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Research Article

Phorbol ester exposure activates an AP-1-mediated increase in ERCC-1 messenger RNA expression in human ovarian tumor cells

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Abstract. *ERCC-1* is an essential gene in the nucleotide excision repair pathway, and may be essential for life. However, the mechanism of transcriptional activation and regulation of *ERCC-1* gene expression is unclear. We therefore investigated the effect of the phorbol ester 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) on the expression of the *ERCC-1* gene in A2780/CP70 human ovarian carcinoma cells. TPA induced a four- to sixfold increase in steady-state ERCC-1 messenger RNA (mRNA) levels that was time- and concentration-dependent. Nuclear run-on experiments demonstrated that the rate of transcription of *ERCC-1* was approximately 2.8-fold higher in TPA-treated cells than in the controls. TPA stimulation of A2780/CP70 cells also resulted in a rapid but transient induction of c-*jun* and c-*fos* as determined by Northern and Western blot analyses, which peaked about 2 h before the peak in *ERCC-1* expression. Electrophoretic mobility shift assays of nuclear extracts from TPA-treated cells revealed an increase in DNA-binding activity specific for the AP-1-like binding site in the 5′-flanking region of *ERCC-1*. c-Jun and c-Fos proteins were confirmed to be the components of the activated AP-1 complex by supershift analysis. The increase in AP-1 activity occurs immediately before the increase in *ERCC-1* transcription. The increase in AP-1 DNA-binding activity and the increase in ERCC-1 mRNA expression were prevented by pretreatment with cycloheximide. These data suggest that AP-1 may contribute to the upregulation of *ERCC-1* in response to TPA in human ovarian cancer cells.

Key words. AP-1; ERCC-1 mRNA; *ERCC-1* gene transcription; nucleotide excision repair; ovarian cancer; phorbol ester.

DNA repair acts as a key line of defense to remove injuries to DNA and increase cell survival by allowing resumption of transcription or replication [1, 2]. DNA repair processes also minimize the mutagenic consequences of DNA damage, thereby preventing the accumulation of mutations at critical positions in DNA and reducing the incidence of cancer or inherited diseases [3–6]. Multiple DNA damage processing pathways

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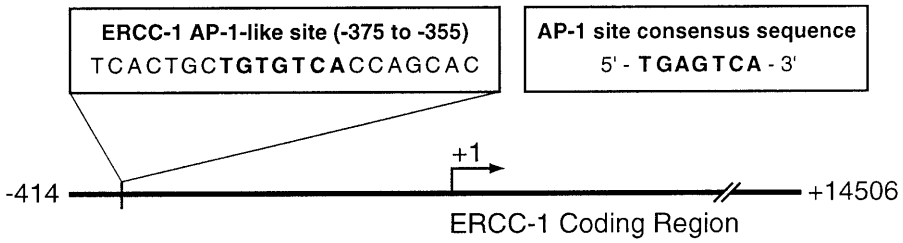

Figure 1. Schematic of the 5′-flanking region of the *ERCC-1* gene. The position of the AP-1-like site (boldface) is pictured relative to the transcription start site [14].

have been identified in mammalian cells, all of which modulate the cytotoxic, apoptotic or mutagenic effects of specific DNA lesions.

Nucleotide excision repair (NER) is the principal DNA repair pathway by which cisplatin, ultraviolet (UV)-induced pyrimidine dimers, cross-linking agents, as well as other types of bulky DNA lesions are removed from cellular DNA [4, 5, 7, 8]. The first steps within NER encompass two basic processes: damage recognition and dual incisions on either side of the lesion [9]. ERCC-1 (excision repair cross complementation group 1) is a DNA repair protein that is essential for life [10], and appears to be one of the critical DNA repair proteins in the NER process [4, 5, 11–13]. The roles of ERCC-1 in NER may include: (i) to assist in DNA damage recognition [11–13]; (ii) to incise DNA on the 5′ side of a lesion (such as platinum-DNA adduct) [14]; and (iii) to play a role in homologous recombination [15]. Clinical and experimental studies suggest that enhanced *ERCC-1* and other NER gene expressions correlate with repair of cisplatin-induced DNA damage [16–18]. Conversely, repair of cisplatin-DNA adduct does not occur in cells lacking functional ERCC-1 protein [19]. Therefore, the expression levels of DNA repair genes like *ERCC-1* are potential markers for cellular NER capacity.

The fundamental molecular basis of transcriptional activation and regulation of *ERCC-1* expression is not well elucidated. The AP-1 (activator protein-1) family is a group of transcription factors responsible for the activation of a wide variety of genes in different cell types and tissues [20–22]. The AP-1 transcription factor consists of either Jun homodimers or Fos/Jun heterodimeric complexes which bind the palindromic sequence TGA(C/G)TCA [20–22]. AP-1 binding sites are frequently found in promoters or enhancers of genes that are inducible by a wide range of extracellular signals, most notably by phorbol esters (e.g. 12-*O*-tetradecanoyl-phorbol-13-acetate, TPA) [20, 22–24], and therefore AP-1 binding sites function as TPA-responsive elements (TREs) [23]. AP-1 transcription factors can regulate proliferation, differentiation, oncogenesis, apoptosis or DNA-damage response, depending on the cell type [25–27]. Analysis of the *ERCC-1* gene sequence [14] reveals an AP-1-like binding site within the promoter region of the gene (fig. 1), suggesting that AP-1 might be the transcriptional activator that modulates the expression of *ERCC-1*. In two earlier reports [28, 29], we demonstrated that cisplatin induces ERCC-1 mRNA expression in the A2780/CP70 human ovarian cancer cell line through a DNA damage-response pathway. In this study, we investigate the influence of TPA on *ERCC-1* expression in these cells, and assess whether the induction of AP-1 may mediate the activation of *ERCC-1* gene expression, independent of the occurrence of DNA damage. We show herein that the time course of *ERCC-1* upregulation induced by TPA and by cisplatin are distinct, suggesting the possible existence of two separate signaling pathways that may lead to *ERCC-1* stimulation.

