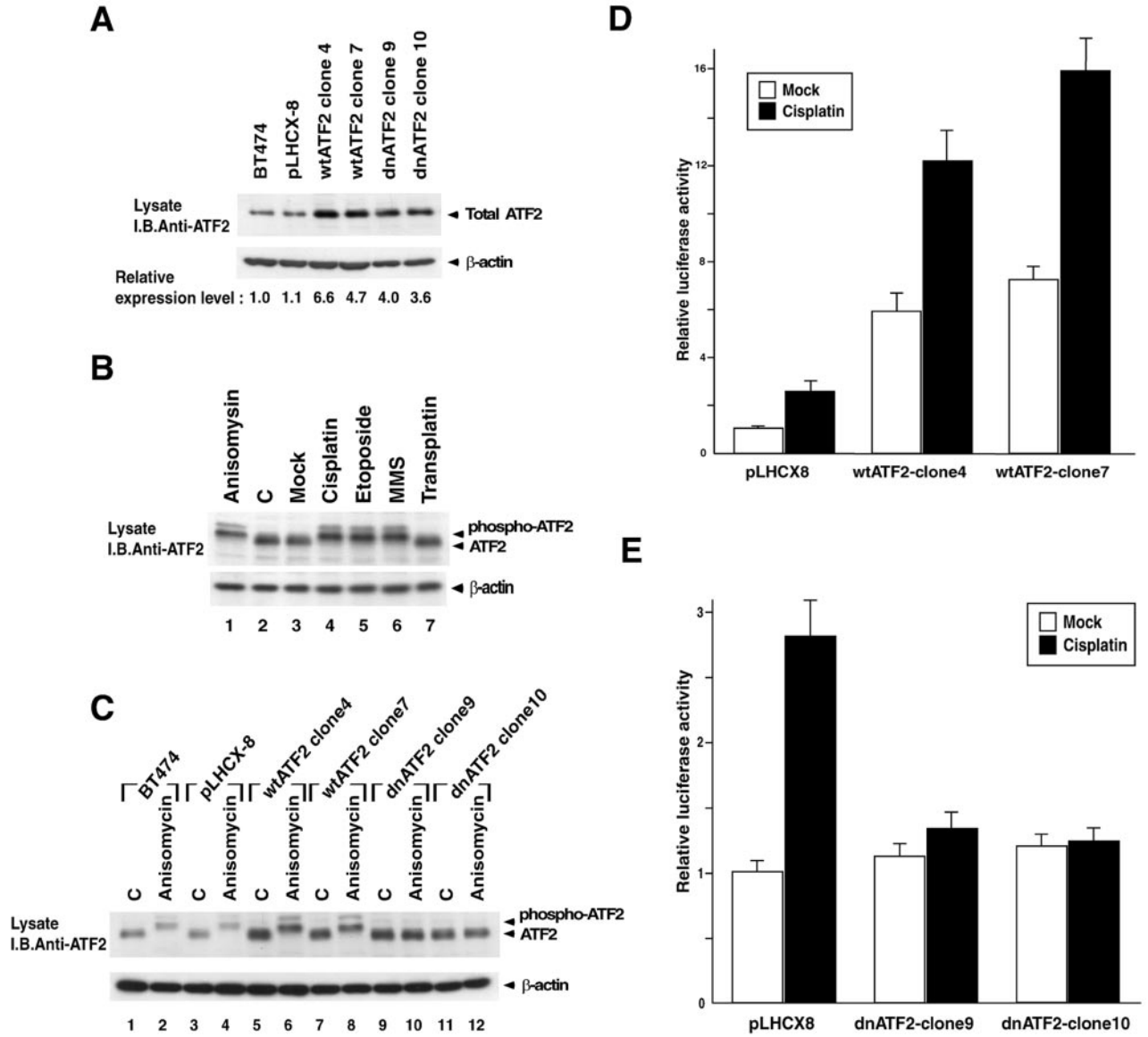

FIG. 3. **Functional characterization: expression of wtATF2 leads to enhanced phospho-ATF2, whereas expression of dnATF2 leads to quantitative inhibition of endogenous phospho-ATF2 formation.** *A*, total ATF2 expression levels in BT474 parental cells (*first lane*), an empty vector expressing clone pLHCX8 (*second lane*), two wtATF2-expressing clones (*third* and *fourth lanes*), and two dnATF2-expressing clones (*fifth* and *sixth lanes*) were subjected to Western analysis using anti-ATF2 antibody, as described under "Experimental Procedures." *I.B.*, immunoblot. *B*, BT474 cells were treated with 10 μg/ml Anisomycin for 45 min (*lane 1*), 100 μM cisplatin for 3 h (*lane 4*), 100 μM etoposide for 3 h (*lane 5*), 1 mM MMS for 3 h (*lane 6*) and 100 μM transplatin for 3 h (*lane 7*) and analyzed by Western blotting using anti-ATF2 antibody, as described under "Experimental Procedures." *C*, the functional properties of the expressed exogenous wtATF2 and dnATF2 of the clonal lines treated with 10 μg/ml anisomycin or the solvent (Me_2SO) for 45 min were analyzed by Western blotting using anti-phospho-ATF2 antibody, as described under "Experimental Procedures." *D* and *E*, wtATF2- and dnATF2-stably expressing cells were transiently cotransfected with the reporter construct p5xjun2tk-Luc and an internal control, pCMVβgal. After transfection, cells were treated with 100 μM cisplatin followed by incubation in drug-free medium for 16 h and subjected to luciferase assay, as described under "Experimental Procedures." Luciferase activity was normalized relative to β-galactosidase activity, and the basal activity of p5xjun2tk-Luc in empty vector-expressing cells (pLHCX8) was set at 1.0. Data are expressed as the mean -fold activation ± S.E. of six transfections.

