available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

MZF1 possesses a repressively regulatory function in *ERCC1* expression

Qing-Wu Yan [1], *Eddie Reed, Xiao-Song Zhong, Keith Thornton, Yi Guo, Jing Jie Yu* *

Department of Biochemistry and Molecular Pharmacology, and Mary Babb Randolph Cancer Center, Robert C. Byrd Health Sciences Center, West Virginia University, 1610-C Health Sciences South, Morgantown, WV 26506-9300, USA

ARTICLE INFO

Article history:
Received 30 August 2005
Accepted 9 December 2005

Keywords:
MZF1
AP1
ERCC1
Repressor
Cisplatin resistance
NER

Abbreviations:
CDDP, cisplatin or *cis*-diamminedichloroplatinum(II)
NER, nucleotide excision repair
ERCC1, excision repair cross-complementation group 1
EMSA, electrophoretic mobility shift assay
RT-PCR, reverse transcriptase-polymerase chain reaction
CAT, chloramphenicol acetyltransferase
ELISA, enzyme-linked immunoassay
CMV, cytomegalovirus
AP1, activator protein 1
MZF1, myeloid zinc finger gene 1

ABSTRACT

ERCC1 is a critical gene within the nucleotide excision repair pathway. Overexpression of *ERCC1* through promoter-mediating transcriptional regulation is associated with repair of cisplatin-induced DNA damage and clinical resistance to platinum-chemotherapy. Several transcriptional repressors and activators within the 5′-flanking region of the *ERCC1* gene may be involved in the up-regulation of this gene. Minimal sequence within the promoter region required for *ERCC1* transcription was analyzed by CAT assay and demonstrated that the region of −220 to −110 is essential to constitutive expression of *ERCC1* gene in ovarian cancer cell line A2780/CP70. A more forward upstream region seems to be responsible for cisplatin-induced expression. Study of the functional cis-element in this region by electrophoretic mobility shift assay indicates that a MZF1-like site as well as an AP1-like site responded in a time-dependent manner to cisplatin stimulation with altered binding activities. EMSA with MZF1 ZN1–4 consensus oligonucleotides suggests that the MZF1 N-terminal domain of zinc finger cluster may bind to the MZF1-like site of the *ERCC1* promoter region. MZF1 mRNA in A2780/CP70 cells decreased upon cisplatin exposure as analyzed by quantitative PCR, suggesting that MZF1 may mediate cisplatin-invoked gene expression in these cells. Overexpression of MZF1 repressed the *ERCC1* promoter activity as determined in co-transfection assay, suggesting that MZF1 might be a repressor of *ERCC1* transcription upon cisplatin exposure. In summary, our studies revealed a core promoter region and adjacent drug-responsible region within the *ERCC1* promoter. The drug-responsible region contains cis-elements of activator, AP1 and repressor, MZF1. In response to cisplatin treatment, decreased MZF1 and increased AP1 binding activities appear to be the leading mechanism of up-regulation of *ERCC1* expression. Our findings imply potential therapeutic strategies to antagonize drug resistant mechanisms in treatment of human ovarian cancer.

* *Corresponding author*. Tel.: +1 304 293 8661; fax: +1 304 293 5244.
E-mail address: jyu@hsc.wvu.edu (J.J. Yu).
[1] Present address: Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA.
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2005.12.015

1. Introduction

Platinum-based chemotherapeutic agents such as cisplatin and carboplatin are the mainstream drugs for almost all advanced cancers [1,2]. Unfortunately, many cancer patients eventually relapse following treatment and become refractory to these anticancer agents [3]. Thus, the acquisition or presence of resistance to platinum drugs is a major clinical obstruction to effective treatment. Elucidation of the molecular basis for cisplatin resistance is required to improve the effectiveness of platinum-chemotherapy [4]. Previous studies reported that clinical resistance to platinum-therapy is positively associated with the human DNA repair gene *ERCC1* [5], and is effected through the platinum-induced DNA adduct repair pathway [6,7]. In this process, cisplatin covalently binds to DNA, which results in intrastrand adducts and interstrand cross-links that stall transcription and replication [8,9]. In addition, enhanced DNA repair of platinum–DNA adducts has been observed in cisplatin-resistant human ovarian cancer cells, and DNA repair inhibitors in some models can potentate cisplatin cytotoxicity [10–13].

The removal of platinum–DNA adducts is thought to occur by nucleotide excision repair (NER), which is responsible for processing DNA lesions that cause large distortions in DNA helical structure, such as platinum–DNA adducts [14]. During NER, a dual incision is made asymmetrically around a lesion to allow its release as part of a large (24–32 nt) DNA fragment [15]. *ERCC1* with *XPF* (xeroderma pigmentosum group F) forms a heterodimeric protein complex (a structure-specific endonuclease) that catalyses incision on the 5′-side of the lesion during NER [16,17]. Chinese hamster cell lines defective in either *ERCC1* or *XPF* show hypersensitivity to UV light and to DNA interstrand cross-linking agents [18,19]. The above findings led us to focus on the most important DNA repair NER pathway and, specifically, on the *ERCC1* gene.