Materials and methods

Cell line and cell culture conditions. The human ovarian cancer cell line A2780/CP70 has been described previously [30] and was used in all experiments. Cells were cultured in monolayer using RPMI 1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.2 U/ml of human insulin, 50 U/ml of penicillin, 50 μg/ml of streptomycin (GIBCO BRL, Gaithersburg, MD, USA). Cells were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2:95% air. Cells were routinely tested for mycoplasmal infection using a commercial assay system (MycoTect; GIBCO BRL), and new cultures were established monthly from frozen stocks. All media and reagents contained < 0.1 ng/ml of endotoxin as determined by *Limulus polyphemus* amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Cell viability was determined in triplicate by trypan blue dye exclusion. Before starting the experiments, the cells were

grown to ~90% confluence after subculturing. α-Amanitin (Calbiochem, San Diego, CA) and cycloheximide (Calbiochem) were dissolved in water. TPA (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) and diluted with the cell culture medium. The final concentration of DMSO in culture flasks was 0.002–0.02% (v/v). Drug cytotoxicity was determined using the colony growth assay and the crystal violet staining assay, as described previously [31]. The concentrations of DMSO, TPA and other drugs used in the studies were not toxic to the cells as confirmed by cell recoveries, trypan blue dye exclusion and cytotoxicity assay.

RNA isolation and Northern blot analysis. Total RNA was isolated from cells by acid guanidinium thiocyanate-phenol-chloroform extraction [32], or using a commercial total RNA isolation reagent kit (GIBCO BRL) according to the manufacturer's instructions. Thirty micrograms of denatured RNA per lane were separated by electrophoresis (GIBCO BRL) through 1% agarose-formaldehyde and transferred to nylon membrane (Zeta-Probe GT; Bio-Rad Laboratories, Hercules, CA) by electrophoretic transfer (Trans-Blot Cell; Bio-Rad Laboratories). Membranes were prehybridized in Quik-Hyb (Stratagene, Menasha, WI) for 15 min at 68 °C and then hybridized for 1–2 h at 68 °C in Quik-Hyb containing 0.67 μg/ml of denatured salmon testes DNA (Stratagene) and ^{32}P-labeled complementary DNA (cDNA) probe. After washings of increasing stringency, the membranes were air-dried, exposed to Kodak XAR-5 X-ray film with intensifying screens at −80 °C and then analyzed by Collage Analysis (Fotodyne, New Berlin, WI) and quantitated by densitometrical scanning. Before hybridization with a second labeled cDNA probe, the first probe was removed by washing for 2 h at 75 °C in 1 mM tris (hydroxymethyl) aminomethane (Tris)·HCl (pH 8.0) containing 1 mM EDTA and 0.1 × Denhardt's solution [33]. The entire sequence of experiments (including growth of A2780/CP70 cells, drug treatment, and Northern blotting and hybridization) was performed, and the results were reproduced in two or more separate experiments. Equal RNA loading was determined by visualization of 18S and 28S ribosomal RNA bands in ethidium bromide-stained gels and quantification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene transcript on Northern blots.

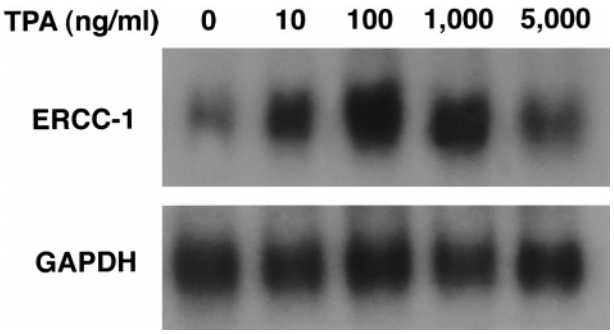

Figure 2. Concentration response of TPA on *ERCC-1* mRNA levels in A2780/CP70 ovarian cancer cells in culture. A2780/CP70 cells were exposed to TPA for 6 h at the indicated concentrations. Total RNA was then isolated and analyzed by Northern blotting as described in 'Materials and methods'. Loading of RNA was monitored by hybridization to labeled GAPDH probe. *ERCC-1* band densities were quantified by densitometry and expressed as a ratio to GAPDH. An autoradiograph from one representative experiment is shown.

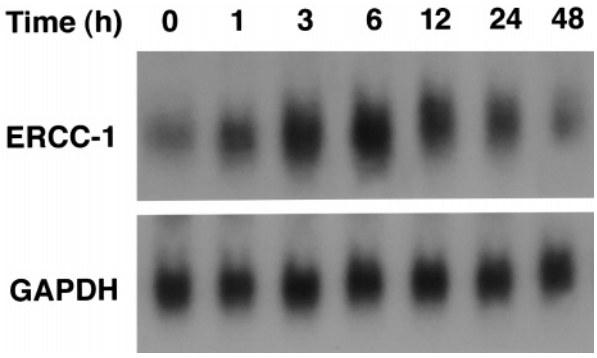

Figure 3. Time course of TPA effect on *ERCC-1* mRNA levels in A2780/CP70 cells in vitro. Cells were incubated in the presence of medium containing 100 ng/ml of TPA, and RNA was sequentially isolated at different times after exposure to the drug and analyzed by Northern blotting as described in 'Materials and methods'. Total cellular RNA (30 μg) was blotted and hybridized with a ^{32}P-labeled *ERCC-1* probe. Equal loading was confirmed by hybridization to labeled GAPDH cDNA probe. *ERCC-1* band densities were quantified by densitometry and expressed as a ratio to GAPDH.

Preparation of cDNA probes. A 1.05-kb cDNA probe for human *ERCC-1* was obtained from Dr. Aziz Sancar (University of North Carolina, Chapel Hill, NC). A 0.8-kb cDNA for human GAPDH was obtained from Dr. Mitchell Olman (University of California, San Diego, CA). A GAPDH probe was also obtained commercially from Oncogene Research Products (Cambridge, MA). cDNA inserts were excised using appropriate restriction enzymes, isolated by electrophoresis through 1% agarose onto DEAE-membrane (NA-45; Schleicher and Scheull) [33] and purified by using the Geneclean II Kit (BIO 101, La Jolla, CA). cDNA was labeled with ^{32}P using a commercial random primer kit (GIBCO BRL) according to the manufacturer's instructions.