expressed dnATF2 indeed quantitatively impedes activation *of all endogenous* ATF2.

To determine whether the exogenous dnATF2 might influence gene regulation, the transactivation potential was tested using the clonal lines (Fig. 3, *D* and *E*). The wtATF2-expressing clones exhibited significantly up-regulated basal and cisplatin-induced transactivation up to 12-fold, consistent with increased steady-state ATF2 expression and further increased activation of ATF2 upon treatment with cisplatin (Fig. 3*D*). In contrast, the dnATF2-expressing clones exhibited significantly inhibited cisplatin-induced transactivation compared with empty vector control cells, which exhibited normal activation of the reporter upon addition of cisplatin (Fig. 3*E*). These results are consistent with the Western analysis indicating that stable expression of dnATF2 effectively impedes activation of endogenous ATF2, thereby blocking the effects of cisplatin-activated JNK on the report construct. The sum of results indicate that the clonal lines with stable expression of wtATF2 and dnATF2 are valid models for examination of the consequences of enhanced and eliminated ATF2-dependent function, respectively.

ATF2 Protects, and Dominant Negative ATF2 Sensitizes Cells to Cell Killing Effects of DNA-damaging or Repair-interfering Drugs—To determine whether the enhanced transactivation potential associated with activated ATF2 had phenotypic consequences, the viability of wtATF2-expressing clones was examined before and after exposure of the cells to various