ERCC1 is highly conserved in nature; it is found in plants, in *E. coli*, and in almost all mammalian cells [20]. *ERCC1* expression reflects DNA repair capacity and clinical resistance [18,21]. The expression of *ERCC1* was elevated in tissues from patients refractory to cisplatin therapy [5]. In in vitro studies, overexpression of the *ERCC1* gene is associated with a platinum-resistant phenotype in ovarian cancer cells [11,22]. In contrast, the levels of expression of *ERCC1* in cisplatin hypersensitive, repair-deficient cells are 30- to 50-fold lower than in inherently resistant cells [18]. Cisplatin treatment in human ovarian cancer A2780/CP70 cells led to a six-fold increase in *ERCC1* mRNA expression, and *ERCC1* mRNA induction by cisplatin is transcriptionally regulated [22]. Therefore, understanding the molecular mechanism of regulation and control of the *ERCC1* expression especially in transcriptional level will provide a possible way to change a platinum-resistant phenotype into one that is platinum-sensitive.

The 414-bp sequence within the 5′-flanking region of the *ERCC1* gene has been studied in detail by Van Duin and colleagues [20], who have shown that constructs of this region may drive transcription of *ERCC1* gene. By means of a transfection assay the *ERCC1* promoter was confined to a region of ±170 bp upstream of the transcriptional start site [20]. Classical promoter elements like CAAT, TATA and GC-boxes are absent in this region of the *ERCC1* gene [20].

The human myeloid zinc finger 1 (*MZF1*) gene is located on chromosome 19q, band 13.3 and encodes a protein that acts as transcription factor [23]. It belongs to the Krüppel family of zinc finger proteins and expresses predominantly in myeloid progenitor cells and in totipotent hemopoietic cells [24]. MZF1 is involved in growth, differentiation, and apoptosis of myeloid progenitors and was found to play a key role in regulating transcription during differentiation along the myeloid lineage [25–27]. It is believed that MZF1 overexpression inhibits hematopoiesis [28], raising the possibility that MZF1 functions as a transcription repressor. This gene contains 13 C_2H_2 zinc finger domains divided into two groups, which can bind DNA independently [23]. MZF1 has been reported as a bi-functional transcription regulator, repressing transcription in non-hematopoietic cells, and activating transcription in cells of hematopoietic origins [24–28]. Consensus MZF1 binding sites were found in the promoters of several hematopoietic cell-specific genes, including CD34, c-Myc, lactoferrin, and myeloperoxidase [25,26].

In this investigation, we analyzed the function of *ERCC1* promoter in both constitutive and drug-induced manners. We identified functional elements corresponding to trans-factors MZF1 and AP1 that regulate *ERCC1* transcription in response to cisplatin-induced DNA damage/repair pathway.

2. Material and methods

2.1. Computer-based analysis of ERCC1 promoter

DNA sequence of the 5′-flanking region of human *ERCC1* gene (−415 to +32 bp) was obtained from GenBank™ (Accession ID: X06581). MatInspector professional software (Genomatix GmbH, Germany) with family matches was utilized to analyze the putative binding sites of transcription factors in the above region of the *ERCC1* gene [29]. Searching parameters were set at 0.75–1.00 for core similarity. Quality library from vertebrates (Matrix family library) was adopted in searches. Transcription factors were selected to match those existing in humans and subjected to functional analysis with TRANSFAC [30].

2.2. Cell line and cell culture conditions

The human ovarian cancer cell line A2780/CP70 was used in all experiments and has been described previously [11]. Cells were cultured in monolayer using RPMI 1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.2 units/ml human insulin, 50 units/ml penicillin and 50 μg/ml streptomycin (Life Technologies Inc., Gaithersburg, MD). Cells grew in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air, and were routinely tested for mycoplasmal infection using a commercial assay system (MycoTect; Life Technologies Inc.). New cultures were established monthly from frozen stocks. All media and reagents contained <0.1 ng/ml endotoxin as determined by Limulus polyphemus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Cell viability was determined in triplicate by trypan blue dye exclusion, and cells were collected when they reached ~90% confluence.

Cisplatin (provided by Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) was initially dissolved in PBS (phosphate-buffered saline, w/o Ca^{2+} or Mg^{2+}) at 1.0 mg/ml (3.33 mM), and then diluted in media to obtain the desired concentrations. A2780/CP70 cells were treated with IC_{50} dose of cisplatin (40 μM unless otherwise indicated) for 1 h and washed twice with PBS. Fresh drug-free media were replenished after drug removal, and cells were continuously incubated for 24–48 h or for the time indicated.