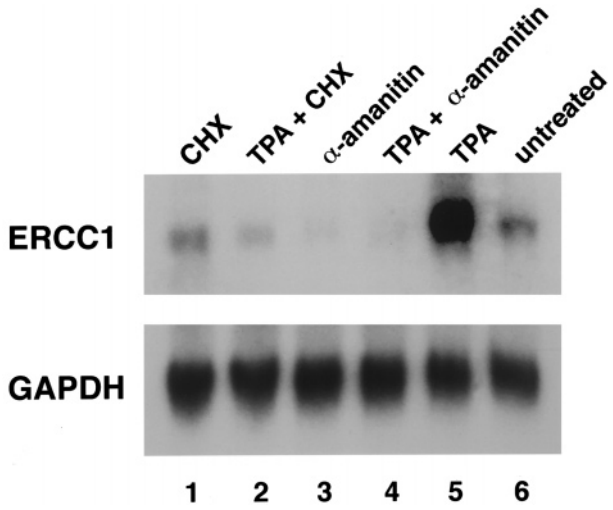

Figure 4. Effect of α-amanitin or cycloheximide on TPA-mediated increase in *ERCC-1* mRNA level in A2780/CP70 ovarian cancer cells in culture. Northern blot analysis of RNA acquired from cells treated with 10 μM cycloheximide (CHX) alone (lane 1); cells preincubated for 2 h with 10 μM CHX prior to treatment with 100 ng/ml of TPA for 6 h (lane 2); cells treated with 5 μg/ml of α-amanitin alone (lane 3); cells incubated for 2 h in the presence of 5 μg/ml of α-amanitin, followed by exposure for 6 h to 100 ng/ml of TPA (lane 4); cells treated 6 h with 100 ng/ml of TPA (lane 5); and untreated A2780/CP70 cells (lane 6). RNA was harvested at 6 h TPA treatment or at 6 h for non-TPA-treated cells. Loading of RNA was monitored by hybridization to labeled GAPDH cDNA probe. *CHX*, cycloheximide.

Measurement of transcription rate. *ERCC-1* transcription rate was measured using a modification of previously described nuclear run-on analysis [34]. A2780/CP70 cells grown to ~90% confluence were lysed in 10 ml of lysis buffer, containing 10 mM Tris (pH 8.0), 2.5 mM $MgCl_2$, 0.25% Triton X-100, 0.3 M sucrose and 1 mM dithiothreitol (DTT), and nuclei were collected by centrifugation for 5 min at 500*g*. Isolated nuclei were incubated with 250 μCi of [α-^{32}P]UTP (New England Nuclear, Wilmington, DE) for 30 min at 37 °C. cDNA for *ERCC-1* and GAPDH, or vector DNA (the plasmid without *ERCC-1* cDNA insert) (5 μg of DNA per blot) used in the run-on assay was heat-denatured and transferred to supported nitrocellulose (GIBCO BRL) by vacuum filtration using a 24-well manifold (Hybri-Dot; GIBCO BRL). The membrane was rinsed with 6× sodium chloride-sodium citrate (SSC), air-dried and baked at 80 °C for 2 h in a vacuum oven. Membranes were prehybridized for 1 h at 42 °C in 50% formamide, 6× SSC, 5× Denhardt's solution, 0.5% sodium dodecyl sulfate, and 100 μg/ml of denatured salmon testes DNA and then hybridized at 42 °C for 3 days in prehybridization buffer containing run-on reaction mixtures adjusted to equalize radioactivity added in all reactions. Membranes were extensively washed with increasing stringency and then treated with 1 μg/ml of ribonuclease for 30 min at 37 °C. Membranes were air-dried, exposed to XAR-5 film, and quantitation of the results was achieved by densitometric scanning normalized to the signal for GAPDH.

Cell extract preparation and Western blot analysis. To prepare whole cell lysates, 2×10^7 cells were washed three times in ice-cold phosphate-buffered saline (PBS), and resuspended in 500 μl of buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 μg/ml of leupeptin, 1 μg/ml of pepstain, 0.5 mM phenylmethylsufonyl fluoride (PMSF), 1 mM sodium orthovandate, 1 mM NaF at 4 °C. Lysates were sheared through a 21-gauge needle and clarified at 4 °C by microcentrifugation. Protein content in the supernatants was determined by means of the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

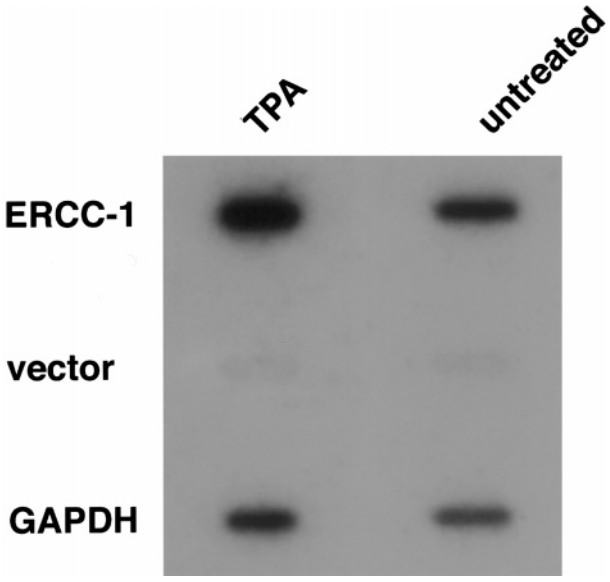

Figure 5. Nuclear transcript elongation (run-on) assay of TPA effect on *ERCC-1* gene transcription in A2780/CP70 ovarian cancer cells in culture. A2780/CP70 cells were incubated in the presence of 100 ng/ml of TPA for 3 h. Nuclei were then isolated, and nuclear run-on reactions were performed as described in 'Materials and methods'. Cells without treatment of TPA were controls (untreated). Run-on reaction mixtures were hybridized to immobilized probes on the membrane. *ERCC-1* band densities were quantified by densitometry and expressed as a ratio to GAPDH. *Vector*, the plasmid without *ERCC-1* cDNA insert.