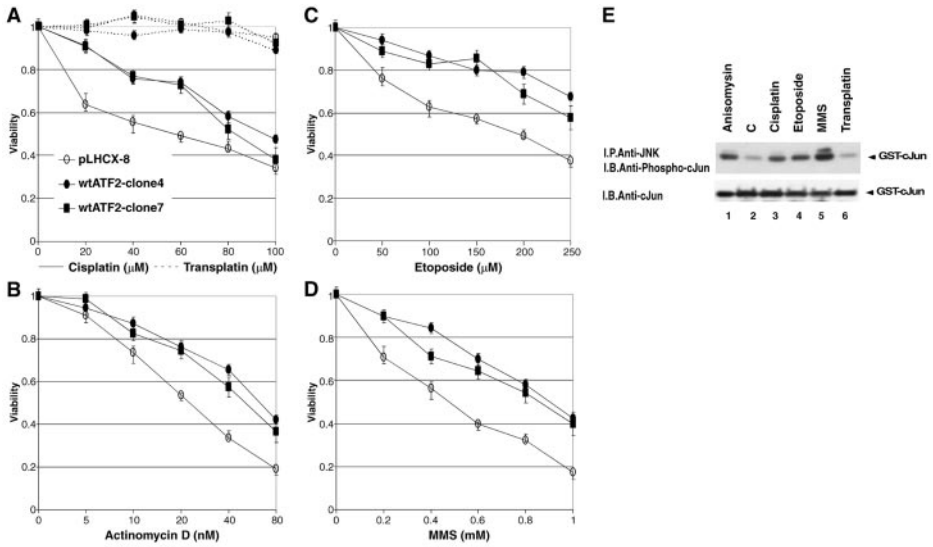

FIG. 4. **Expression of wtATF2-conferred resistance to various DNA-damaging agents.** Cell viability assays using empty vector pLHCX8 (*open circles*), wtATF2-clone 4 (*closed circles*), and wtATF2-clone 7 (*closed squares*) after the indicated concentrations of cisplatin/transplatin (*A*), actinomycin D (*B*), etoposide (*C*), and MMS (*D*) treatments were carried out as described under "Experimental Procedures." *E*, BT474 cells were treated with 10 μg/ml anisomycin for 45 min (*lane 1*), 100 μM cisplatin for 3 h (*lane 3*), 100 μM etoposide for 3 h (*lane 4*), 1 mM MMS for 3 h (*lane 5*), and 100 μM transplatin for 3 h (*lane 6*) and analyzed by JNK kinase assay, as described under "Experimental Procedures." *I.P.*, immunoprecipitation; *I.B.*, immunoblot.

TABLE I

Sensitization of wt/ATF2- and dnATF2-expressing cells to DNA-damaging agent-induced cytotoxicity

IC_{50} values were determined by direct titration of viability as described under "Experimental Procedures."

Agents	Control[a]		wt ATF2-expressing		dnATF2-expressing		$IC_{50}^{wtATF2}/IC_{50}^{dnATF2}$
	Cells	IC_{50}	IC_{50}	Sensitization[b]	IC_{50}	Sensitization[b]	
		μM	μM		μM		
Cisplatin	Parental	61.4 ± 1.4					
	Empty vector pLHCX	62.0 ± 1.5	96.0 ± 2.2	1.55	45.3 ± 1.0	0.73	2.12
	SB202190 (10 μM)	64.2 ± 1.5	N/A[c]		N/A[c]		N/A
	SB202190 (50 μM)	46.1 ± 1.0	N/A		N/A		N/A
	SP600125	43.1 ± 0.9	N/A		N/A		N/A
	dncjun-clone10	45.0 ± 1.0	N/A		N/A		N/A
	Parental	184.9 ± 4.2					
Etoposide	Empty vector pLHCX	183.5 ± 3.8	400.2 ± 8.6	2.18	108.6 ± 2.2	0.59	3.69
	Parental	0.041 ± 0.001					
Actinomycin D	Empty vector pLHCX	0.038 ± 0.001	0.064 ± 0.002	1.65	0.023 ± 0.001	0.58	2.85
	Parental	550.4 ± 18					
MMS	Empty vector pLHCX	535.7 ± 15	914.0 ± 32.8	1.71	401.3 ± 12	0.74	2.27

[a] Parental and empty vector cells were analyzed in parallel and with equal concentrations of cisplatin, etoposide, actinomycin D, MMS and transplatin in the range 0-1 mM in octruplicate. Transplatin had no effect on the viability of any cells.

[b] Sensitization is defined as the ratio of the IC_{50} value for the empty vector cells to the IC_{50} value for the wtATF2-or dn-ATF2-expressing cells.

[c] N/A, not applicable.

concentrations of cisplatin under conditions that lead to activation of JNK (Fig. 4). Cisplatin treatment greatly impedes the growth and survival of BT474 cells with an IC_{50} of ~60 μM (Table I). However, cells that express increased steady-state levels of wtATF2 exhibit a readily detectable "right" shift of the viability curve (Fig. 4*A*), leading to an increased IC_{50} of ~100 μM, a significant increase ($p < 0.0001$). Treatment with transplatin had no significant effect on the viability in parental, wtATF, and dnATF2 clones at any concentration (Figs. 4*A* and 5*A*, *dashed lines*) further indicating that cisplatin-induced DNA damage is required for observation of the effects of wtATF2 and dnATF2. Further, these observations indicate that activated ATF2 mediates an effect leading to resistance to the cytotoxic effects of cisplatin treatment.

To determine the generality of the resistance effect, additional DNA-damaging agents or inhibitors of DNA replication were examined (Fig. 4, *B–D*). Etoposide complexes with topoisomerase II and DNA and blocks ligation leading to an accumulation of double-stranded and single-stranded breaks (54). Actinomycin D blocks replication by intercalation (55). MMS is a DNA-alkylating agent (56). Moreover, all of these agents lead to the activation of JNK (Fig. 4*E*). Expression of wtATF2 leads to right shifts in the viability curves for all four of the DNA-damaging and synthesis-blocking drugs tested, leading to significantly increased IC_{50} values in all cases (Fig. 4, *B–D*, and Table I). Because the agents represent distinct mechanisms of DNA damage repair, these results strongly indicate that increased ATF2 reliably facilitates a general response of DNA damage and DNA synthesis blockade.