2.3. Construction of reporter plasmids

Cellular DNA was extracted and purified from A2780/CP70 cells by hot phenol/chloroform method [31]. Oligonucleotides were selected to amplify fragments of 410, 330, 220 and 110 base pairs specific to the 5′-untranslated region of the *ERCC1* promoter [20] and to include restriction enzymes *Xho*I and *Sac*I (for facilitating cloning into the CAT expression vector). The primers listed in Table 1 were designed using the Amplify 2.1 software (HARDI Electronics AB, Sweden) and synthesized by Genosys Fisher Scientific (Pittsburgh, PA). PCR was performed at 35 cycles under optimal PCR conditions using the PCR amplification kit (BRL, Gaithersburg, MD). Aliquots of the PCR products were electrophoresed on a 1% agarose gel, and the DNA fragments were purified using the QIAEX® II kit. The purified fragments were digested with restriction enzymes *Xho*I and *Sac*I, and then ligated into the pCAT3 expression vector (Promega, Madison, WI). Ligated DNA-vectors were transformed into DH5 alpha cells (BRL, Gaithersburg, MD) and screened for correct inserts by restriction enzyme digestion with *Xho*I and *Sac*I. All constructed plasmids were diluted to a concentration of 0.5 μg/μl and stored at −20 °C until used.

2.4. Transient transfection and CAT assay

A2780/CP70 cells were seeded at 3×10^5 cells/well in a 6-well tissue culture plate (Fisher, Pittsburgh, PA) and allowed to adhere for 30 min at 37 °C. Promoter constructs were transfected using FuGene cationic liposome (F. Hoffmann-La Roche Ltd., Switzerland) according to the manufacturer's instruction. Cells were harvested 24 h post-transfection. CAT ELISA assay was performed with CAT ELISA Assay Kit (Roche). Briefly, cells were washed with PBS, disrupted in lysis buffer, and then centrifuged for 10 min at 13,000 rpm. Samples were diluted 1:400 prior to measurement. According to the manufacturer's instruction, chloramphenicol acetyltransferase was quantitatively determined using a colorimetric enzyme immunoassay.

2.5. Preparation of nuclear extracts

Nuclear extracts were prepared from cisplatin-treated or untreated A2780/CP70 cells using a modified procedure described by Dignam et al. [32]. Briefly, 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 sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, 25 mM HEPES, pH 7.5). The cell mixture was incubated on ice for 20 min and then extracted by adding 1.6 volumes of extraction buffer (0.5 mM EDTA, 20% glycerol, 1.66 M KCl, 0.4 mM sodium fluoride, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 25 mM HEPES, pH 7.5) with constant shaking for 4 h at 4 °C. The extracts were centrifuged at 55,000 × *g* for 1 h at 4 °C, and the supernatant was dialyzed for 4 h at 4 °C in a buffer containing 20 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM sodium fluoride, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride and 1.0 mM dithiothreitol. Nuclear extract content was determined by BCA protein assay (Pierce) and samples were stored at −80 °C.

2.6. Preparation of oligonucleotide probes

The design of oligonucleotides for the following electrophoretic mobility shift assay was based on sequence analysis of the 5′-flanking region of *ERCC1* gene. The oligonucleotides including *ERCC1* promoter fragments, MZF1 consensus and mutated *ERCC1* MZF1-like binding motifs are listed in Table 1. These oligonucleotides were synthesized by Gene Probe

Table 1 – Oligonucleotides used in EMSA and quantitative PCR experiments

Oligonucleotides	Sequence
ERCC1 USF-like site (−400 to −380, Accesion ID: X06581)	5′-GAGGACAACACGGGGCTGTCG-3′
ERCC1 AP1-like site (−375 to −355, Accesion ID: X06581)	5′-TCACTGCTGTGTCACCAGCAC-3′
ERCC1 Ets1/Myc-like site (−340 to −316, Accesion ID: X06581)	5′-CCGGGAAGAGAGGAAGCGCGTGGGG-3′
ERCC1 NF1-like site (−196 to −170, Accesion ID: X06581)	5′-CTGTTCTCCACTGAAGCCCTGCCAAGA-3′
ERCC1 MZF1-like site (−324 to −298, Accesion ID: X06581)	5′-CGCGTGGGGGGAATAGGTGTGGAATAA-3′
ERCC1 MZF1-like site mutation 1	5′-CGCGT**aatc**GGAATAGGTGTGGAATAA-3′
ERCC1 MZF1-like site mutation 2	5′-CGCGTGGGGGGAATAGGT**a**T**t**GAATAA-3′
MZF1 ZN1–4 consensus oligos (TRANSFAC Accesion ID: M00083)	5′-GATCTAAAAGTGGGGAGAAAA-3′
MZF1 ZN5–13 consensus oligos (TRANSFAC Accesion ID: M00084)	5′-GATCCGGCTGGTGAGGGGGAATCG-3′
MZF1 sense primer (464–483, Accesion ID: AF055077)	5′-GTCCAGAGGTACGCTCCAAG-3′
MZF1 antisense primer (762–781, Accesion ID: AF055077)	5′-CCAGTGGTGATTCCTGCATA-3′
18S rRNA sense primer (1780–1800, Accesion ID: M10098)	5′-AGTCCCTGCCCTTTGTACACA-3′
18S rRNA antisense primer (1828–1848, Accesion ID: M10098)	5′-GATCCGAGGGCCTCACTAAAC-3′

Underlines indicate the core sequence of binding matrix of the corresponding transcription factor; and the lowercase and bold letters indicate mutated bases.

Technologies Inc. (Gaithersburg, MD) and purified with reverse-phase cartridge chromatography. The double-stranded oligonucleotides were labeled with [^{32}P]ATP through phosphorylation with bacteriophage T4 polynucleotide kinase; and unincorporated precursors were removed using G-25 Sephadex columns (F. Hoffmann-La Roche Ltd., Switzerland). The other oligonucleotides that were used as controls in our experiments were purchased from Promega Corp. (Madison, WI).