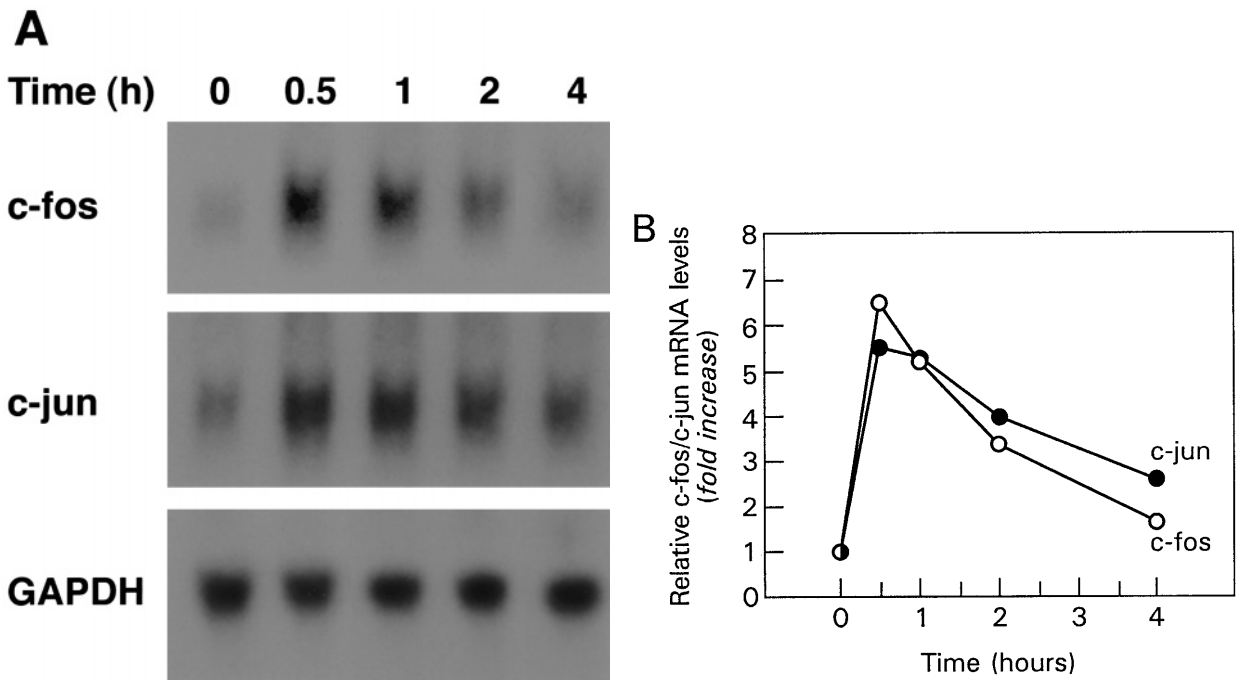

Figure 6. Time course of TPA effect on c-*fos* and c-*jun* mRNA accumulation in A2780/CP70 cells. A2780/CP70 cells were incubated in the presence of medium containing 100 ng/ml TPA, and RNA was extracted at different times of exposure to the drug and analyzed by Northern blotting as described in 'Materials and methods'. Cells without treatment of TPA were controls (0 h). Total cellular RNA (30 μg) was blotted and hybridized with a ^{32}P-labeled c-*fos* or c-*jun* probe. Loading of RNA was monitored by hybridization to labeled GAPDH probe. c-*fos* and c-*jun* mRNA band densities were quantified by densitometry and expressed as a ratio to GAPDH. The resultant numerical values are shown graphically in panel B.

The cell extract proteins (25 μg) were loaded on a 12% Tris-glycine gel (Novex, San Diego, CA), electrophoresed and transblotted to a Protran (pure nitrocellulose) membrane (Schleicher and Schuell, Keene, NH) by the procedure described by Towbin et al. [35]. The blot was then rinsed with Tris-buffered saline (TBS), and was incubated with a blocking buffer (1 × TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for overnight at 4 °C. After rinsing with TBS-Tween (0.1%) three times, the blot was incubated with c-Jun or c-Fos antibody (1:1000 in TBS-Tween with milk) overnight at 4 °C on a shaker. The blot was washed thrice with TBS-Tween, and incubated with a 1:2000 dilution of secondary antibody coupled with HRP for 2 h at room temperature on a shaker. The blot was washed thrice with TBS-Tween and then incubated with ECL reagents for 1 min at room temperature. The blot was exposed to X-ray film to visualize the results.

Preparation of nuclear extracts. Nuclear extracts were prepared from resting or TPA-treated A2780/CP70 cells by a modification of the procedure described by Dignam et al. [36]. Cells were harvested by scraping and washed once with ice-cold PBS. The cells were then resuspended in 1.5 volumes of lysis buffer [70 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM sodium orthovanadate, 0.4 mM NaF, 0.5 mM PMSF, 1.0 mM DTT, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 7.5]. The mixture was incubated on ice for 20 min and then extracted by adding of 1.6 volumes of extraction buffer (0.5 mM EDTA, 20% glycerol, 1.66 M KCl, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM PMSF, 1.0 mM DDT, 25 mM HEPES, pH7.5) with constant shaking at 4 °C for 4 h. Samples were centrifuged at 55,000*g* for 1 h at 4 °C, and the supernatant was dialyzed at 4 °C for 4 h in a buffer containing 20 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.1 mM PMSF and 1.0 mM DTT. Samples were stored at − 80 °C. Protein content was determined by the BCA protein assay (Pierce).

Preparation of oligonucleotide probes. The oligonucleotide sequence used in the following electrophoretic

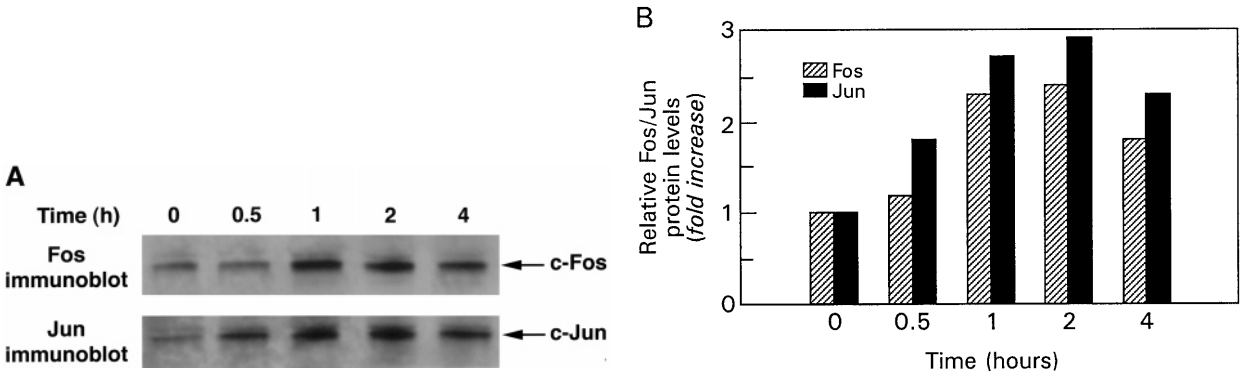

Figure 7. Western immunoblot analysis of the effect of TPA on the levels of c-Jun and c-Fos proteins in A2780/CP70 cells. Cells were incubated with 100 ng/ml of TPA, and cell protein was sequentially extracted at various time points after exposure of the drug. A total of 25 μg of cell extract protein isolated from untreated (0 h) and TPA-treated A2780/CP70 cells was subjected to Tris-glycine gel electrophoresis and immunoblotted with a c-Fos antibody (upper) or with a c-Jun antibody (lower). c-Fos protein band and c-Jun protein band densities were quantified by densitometry. The relative Fos or Jun protein levels were expressed as a ratio to untreated cells and are shown graphically in panel B.