This conclusion predicts that the inhibition of ATF2 activation would sensitize cells to the same DNA repair-interfering agents, *i.e.* expression of dnATF2 would promote the converse result. Thus, the survival curves of both clonal lines expressing dnATF2 were examined after treatment with each of the four compounds (Fig. 5). In the case of cisplatin, both clonal lines that express dnATF2 behave similar to each other and exhibit prominent "left" shifts upon exposure to increasing concentrations of each drug (Fig. 5*A*), indicating that expression of dnATF2 leads to decreased viability after treatment with cisplatin. In contrast, parallel experiments with transplatin showed that viability of dnATF2-expressing cells was not altered in the absence of the DNA cross-linker, cisplatin (Fig. 5*A*, *dashed line*) further supporting the importance of DNA damage in initiating events leading to sensitization. Moreover, similar decreases in viability were observed upon treatment of the cells with etoposide, MMS, and actinomycin D (Fig. 5, *B–D*). Thus, the IC_{50} values are decreased by expression of dnATF2, and in all cases the decrease is significant, $p < 0.0001$ (Table I). The relative increase in sensitivity between wtATF2- and dnATF2-expressing cells is a 4-fold change in IC_{50} (Table I). These

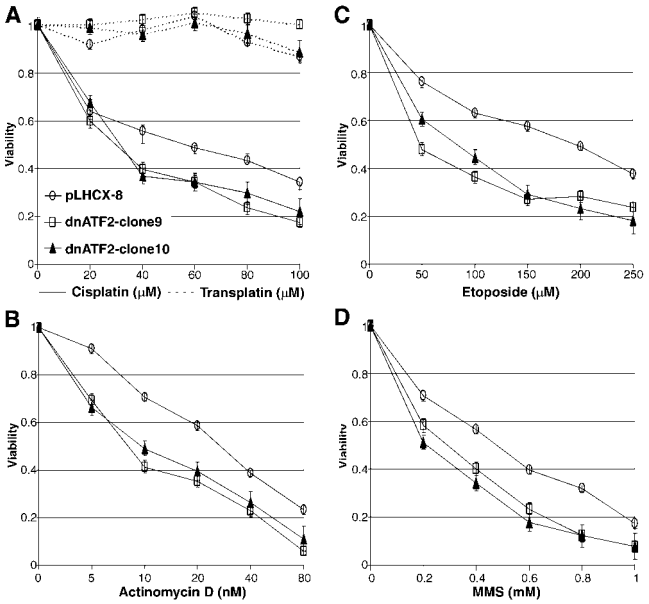

FIG. 5. **Expression of dnATF2-sensitized BT474 cells to various DNA-damaging agents.** Cell viability assays using empty vector pLHCX8 (*open circles*), dnATF2-clone 9 (*open squares*), and dnATF2-clone 10 (*closed triangles*) after the indicated concentrations of cisplatin/transplatin (*A*), actinomycin D (*B*), etoposide (*C*), and MMS (*D*) treatments were carried out as described under "Experimental Procedures."

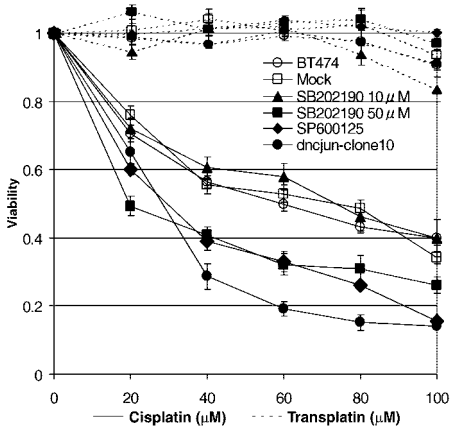

FIG. 6. **Inhibition of JNK by treatment with SB202190 and SP600125 sensitizes BT474 cells to cisplatin.** Cell viability assays using parental cells (*open circles*), dncJun-expressing (c-Jun(S63A, S73A)) cells (*closed circles*), and in cells pretreated with 10 μM SB202190 (*closed triangles*), 50 μM SB202190 (*closed squares*), 30 μM SP600125 (*closed diamonds*), and the solvent (Me_2SO) (*open squares*) for 30 min after the indicated concentrations of cisplatin and transplatin treatments were carried out as described under "Experimental Procedures."

results support a role for activation of ATF2 by JNK in promoting resistance to cell killing by these DNA-damaging and synthesis-blocking agents. Consistent with this, the treatment with parental cells with SB202190 under the conditions that led to inhibition of JNK also causes a marked left shift of the viability curve leading to an IC_{50} for cisplatin of 20 μM (Fig. 6).