2.7. Electrophoretic mobility shift assay (EMSA)

The nuclear extracts were analyzed for binding activity of transcription factors by gel mobility shift assays. Briefly, nuclear extracts were incubated in a 20 μl volume of 1× binding buffer (1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane, 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) for 10 min at room temperature. Then, [^{32}P]ATP-labeled probe was added to the above mixture and incubated for 30 min at room temperature. In some experiments, a 50-fold concentration of unlabeled competitor DNA was included in samples prior to the addition of the radiolabeled probe. After completion of the binding reaction, samples were mixed with 2 μl of 10× gel loading buffer (250 mM Tris–HCl, pH 7.5, 0.2% bromophenol blue, 0.2% xylene cyanol and 40% glycerol) and electrophoresed at room temperature through a 5% nondenaturing polyacrylamide gel in 0.5× 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 for 30 min at 100 V prior to the sample loading. The gels were visualized by autoradiography. For supershift assays, the nuclear extracts were pre-incubated with antiserum for 30 min at room temperature prior to EMSA. The human anti-Fos and anti-Jun antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). These sera specifically detect the presence of the corresponding transcription factor and do not interfere with nuclear factor binding.

2.8. Co-transfection assay with MZF1 expression vector

MZF1 gene was a gift from Dr. Morris F. Jennifer (Medical College of Wisconsin, Milwaukee, WI). The *MZF1* gene was cloned into downstream of the immediate-early promoter of CMV. The plasmids were grown in L-broth + ampicillin. Human ovarian cancer A2780/CP70 cells were seeded into 6-well plate at 300 cells/well, which grew overnight at 37 °C. The MZF1 expression vector (MZF1/CMV plasmids), or the blank vector as control, was transfected into the A2780/CP70 cells together with the pCAT410 reporter plasmids using FuGene cationic liposome. Prior to cisplatin treatment, cells were washed with PBS after removal of transfection-media. Cells were treated with cisplatin at IC_{50} dose for 1 h and continuously in culture until harvest at indicated timepoints for CAT ELISA assay.

2.9. Quantitative PCR

MZF1 mRNA levels of cisplatin-treated and untreated cells were analyzed by quantitative PCR. Briefly, total RNA was extracted from A2780/CP70 cells using RNeasy Mini Kit (Qiagen). cDNAs obtained from 2 μg of each RNA sample via SuperScript II reverse transctiptase (Invitrogen) were amplified in triplicate with 2× SYBR Green Master Mix in 50 μl reaction volumes on an ABI 7700 Sequence Detection System (Applied Biosystems). Human 18S rRNA was also amplified as an endogenous control for normalization purpose. *MZF1* primers and 18S rRNA primers used for this experiment are listed in Table 1. Validation experiment was performed to demonstrate the equal efficiencies of target *MZF1* and of internal control 18S rRNA. The relative amount of *MZF1* transcripts was determined using comparative C_t method with the expression level of untreated control as 1.

3. Results

3.1. Potential ERCC1 promoter transcription factors

ERCC1 is a critical enzyme in the nucleotide excision repair pathway, and appears to be essential for life. *ERCC1* mRNA expression has been linked to cellular and clinical resistance to platinum compounds. We therefore investigated the upstream region of transcription start site, *ERCC1* promoter region (448-bp, from −415 to +32) using MatInspector professional software with family matches. A total of 24 matching binding sites of transcription factors were found using parameter of 0.75 set as core similarity. A stringent search with core similarity as 1.00 resulted in 16 matches. Thirteen transcription factors of the 16 binding sites were verified to exist in human beings (Fig. 1), and these 13 transcription factors were subjected to further analysis with TRANSFAC transcription factors database. Binding matrices of seven transcription activators (USF, HEN1, AP1, Ets1, MYC/MAX, PIT1 and NF1) and of one transcription repressor (MZF1) were found in −415 to −170 region of *ERCC1* promoter sequence. Binding sites of c-Ets1 (P54, activator), NRSE and CDP3 (repressors), ZID and VDR/RXR (function depending on the context) were found in the region of −170 to +32 of *ERCC1* promoter (Fig. 1). The promoter was found to lack TATA, CAAT or GC boxes in the upstream region.