mobility shift assay was based on sequence analysis of the 5′-flanking region of *ERCC-1* gene as described previously [14]. Two duplex 21-bp oligonucleotides which encompassed an *ERCC-1* AP-1-like site (5′-TCACTGC**TGTGTCA**CCAGCAC-3′, within -355 to -375 from the transcriptional start site at $+1$ in the *ERCC-1* promoter region) (see fig. 1) and an altered *ERCC-1* AP-1-like site (5′-TCACTGC**TGAGTCA**CCAGCAC-3′, -355 to -375) were synthesized by Lofstrand Labs Limited (Gaithersburg, MD) and were purified by reverse phase cartridge chromatography. The altered *ERCC-1* AP-1-like site contains a consensus AP-1 element produced by a 1-bp substitution from thymidine residue to adenosine as indicated by the underline. The double-stranded oligonucleotides were labeled with (α-^{32}P) ATP by phosphorylation with bacteriophage T4 polynucleotide kinase and unincorporated precursors were removed using G-25 Sephadex columns (Boehringer Mannheim, Indianapolis, IN). Twenty-one base-pair oligonucleotides that contained the accepted consensus sequence for AP-1, AP-2, CREB, TFIID and NF-κB were obtained from Promega Corporation (Madison, WI) and used in binding or competition studies described below.

Electrophoretic mobility shift assay (EMSA). The nuclear extracts were analyzed for transcription factor binding activity by gel mobility shift assays. Briefly, nuclear extracts were incubated in 20-μl volume with $1\times$ binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DDT, 50 mM NaCl, 4% glycerol, 10 mM Tris-HCl, pH 7.5) and 2 μg of poly (dI-dC)-poly (dI-dC) (Pharmacia, Piscataway, NJ) at room temperature for 10 min. The ^{32}P-labeled target DNA was then added, and the mixture was incubated for 20–30 min at room temperature. In some experiments, a 50-fold concentration of unlabeled competitor DNA was included in the sample prior to the addition of the radiolabeled probe. After the completion of the binding reaction, 2 μl of $10\times$ gel loading buffer (250 mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol) was added, and samples were electrophoresed at room temperature through a 4% nondenaturing polyacrylamide gel in $0.5\times$ TBE running buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) for 4 h at 100 V which had been pre-run at 100 V for 30 min prior to the sample loading. The gels were dried under vacuum and visualized by autoradiography.

Supershift assay. The nuclear extracts were preincubated with antiserum at room temperature for 20–30 min before analysis by EMSA as described above. The human anti-Fos and anti-AP-2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); the affinity purified human anti-Jun antibody has been previously described [37]. These sera specifically detect the presence of the corresponding transcription factor and do not interfere with nuclear factor binding.

Results

Concentration response and time course of induction of ERCC-1 mRNA expression in response to phorbol ester in human ovarian carcinoma cells. We determined first whether TPA can directly stimulate ERCC-1 mRNA expression in the human ovarian cancer cell line, A2780/CP70. Time and dose response studies were conducted. A2780/CP70 cells were stimulated for 6 h with

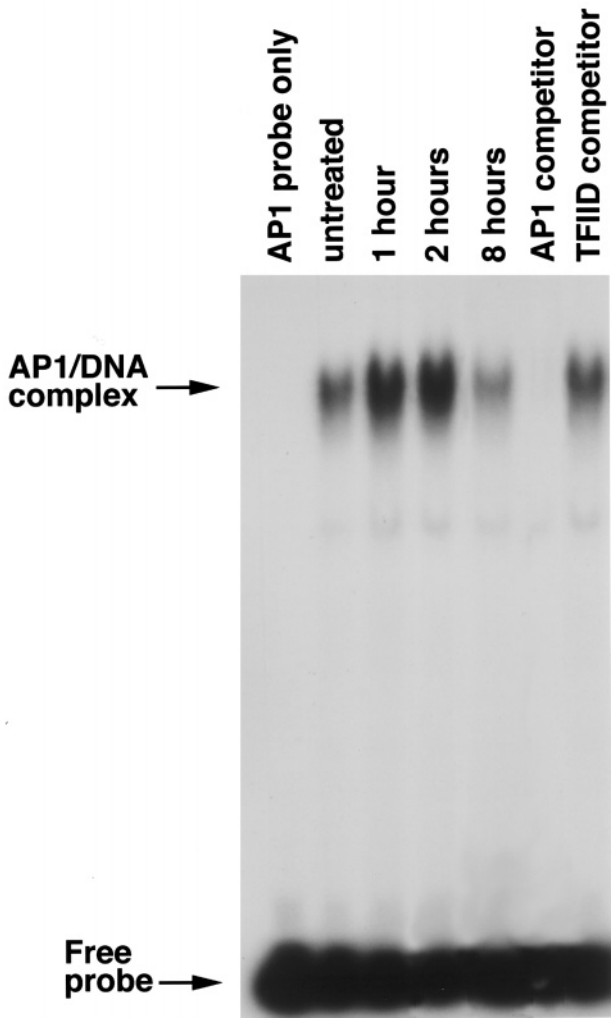

Figure 8. EMSA of AP-1 binding activity in nuclear extracts from TPA-treated and untreated A2780/CP70 cells. Cells were incubated with 100 ng/ml of TPA, and nuclear extract was prepared at different times after the drug exposure. EMSAs were performed by using a ^{32}P-labeled synthetic double-stranded oligonucleotide containing the *ERCC-1* promoter sequence of AP-1-like binding site as probe. The protein-DNA complexes formed are indicated (arrow). The unbound (free) probe in the gel is shown at the bottom. TPA-inducible DNA binding activity was abolished by competition with a 50-fold molar excess of unlabeled AP-1 oligonucleotide (lanes labeled 'AP-1 competitor') but not by an identical concentration of TFIID oligonucleotide (lanes labeled 'TFIID competitor').