These results suggest that a JNK-dependent activation of ATF2 mediates resistance to multiple DNA-damaging drugs. To test this possibility further, two additional inhibitors that are said to be specific to the JNK pathway have been examined. First, we examined a clonal lines of BT474 cells that were stably transfected with pLHCcJun(S63A,S73A) which has been used previously (19, 42) to examine the consequences of disrupting NH_2-terminal phosphorylation of c-Jun, the transcriptional coactivator of ATF2. Viability assays of these clones were very similar to those of BT474 cells treated with high concentrations of SB202190 (Fig. 6). Second, pretreatment of BT474 cells with SP600125 resulted in a viability assay that is nearly indistinguishable from the effects of treatment with high concentrations of SB202190 (Fig. 6). These results strongly support the conclusion that a JNK-dependent activation of ATF2 facilitates a resistance mechanism that applies to multiple modes of DNA damage either by direct interaction or inhibition of DNA replication.

ATF2 Enhances and Dominant Negative ATF2 Inhibits DNA Repair—DNA damage repair represents a highly complex cellular response utilizing several multicomponent systems (17, 18, 57). A number of the component genes contain functional AP-1 and/or ATF/CREB binding sequences. One or more of these genes may, therefore, be regulated, in part, by ATF2, thereby explaining the general role of activated ATF2 in controlling the viability of BT474 cells. To test this hypothesis, we used an assay that is sensitive to all modes of DNA damage, including the requirement for replication for repair, *i.e.* a modification of the general assay of Eastman *et al.* (19, 20, 43, 44). In this assay a representative portion of the *HPRT* gene is PCR amplified, and the effects of disruption of the chromatin template by DNA adduct formation are measured as inhibition of PCR amplification efficiency (see "Experimental Procedures"). DNA is isolated from BT474 cells immediately after a 1-h treatment with cisplatin. The ability to PCR amplify a 2.7-kb fragment of the *HPRT* gene is used to measure repair of cisplatin-DNA adducts because these adducts block the PCR amplification reaction. A 0.15-kb fragment that is too short to suffer cisplatin adduct formation at the doses used here is used as an internal control for PCR amplification.

DNA repair is assessed by comparing inhibition of PCR amplification measured immediately after a DNA-damaging event with that after a recovery period during which repair and restored PCR efficiency occur. Treatment with transplatin had no effect on the PCR efficiency (Fig. 7*B*, *solid bars*). This observation indicates that direct DNA damage induced by platination effects the PCR efficiency. Treatment of BT474 cells for 1 h with cisplatin leads to a 50% inhibition of PCR product formation, which is significantly restored after a 16-h "recovery" period (Fig. 7*B*), indicating substantial DNA repair during the recovery period. Moreover, inhibition of the p38 MAPK pathway has little effect, whereas inhibition of the JNK pathway by use of 50 μM SB202190 entirely precludes DNA repair (Fig. 7*C*). Similarly, pretreatment with 30 μM of the JNK-specific inhibitor SP600125 nearly completely inhibits DNA repair (Fig. 7*C*). These results indicate that active JNK is required for repair of cisplatin-induced DNA damage.

As for the 4-fold change in viability (Table I and Fig. 6), it would be expected that disruption of complete activation of an ATF2·c-Jun complex also would impede DNA repair. Indeed, expression of a nonphosphorylatable c-Jun, c-Jun(S63A,S73A), in BT474 cells completely blocked recovery of DNA repair (Fig. 7*C*). These results confirm our previous observations that JNK and wild type c-Jun are required for DNA repair of cisplatin-induced adduct formation in a variety of other human tumor lines (19). Further, the effect of cisplatin treatment on DNA repair is dependent on the dose of cisplatin (Fig. 7*A*, *inset*) and dependent on the length of the recovery period (Fig. 7*B*). These observations are in agreement with previous studies (19, 20). The sum of results indicate that active JNK *and* phosphorylation of c-Jun are both required for effective mediation of DNA repair by ATF2.