3.2. Minimal 5′-flanking sequence required for ERCC1 promoter activity

We utilized polymerase chain reaction (PCR) to develop DNA fragments of variable lengths and named pCAT110, pCAT220, pCAT330 and pCAT410 according to their approximate lengths from the 5′-flanking region of *ERCC1* gene (Fig. 1). We cloned these fragments into the pCAT 3 expression plasmid. Each construct was transfected into A2780/CP70 cells to assess the ability of protein generation. In our experiments, we found that the 410 base segment generated a high level of CAT expression (~14-fold higher than the pCAT control vector). Sixty-nine percentage of CAT expression was lost between the pCAT220 construct and the pCAT110 construct. The 330 and 220 base segments were as active as the 410 base segment (Fig. 2). These results suggest that the sequence between −220 and −110 of *ERCC1* promoter region is required for full promoter activity under normal cellular conditions. Interestingly, this essential fragment lacks the ability to respond to cisplatin treatment (data not shown), which suggests that the cisplatin-induced

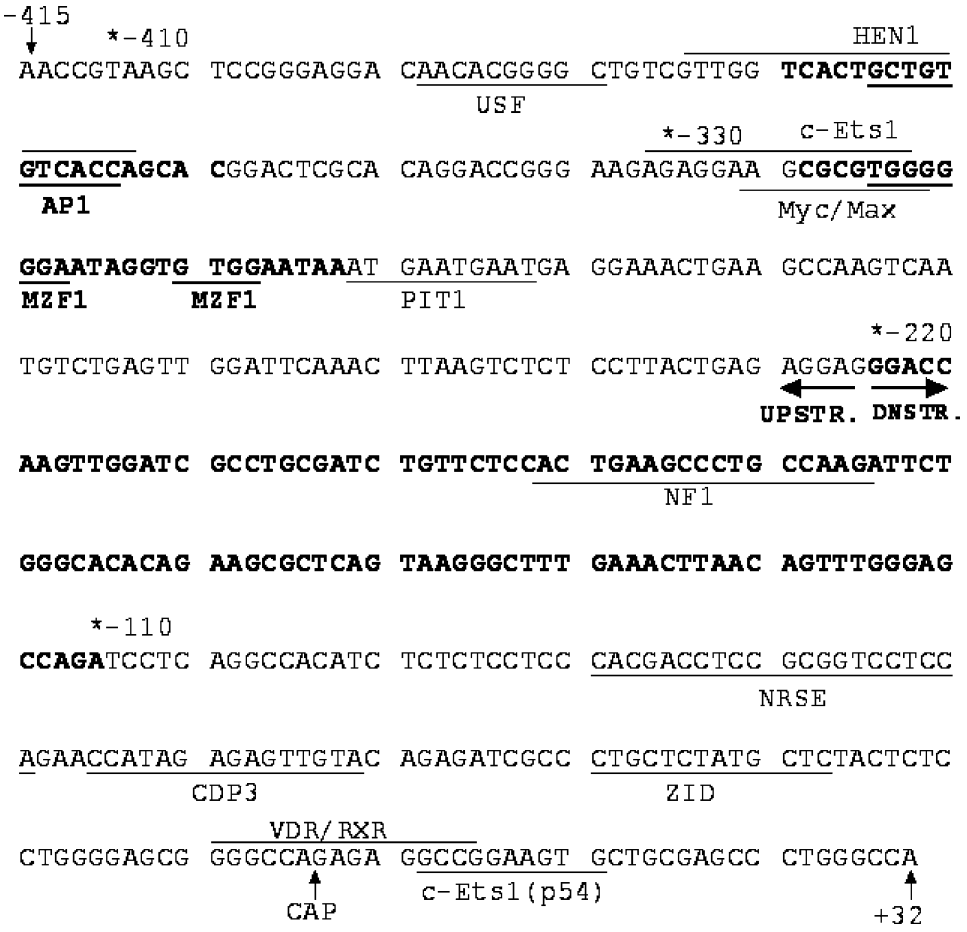

Fig. 1 – Nucleotide sequence of the *ERCC1* promoter region. The binding consensus sites of transcription factors are labeled. The 5′-ends of various constructs for the CAT assay are indicated with asterisks. The numbers represent the nucleotide position relative to the transcription initiation site. The upstream region (UPSTR) and the downstream region (DNSTR) of the *ERCC1* promoter are depicted with horizontal arrows. Bold letters show the essential sequences of fundamental transcription of the *ERCC1* gene and regulatory elements within this region.

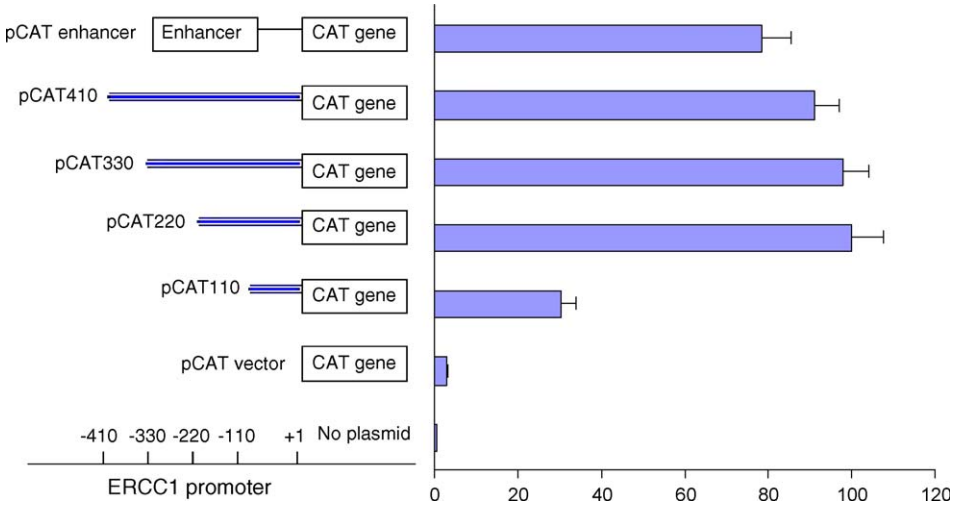

Fig. 2 – Functional analysis of 5′-flanking region of *ERCC1* gene. Left panel, various lengths of the *ERCC1* promoter region were inserted into the pCAT 3 basic plasmid. Right panel, CAT activity of a series of the deletion constructs was presented relative to the pCAT220. The CAT activity was normalized to β-galactosidase activity. The data are presented as mean ± S.D. of three independent experiments.

enhancement of *ERCC1* expression may involve other mechanisms or other transcription factors beyond this region.