varying concentrations of TPA (10–5000 ng/ml), total cellular RNA was then isolated, and ERCC-1 mRNA expression was assessed by Northern blot analysis. TPA was found to induce ERCC-1 mRNA expression in a concentration-dependent manner (fig. 2). Dose response experiments showed that the effect of TPA was maximal at 100 ng/ml with about sixfold increase in the ERCC-1 mRNA level. In addition, the effect of TPA on *ERCC-1* induction was found to be time-dependent (fig. 3). ERCC-1 mRNA levels started to increase when the cells were treated with 100 ng/ml of TPA for 1 h and peaked at 3–6 h with the exposure of cells to TPA, and then the levels of ERCC-1 mRNA decreased gradually as the incubation time with TPA increased. Long-term exposure of A2780/CP70 cells to TPA (100 ng/ml) for up to 48–72 h caused further downregulation of *ERCC-1* to levels comparable to the untreated cells in this system. Our results also show that the phorbol ester has the same effect on other human ovarian tumor cell lines (data not presented). The *ERCC-1* increase was not associated with changes in the steady-state levels of GAPDH mRNA (figs 2 and 3).

Effect of cycloheximide or α-amanitin on the phorbol ester-dependent increase in ERCC-1 mRNA content. We next examined the role of ongoing protein synthesis in the induction of ERCC-1 mRNA in response to TPA. The effect of cycloheximide, an inhibitor of protein synthesis, was investigated. When the cells were preincubated for 2 h with cycloheximide at 10 μM prior to treatment with TPA for 6 h, the expected increase in ERCC-1 mRNA did not occur (fig. 4; lanes 2 and 5). Cycloheximide alone had little effect on baseline ERCC-1 mRNA levels (fig. 4; lanes 1 and 6), suggesting that new protein synthesis is required for TPA-dependent ERCC-1 mRNA induction. To determine whether the TPA effect on *ERCC-1* expression was at the transcriptional level, TPA-induced cells were cultured with the transcriptional inhibitor α-amanitin at a concentration of 5 μg/ml. Again the expected increase in *ERCC-1* was not seen (fig. 4; lanes 4 and 5). The same results were observed when another widely used intercalating transcription inhibitor actinomycin D was used at a concentration of 5 μg/ml (data not shown). Moreover, α-amanitin repressed the basal expression of ERCC-1 mRNA (fig. 4; lane 3). These data suggest that ERCC-1 mRNA induction by TPA is transcriptionally regulated. Since α-amanitin has been shown to be a specific inhibitor for RNA polymerase II [38], this suggests that these genes are normally transcribed by RNA polymerase II.

Transcriptional regulation of the *ERCC-1* gene by phorbol ester. To determine more directly whether TPA can induce the transcription of the *ERCC-1* gene, in vitro transcript elongation (nuclear run-on) assays were performed using purified A2780/CP70 cell nuclei following incubation with TPA. As seen in figure 5, transcription activity increased to about 2.8 times control (untreated cells) by 3 h after incubating A2780/CP70 cells with 100 ng/ml of TPA. No signal was detected for the plasmid vector control sequences (fig. 5). Transcription of the *ERCC-1* gene in these assays was blocked by 5 μg/ml of α-amanitin (data not shown).

Induction of nuclear factor AP-1 binding activity by phorbol ester exposure. We examined the effect of TPA on the expression of the components of AP-1 in A2780/CP70 cells. As shown in figure 6, TPA exposure induced a rapid but transient expression of c-*fos* and c-*jun* mRNA in a time-dependent manner with peak levels of five- to sixfold increase at 0.5 to 1 h in this system.

We next determined whether TPA treatment induced an increase in c-Jun or c-Fos protein level in these cells by Western blot (immunoblot) analysis (fig. 7). Consistent with the Northern blot (RNA) analysis (fig. 6), the TPA-induced increases in c-*jun* and c-*fos* mRNAs were paralleled by increases in c-Jun and c-Fos proteins. Protein levels peaked at 1–2 h following TPA administration with about a two- to threefold increase (fig. 7).

We analyzed the 5′-flanking region of the *ERCC-1* gene [14] and found an AP-1-like site located between −361 and −367 bp in the *ERCC-1* promoter which consisted of TGTGTCA (see fig. 1). No consensus sequence for AP-2, CREB or NF-κB was found in this region. We constructed two oligonucleotides of 21 bp (−355 to −375), as described in 'Materials and methods', encompassing this potential binding region and performed EMSA with these oligonucleotides. Induction of AP-1 nuclear factor binding to the *ERCC-1* AP-1-like site (5′-TGTGTCA-3′) was found as an early event after TPA exposure, and binding activity peaked at 2 h and decreased to the baseline level by 8 h (fig. 8). Identical results were observed with an *ERCC-1* AP-1-like site altered to contain a consensus AP-1 site (5′-TGAGTCA-3′), and with an oligonucleotide which contained the consensus AP-1 sequence (5′-CGCTTGA**TGAGTCA**-GCCGGAA-3′) of the collagenase promoter (Promega) (data not shown). To ascertain whether the binding specificity observed was consistent with transcription factor binding to a classical AP-1-binding sequence, a 21-bp oligonucleotide containing the accepted consensus sequence for AP-1 was used as a competitor in the EMSA. This oligonucleotide, at 50-fold excess, abolished binding to the experimental ^{32}P-labeled AP-1-like site (fig. 8), whereas a 21-bp oligonucleotide for TFIID did not (fig. 8). Other oligonucleotides used included AP-2, CREB and NF-κB, and these oligomers also showed no effect (data not shown).

We tested the effect of protein synthesis inhibition on the induction of AP-1 binding activity by TPA. Exposure to TPA for 2 h is associated with peak AP-1 binding activity (fig. 8). Figure 9 shows that the increase in AP-1 binding activity induced by TPA is inhibited by a 2-h preincubation with the protein synthesis inhibitor cycloheximide. To further characterize the proteins binding to the AP-1-like site in TPA-treated cells, we used supershift assays with antibodies specific to Fos, Jun or AP-2. In figure 10, nuclear extracts show binding to the 21-bp AP-1-like oligomer from *ERCC-1*. Also, migration of this oligomer-protein complex is retarded by antibodies to Fos or to Jun, but not by antibody to AP-2.