When the DNA repair of wtATF2-expressing cells was examined after a cisplatin challenge, *accelerated* DNA repair was observed (Fig. 8*A*). Treatment with cisplatin caused an ~50%

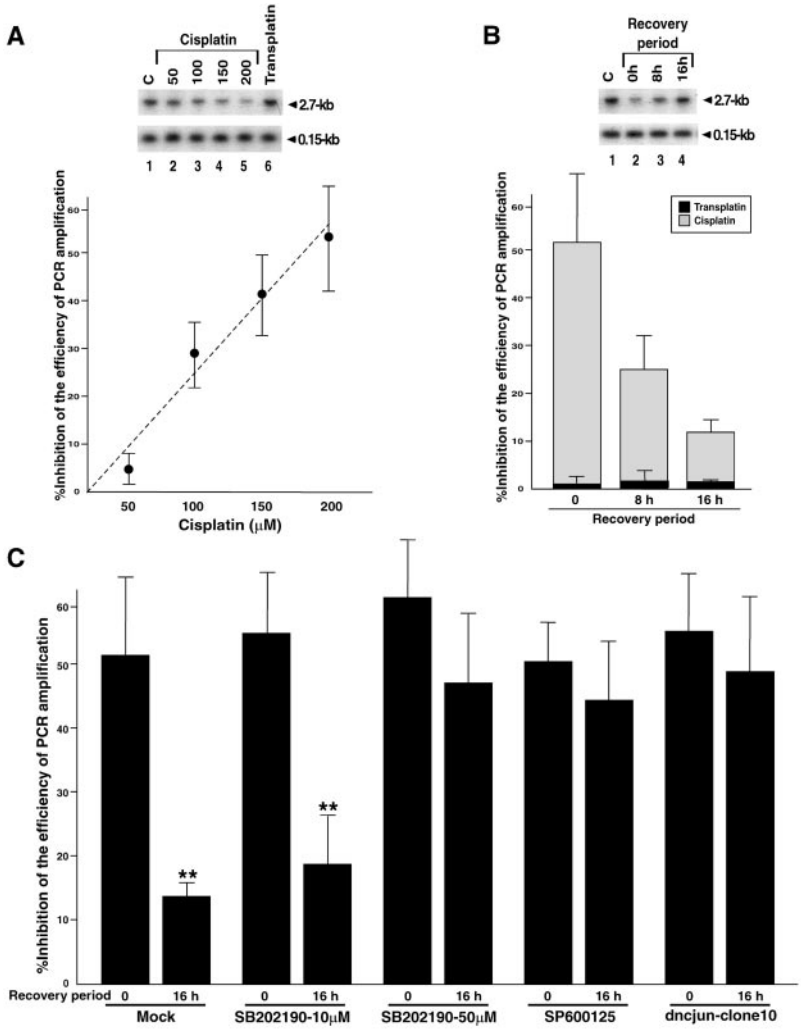

FIG. 7. **Inhibition of JNK by treatment with SB202190 and SP600125 inhibits DNA repair.** *A*, BT474 cells were treated with indicated concentrations of cisplatin for 1 h and subjected to PCR stop assay, as described under "Experimental Procedures." *B*, BT474 cells were treated with 200 μM cisplatin for 1 h followed by incubation in drug-free medium for 0 or 16 h and subjected to PCR stop assay, as described under "Experimental Procedures." *C*, BT474 cells were incubated with SB202190, SP600125, or the solvent (Me_2SO) for 30 min as indicated and then treated with 200 μM cisplatin or 200 μM transplatin for 1 h followed by incubation in drug-free medium for 0 or 16 h and subjected to PCR stop assay, as described under "Experimental Procedures." *Bars*, the results was expressed as inhibition of PCR amplification after the normalizing the averaged PCR efficiency of untreated cells set as 1 minus that of a given treatment or a given treatment with recovery period × 100 (% inhibition of the efficiency of PCR amplification). The level of DNA repair indicates a comparison of inhibition of PCR amplification measured immediately after cisplatin for 1 h and again after a recovery period. The results are the averages of three assays for each of two independent preparations of DNA. ** indicates $p < 0.001$ compared with the results immediately after cisplatin treatment (0 time).

inhibition of PCR amplification of control cells. However, for both wtATF2-expressing clones, the value had fallen to 10% by 16 h. Moreover, for both of the wtATF2-expressing clones the reduction in inhibition was readily recognized after only 8 h of recovery which was reduced significantly compared with the response of the empty vector control cells ($p \leq 0.001$) (Fig. 8*A*) thereby indicating that expression of wtATF2 promotes an accelerated DNA repair.