3.3. *Functional cis-elements in the upstream promoter region responding to cisplatin stimulation*

To investigate the mechanism of drug induced *ERCC1* expression we synthesized several fragments containing transcription binding motifs within the upstream region of *ERCC1* promoter, according to the results of computer analysis. These synthesized nucleotide fragments included −400 to −380 (USF), −375 to −355 (AP1), −340 to −316 (c-Ets1/c-Myc), −324 to −298 (MZF1) and −196 to −170 (NF1) (Table 1). The A2780/CP70 cells were treated with 40 μM cisplatin for 1 h, and then continuously cultured to specific timepoints (6, 12 and 48 h). EMSA with nuclear extracts from above cisplatin treated and untreated samples illustrated that synthesized fragments USF, c-Ets1/c-Myc and NF1 displayed no significant band of DNA–protein complex (data not shown).

A shifted band was clearly identified in the samples of cisplatin-treated and incubated with AP1 fragment, while the corresponding band of untreated samples was very weak (Fig. 3). This result is consistent with the findings reported by Li et al., who showed that AP1 was responsible, at least partially, for the cisplatin-induced *ERCC1* expression [22]. To further characterize the proteins corresponding to this AP1-like site, we employed supershift assays with antibodies specific to Fos and Jun. The results demonstrated that each of the two antibodies caused an altered gel migration of the AP1·DNA complex (data not shown). Our data suggest that both c-Fos and c-Jun may be involved in cisplatin-induced response.

Another fragment, a putative DNA–protein complex formed by transcription binding motif MZF1, drew our attention. This complex band was strongly displayed in untreated samples and decreased in amount at 6 h and more decreased at 12 h after treatment (Fig. 3). These results suggest that the ERCC1 MZF1 fragment (27-mer) within the *ERCC1* promoter may function as a repressor of *ERCC1* transcription following cisplatin exposure.

To further confirm the above observations, we performed EMSA to measure the binding activity of AP1 and MZF1 within *ERCC1* promoter after cisplatin treatment. This detailed investigation of the AP1-like site and MZF1-like site demonstrated that the binding activity of the AP1-like site stayed at a high level 6–24 h post-treatment, whereas the binding activity of the MZF1-like site remained at its baseline level (Fig. 4). Our data suggest that the cisplatin-induced *ERCC1* promoter activity depends on synergistic interaction between activator AP1 and MZF1, which is located closely to the AP1 site within the 5′-flanking region of the *ERCC1* gene. Collectively, these data illustrate that decreased MZF1 may contribute to the increased transcription of *ERCC1* gene, synergistically associated with increased activator AP1 level after cisplatin-treatment in human ovarian cancer A2780/CP70 cells.

3.4. *MZF1 ZN1–4 may be responsible for the depression of binding activity*

To determine what region of MZF1 plays the essential role of transacting factor corresponding to the MZF1-like site, we

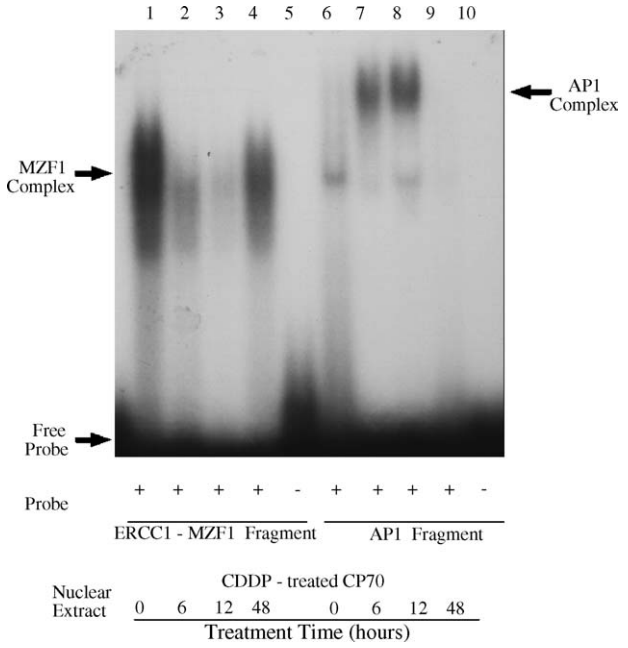

Fig. 3 – EMSA of potential binding sites of AP1 and MZF1 in nuclear extracts from cisplatin-treated and untreated A2780/CP70 cells. Cells were incubated with 40 μmol/l of cisplatin for 1 h, and nuclear extracts were prepared at different timepoints after the drug exposure. Ten micrograms of nuclear protein was incubated with [^{32}P]ATP-labeled double-stranded oligonucleotides corresponding to AP1 element at the −375 to −355 region or MZF1 element at the −324 to −298 region of the *ERCC1* promoter, respectively. The gel was visualized by autoradiography. The formed protein–DNA complexes are indicated (top arrows). The unbound (free) probe is shown at the bottom.