Discussion

The aim of this work was to evaluate the effect of TPA on the induction of mRNA expression of *ERCC-1* in

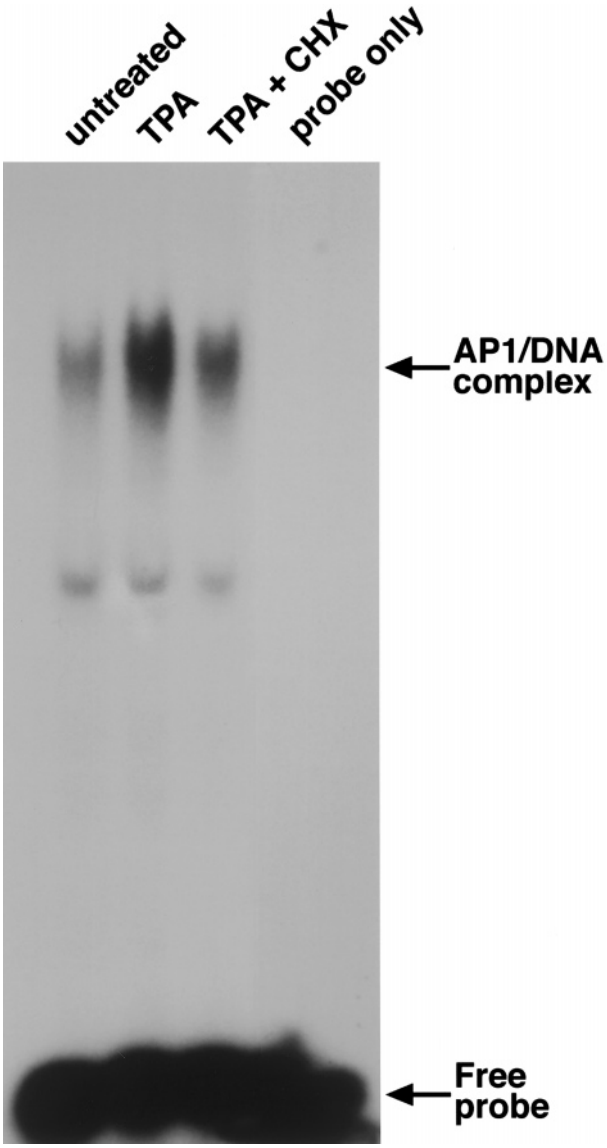

Figure 9. Effect of protein synthesis inhibition on the induction of AP-1 binding activity by phorbol ester. A2780/CP70 cells were incubated for 2 h in the presence or absence of 10 μM cycloheximide (CHX), followed by exposure to TPA at 100 ng/ml for 2 h. Nuclear extracts were incubated with ^{32}P-labeled AP-1-like oligonucleotide probe and analyzed by gel mobility shift assay.

human ovarian cancer cells, and the mechanism involved in the process. In view of the fact that TPA may act through AP-1 [20, 22], we analyzed the potential role of this transcription factor in mediating the activation of *ERCC-1* transcription after phorbol ester exposure [20, 22, 23]. In the present study, we demonstrate here for the first time that TPA induces increases in steady-state levels of ERCC-1 mRNA in A2780/CP70 human ovarian carcinoma cells (figs 2 and 3). We have investigated TPA induction of *ERCC-1* in several additional human ovarian cancer cell lines. In each case, the TPA effect is virtually identical to the effect seen in A2780/CP70 cells (E. Reed and Q. Li, unpublished observations). Our results also show that this induction of ERCC-1 mRNA by TPA is regulated at the transcriptional level (figs 4 and 5). In vitro run-on assays in nuclei isolated from control and TPA-stimulated A2780/CP70 cells revealed that the increase of ERCC-1 mRNA occurred in large part because of an increase in the level of de novo transcription. This finding suggests that the most prominent action of TPA on ovarian cancer cell *ERCC-1* expression is by activation of *ERCC-1* gene transcription.

As has been reported with other TPA-inducible genes [22–25], the transcriptional activator AP-1 appears to contribute to the elevated levels of *ERCC-1* gene transcription. The binding activity for nuclear factor AP-1 is substantially increased in response to TPA within 2 h (fig. 8). This TPA-related AP-1 activation is followed by an increase in the *ERCC-1* transcription rate (fig. 5), and an increase in the *ERCC-1* steady-state mRNA levels (fig. 3). By contrast, binding activity to AP-2, CREB or NF-κB elements were not induced by TPA in these cells (Q. Li and E. Reed, unpublished results). The inducible increase of the AP-1 binding activity by TPA appears to involve at least two steps: an induction of c-*jun* and c-*fos* gene expression with the accumulation of increased levels of the respective mRNAs (fig. 6), and a de novo synthesis of the AP-1 component proteins (figs 7 and 9). Our data suggest that the activated AP-1 heterodimer may complex to the AP-1-like site (5′-TGTGTCA-3′) of *ERCC-1* in ovarian cancer cells, since antibodies directed to either c-Jun or c-Fos significantly altered the mobility of AP-1 binding complexes in supershift analysis (fig. 10).

Cycloheximide produced an inhibitory effect on the TPA induction of AP-1 binding activity (fig. 9) and ERCC-1 mRNA levels (fig. 4). The cycloheximide effect probably reflects a decreased synthesis of c-Fos and c-Jun proteins. Newly synthesized AP-1 proteins may be necessary for the upregulation of *ERCC-1* gene expression. However, such a mechanism does not exclude posttranslational modifications of the AP-1 component proteins which may also occur upon exposure of cells to phorbol esters [22, 23]. TPA has been reported to induce AP-1 binding activity by a mechanism independent of de novo protein synthesis [39], which is thought to be mediated by AP-1 component protein phosphorylation [22, 23]. It appears likely that both processes are involved in TPA-induced *ERCC-1* gene expression in A2780/CP70 cells.