This conclusion suggests that dnATF2-expressing cells would be expected to exhibit the converse result, *i.e.* retarded or eliminated DNA repair. This effect is shown in Fig. 8*B*. As for the empty-vector control cells, treatment of the dnATF2-expressing cells with cisplatin promotes considerable inhibition of PCR product formation when measured immediately after cisplatin treatment (Fig. 8*B*). However, unlike control or parental cells, little improvement in DNA repair is detected at any time after the single treatment in either dnATF2-expressing clonal line. Moreover, the application of non-DNA-damaging transplatin isomer had no effect on the PCR amplification efficiency for any cell line (Fig. 8, *solid bars*). Thus, these results confirm the expectations based on wtATF2-expressing cells. The sum of results indicate that activated ATF2 functions to facilitate DNA repair in a JNK-dependent manner and that this effect is associated with corresponding increased viability of wtATF2-expressing cells after exposure to DNA-damaging drugs. The dnATF2-expressing cells exhibit reciprocal responses characterized by blocked DNA repair and decreased viability after treatment with all agents examined.

DISCUSSION

The goal of this study is to define the role of ATF2 in mediating DNA-damaging agents and the phenotypic consequences of activation of ATF2 in human breast cancer BT474 cells. Increased expression of ATF2 led to an increased transcriptional activity of ATF2 in response to DNA damage. Moreover, viability assays indicated that activation of ATF2 leads to a specific phenotype of a general resistance to DNA-damaging agents. In contrast, inhibition of ATF2 phosphorylation using dnATF2 or a pyridinyl imidazole inhibitor, SB202190, and the JNK-specific anthrapyrazolone inhibitor SP600125 markedly decreases transcriptional activity of ATF2 and the cell viability after treatment with DNA-damaging agents. These observations reproducibly identify ATF2 and in particular NH_2-terminal phosphorylation of ATF2 as an important effector mechanism of viability and survival after DNA damage.

Additional studies were carried to understand a mechanistic basis of the reciprocal effects of increased and decreased activation of ATF2. ATF2 is a common coactivator with c-Jun, a JNK substrate previously implicated in mediating DNA damage in a NH_2-terminal phosphorylation-dependent mechanism (19, 21). Here it was found that increased expression of ATF2 promotes increased phospho-ATF2 and accelerated DNA repair. Conversely, it was observed that expression of dnAFT2 effectively, indeed quantitatively, inhibited phosphorylation of endogenous ATF2. This effect was associated with blocked DNA repair after treatment with DNA-damaging agents as

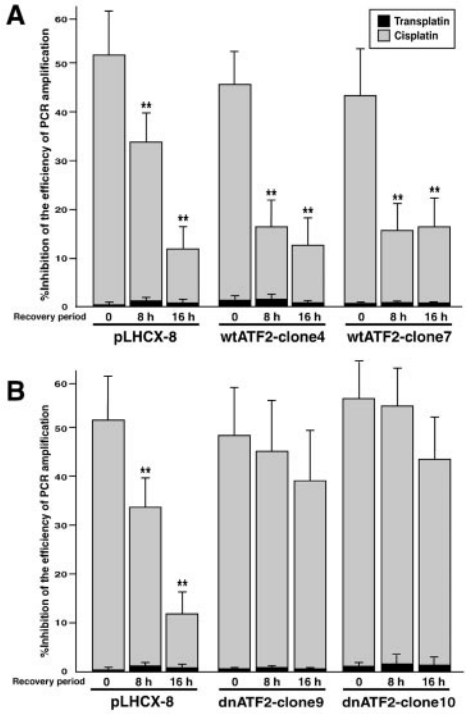

FIG. 8. **wtATF2 enhances and dnATF2 inhibits DNA repair.** Cells were treated with 200 μM cisplatin or 200 μM transplatin for 1 h followed by incubation in drug-free medium for 0 or 16 h and subjected to PCR stop assay, as described under "Experimental Procedures." *Bars*, the results were expressed as inhibition of PCR amplification after normalizing the averaged PCR efficiency of untreated cells set as 1 minus that of a given treatment or a given treatment with recovery period $\times$ 100 (% inhibition of the efficiency of PCR amplification). The level of DNA repair indicates a comparison of inhibition of PCR amplification measured immediately after 200 μM cisplatin for 1 h and again after a recovery period. The results are the averages of three assays for each of two independent preparations of DNA. ** indicates $p < 0.001$ compared with the results immediately after cisplatin treatment (0 time).

judged by quantitative PCR. Thus, our results suggest that JNK-activated ATF2 plays the important role in determination of chemosensitization to DNA-damaging agents through modulation of DNA repair in breast cancer cell lines.