synthesized two MZF1 consensus oligonucleotides: the MZF1 ZN1–4 (21-bp fragment, 5′-GAT CTA AAA GTG GGG AGA AAA-3′), which can bind to zinc fingers 1–4 of MZF1 protein, and the MZF1 ZN5–13 (24-bp fragment, 5′-GAT CCG GCT GGT GAG GGG GAA TCG-3′), which can bind to zinc fingers 5–13 of MZF1 protein. Competing EMSA using three DNA fragments (ERCC1 MZF1, MZF1 ZN1–4 and MZF1 ZN5–13) showed that the ERCC1 MZF1–protein complex was competed off completely at 100-fold molar excess by unlabelled MZF1 and by MZF1 ZN1–4, but not by MZF1 ZN5–13 (Fig. 5). In addition, protein–MZF1 ZN1–4 complex shifted to the same position as ERCC1 MZF1–protein complex, suggests that the sequence encompassing the MZF1 ZN1–4 binding site within upstream region of the *ERCC1* promoter participates in the DNA–protein interaction. To further illustrate the role of the essential nucleotides ERCC1 MZF1-like site (27-mer fragment), we synthesized two mutated oligonucleotides within the MZF1 core sequence (ERCC1 MZF1-like site mutation 1 and ERCC1 MZF1-like site mutation 2) and performed EMSA analyses as shown in Fig. 6. The corresponding DNA–protein complexes disappeared completely when either mutated probe was added. These data suggest that MZF1 ZN1–4 may be responsible for the depression of binding activity, and

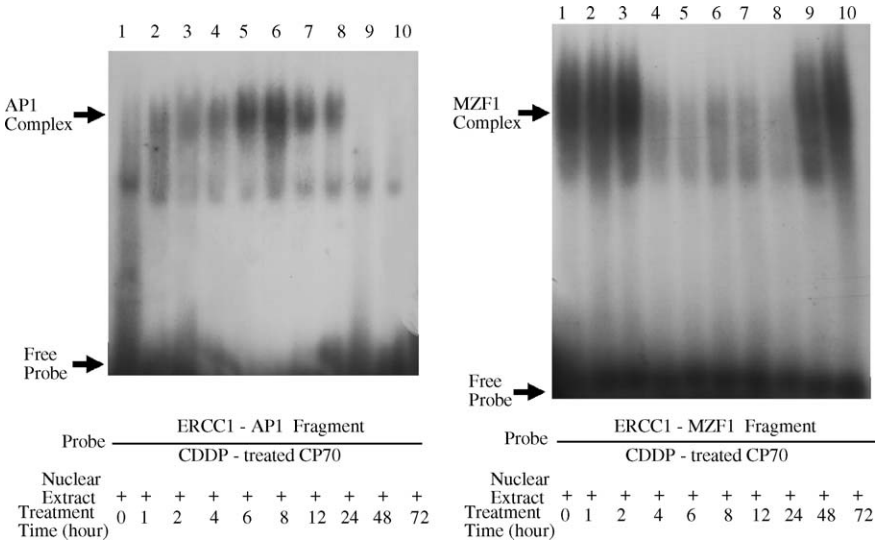

Fig. 4 – Binding activities of AP1 element and MZF1 element within the *ERCC1* promoter during time course after cisplatin treatment. A2780/CP70 cells were incubated for 1 h in the presence of medium containing 40 μmol/l cisplatin, and continuously to be cultured in fresh medium (w/o drug) to the indicated timepoints. EMSAs were performed with nuclear extracts from above samples and [^{32}P]ATP-labeled oligonucleotides as described in Fig. 3.