ERCC-1 is an essential gene within the NER pathway, and appears to be a useful marker for NER DNA repair activity [16–18]. Therefore, activation of AP-1, leading to the overexpression of *ERCC-1* and other AP-1-regulated DNA repair genes, may enhance DNA repair capacity in affected cells. This could contribute to alterations in the genetic program of human ovarian cancer cells. Such genetic changes may be associated with phenotypic changes, such as decreased radiosensitivity and chemosensitivity [5, 7, 8, 19, 40]. This hypoth-

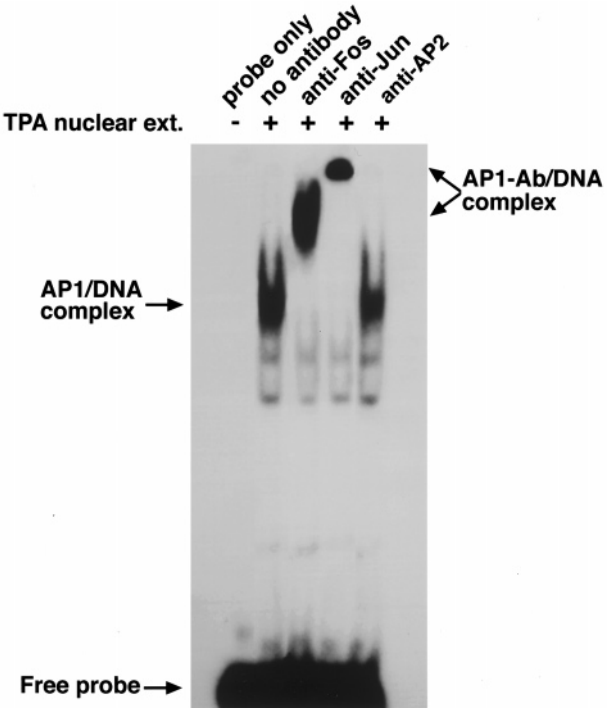

Figure 10. Supershift assay of binding activity in TPA-treated A2780/CP70 cell nuclear extracts to experimental *ERCC-1* AP-1-like binding site. Cells were incubated with 100 ng/ml of TPA for 2 h, and nuclear extract was then prepared. EMSAs were performed by using a ^{32}P-labeled synthetic double-stranded oligonucleotide containing the *ERCC-1* AP-1-like binding site as probe. The AP-1/DNA complexes formed are indicated (arrow). The unbound (free) probe in the gel is shown at the bottom. The use of anti-Fos or anti-Jun antibody before EMSA is seen to result in a super-retardation in band mobility consistent with the formation of protein-antibody (Ab)/DNA complexes (arrow with AP-1-Ab/DNA complex), whereas a supershift in band mobility is not seen when anti-AP-2 antibody was used before EMSA (lanes labeled 'anti-AP-2').

Table 1. Comparison of the time course of the events related to *ERCC-1* induction by TPA and by cisplatin in A2780/CP70 cells.

Event examined	TPA stimulation (h)	Cisplatin stimulation (h)
Peak c-*jun*/*fos* mRNA levels	0.5–1	1–2
Peak Jun/Fos protein levels	1–2	3–5
Peak AP-1 binding levels	1–2	3–5
Peak *ERCC-1* transcription levels	2–3	4–6
Peak ERCC-1 mRNA levels	3–6	24–48
Peak ERCC-1 protein levels	12–24	24–48

esis is supported by several lines of evidence. We have previously demonstrated that cisplatin exposure activates an AP-1 mediated increase in *ERCC-1* expression in human ovarian tumor cells [29]. Others have shown that overexpression of wild-type c-Jun is associated with cisplatin resistance [41], whereas inhibition of AP-1 activity in cells modified by overexpression of a dominant negative mutant of c-Jun, blocks DNA repair, and leads to decrease in viability following treatment with cisplatin [41]. Furthermore, cells in which AP-1 or *ERCC-1* has been genetically inactivated are hypersensitive to genotoxic insults, including antitumor agents or UV irradiation [42–44]. These observations suggest that AP-1 may play a prominent role in modulating DNA repair processes in both the physiological and pathophysiological conditions, and AP-1-dependent DNA repair activities may provide important stress-protective functions in cells, which effectively reduce the cytotoxic, mutagenic and carcinogenic consequences of DNA damage. In addition, this may also explain, at least partially, the observed increase in ERCC-1 mRNA in clinical specimens that are platinum-resistant [16–18].

AP-1 DNA binding sites are the cis-acting elements of *ERCC-1* and some other DNA repair genes; therefore, signal transduction pathways that modulate AP-1 may be important in the mediation of DNA repair. We show that cisplatin or TPA both induce an AP-1-mediated increase in ERCC-1 mRNA expression in this system. However, their pathways leading to AP-1 activation may not be the same. Table 1 shows the time course of the events related to *ERCC-1* induction by TPA and by cisplatin. Generally, each step in the process of *ERCC-1* induction occurs more quickly after TPA exposure. Upregulation of c-*jun* and of c-*fos* mRNA is followed by increased protein levels. This is followed by increased AP-1 binding activity to the *ERCC-1* promoter, which is followed by increases in ERCC-1 mRNA, and then protein. The differences in time course outlined in the table is consistent with the previously proposed model that DNA damage may cause activation of the JNK/SAPK (c-Jun NH_2-terminal kinase/stress-activated protein kinase) pathway, whereas non-DNA-damage types of cellular stress may activate the ERK (extracellular signal-regulated kinase) pathway [22, 23].

Recent work suggested that cisplatin activates JNK/SAPK (c-Jun NH_2-terminal kinase/stress-activated protein kinase) in human ovarian carcinoma cells and other tumor cells [41, 45]. We have also shown that JNK/SAPK may directly phosphorylate c-Jun at serine residues 63 and 73, and thereby activate *ERCC-1* transcription in A2780/CP70 ovarian cancer cells upon stimulation with cisplatin [29]. In contrast, a rather large body of literature indicates that TPA exerts its action on AP-1 activation through an ERK (extracellular signal-regulated kinase) pathway in a wide variety of different cell types [22–24]. This appears to suggest that the pathways induced by cisplatin and TPA may differ from each other, although they may converge and act on the same cis-regulatory elements within the *ERCC-1* promoter. Investigations are in progress to study the role of MAP kinases (ERK and JNK/SAPK) in the regulation of *ERCC-1* expression in response to phorbol esters or DNA-damaging agents, and delineate the signal transduction pathways involved in both the transcriptional and posttranscriptional processes in human ovarian carcinoma cells. The molecular steps between the signaling events and damage response/DNA repair in human tumors are complex. However, further understanding of this mechanism may give insights into the development of new and potentially useful strategies for practical applications.

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