Extensive previous studies have examined the role of ATF2 after irradiation by UV-C as a model of DNA damage. Irradiation of human melanoma LU1205 cells, a model of late stage melanoma, leads to activation of ATF2, down-regulation of TNF-α, a known target gene of ATF2, and increased apoptosis (32). Thus, ATF2 mediates a cell death response in these cells (32). Subsequent studies utilizing human 293T fibroblasts and murine K1753 melanoma cells identified TIP49b as a new ATF2-binding protein that also is increased upon UV ionizing irradiation and serves to oppose the action of ATF2 thereby promoting decreased apoptosis and resistance to irradiation (26). There is evidence that increased resistance also follows exposure to DNA-damaging drugs (31). The results observed here compare well with the latter studies (26, 31), whereas comparison with the results based on UV-C irradiation is limited. In studies utilizing UV-C, the activation of ATF2 by p38 and JNK was not distinguished, DNA damage was not directly assessed, and a non-DNA-damaging activator of MAPK was not assessed. Moreover, the cell types examined differed, for example, in the case of Caov-3 cells, we observed that *both* ERK and JNK were required for resistance to treatment with cisplatin (21), whereas the present studies with BT474 cells consistently indicated that JNK is the major mediator. In the results summarized here, four DNA-damaging or repair-interfering drugs led to similar results of increased viability after expression of wtATF2, whereas non-DNA-damaging transplatin had no effect. Conversely, inhibition of NH_2-terminal phosphorylation of ATF2 led to reciprocal results of sensitization to all four DNA-damaging agents. Direct assessment of DNA damage by a PCR-based protocol confirmed that inhibition of ATF2 activation is associated with increased sensitization. Kinase inhibitor studies pointed to JNK and not p38 or ERK as the predominate kinase. Thus, our studies of BT474 cells consistently indicate that ATF2 mediates a chemotherapeutic drug resistance mechanism and implicate an ATF2-stimulated DNA repair function in the mechanism of resistance.

The results are consistent with previous studies of four different human tumor lines, T98G and U87 glioblastoma, PC3 prostate carcinoma, and MCF-7 breast carcinoma, all indicating that NH_2-terminal phosphorylation of c-Jun by JNK is required for DNA repair in several human tumor lines (19). A unifying and testable hypothesis that combines these findings with the current results on the phenotype of activated ATF2 is that an activated c-Jun/ATF2 heterodimer is formed which transactivates one or more genes directly involved in DNA repair. Indeed, expression of a nonphosphorylatable c-Jun enhanced cell killing by cisplatin similar to that of JNK inhibition and blocked DNA repair. Functional c-Jun/ATF2-regulated genes are well known and include interferon (58), E-selectin (30, 59, 60), TNF-α (8) as well as c-Jun itself (12). In addition, genes with roles in DNA repair which bear functional octomeric sites are known (9, 10, 61, 63). For example, the DNA repair protein DNA polymerase-β is known to contain a functional octomeric site whose activation is reversibly dependent upon phosphorylation of an activation factor (62). Additional examples include topoisomerase I (9, 64) and proliferating cell nuclear antigen (61, 63). Whether these potential targets or others that are regulated by c-Jun/ATF2 heterodimers mediate drug resistance by BT474 cells is unknown. However, such DNA repair genes provide a testable hypothesis for further study.

The normal cell response after the recognition of DNA repair involves activation of p53 and subsequent cell cycle arrest, effects thought to allow for DNA repair (57). However, in BT474 cells p53 is mutated and nonfunctional (37, 38). The activation of JNK and resultant effects on DNA repair are, therefore, independent of p53. The existence of a separate mechanism that facilitates DNA repair by tumor cells may favor continued proliferation and enhanced resistance to DNA-damaging chemotherapeutic agents, a class of drug that accounts for the majority of chemotherapeutic drugs. Moreover, in recent studies of acute myeloid leukemia, we observed that c-Jun participates in the induction of multidrug resistance protein-1 in a NH_2-terminal phosphorylation-dependent manner (16). These observations strongly argue that agents that disrupt the activation of c-Jun and ATF2 in selected tumor cells may be an important new approach in limiting drug resistance.

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The Activation of c-Jun NH_2-terminal Kinase (JNK) by DNA-damaging Agents Serves to Promote Drug Resistance via Activating Transcription Factor 2 (ATF2)-dependent Enhanced DNA Repair

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