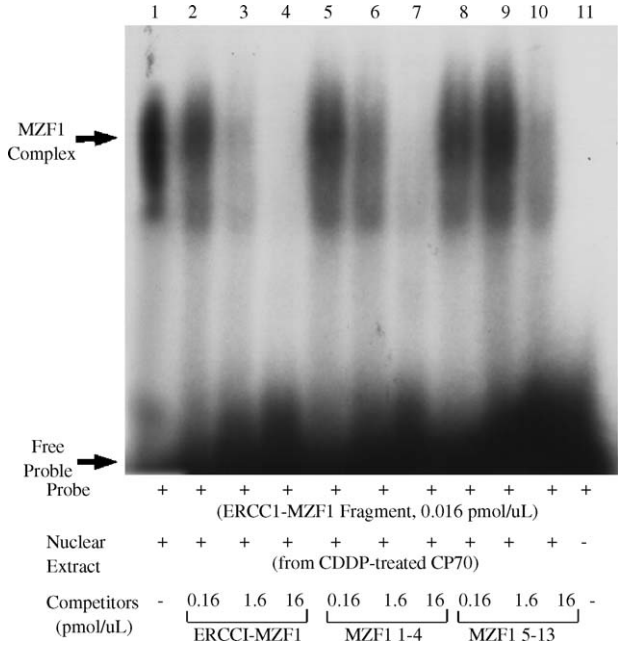

Fig. 5 – MZF1 ZN1–4 consensus oligonucleotides competing with ERCC1 MZF1-like site in EMSA. Nuclear extracts were prepared from cisplatin-treated (for 2 h) A2780/CP70 cells. Sequences of oligonucleotides used in this experiment are listed in Table 1. All of oligonucleotides were double-stranded, and the ERCC1 MZF1-like site was labeled with [^{32}P]ATP. The final concentration of labeled oligonucleotides was 0.016 pmol/l, and the concentrations of competitors are as indicated above. The unlabeled competitors were added to the reactions 10 min before adding the labeled probes. The gel was visualized by autoradiography.

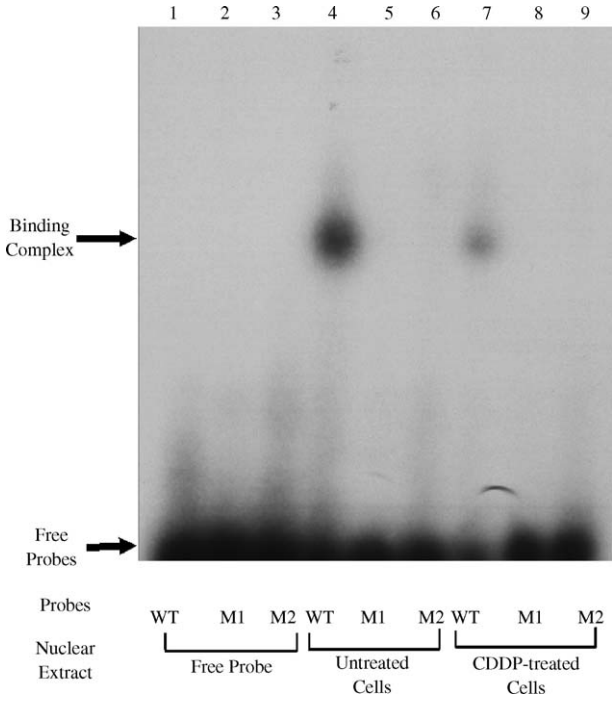

Fig. 6 – EMSA analysis of Mutated MZF1-lile site within *ERCC1* promoter. Sequences of wild type of the ERCC1 MZF1-like site (WT), mutation 1 (M1) and mutation 2 (M2) are listed in Table 1. All of oligonucleotides were double-stranded and labeled with [^{32}P]ATP. Cells were incubated with 40 μmol/l of cisplatin for 1 h, and nuclear extracts were prepared at 8 h after cisplatin exposure. Ten micrograms of nuclear protein were incubated with the labeled probes. The gel was visualized by autoradiography. The protein–DNA complexes are indicated (top arrow). The unbound (free) probe is shown at the bottom.

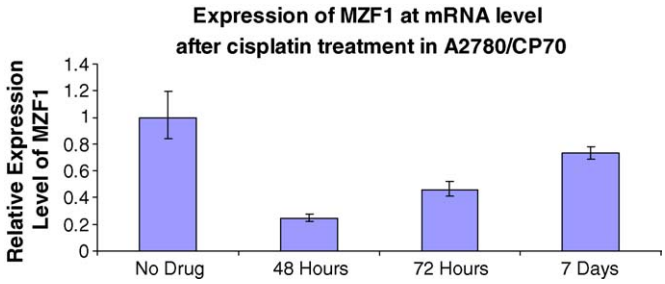

Fig. 7 – Analysis of *MZF1* mRNA expression levels in cisplatin-treated and untreated A2780/CP70 cells by quantitative PCR. Cells were treated with 40 μmol/l cisplatin (IC_{50} dose) for 1 h and then continuously incubated in fresh medium for 48 h, 72 h, or 7 days, respectively. Total RNA was extracted using RNeasy Mini Kit. cDNAs obtained from 2 μg of each RNA sample (via SuperScript II reverse transctiptase) were amplified with 2× SYBR Green Master Mix on an ABI 7700 Sequence Detection System. Primers used to amplify the specific fragments of *MZF1* gene and human 18S rRNA as internal control are listed in Table 1.

MZF1 may possess repressively regulatory function in *ERCC1* expression.

3.5. *Expression of MZF1 mRNA suppressed by cisplatin exposure*

In our quantitative reverse transcriptase-PCR experiments, we employed a pair of primers selected from different exons of the *MZF1* gene (Table 1). The results of quantitative PCR showed that mRNA level of *MZF1* gene expression decreased about 75% in the cells collected at 48 h after cisplatin exposure, compared to the untreated control. The expression levels gradually recovered, but did not show complete recovery even after 7 days (Fig. 7). This data confirmed that *MZF1* mRNA expression is responsive to cisplatin stimulation and that MZF1 may be involved in cisplatin-induced gene regulation in A2780/CP70 cells.

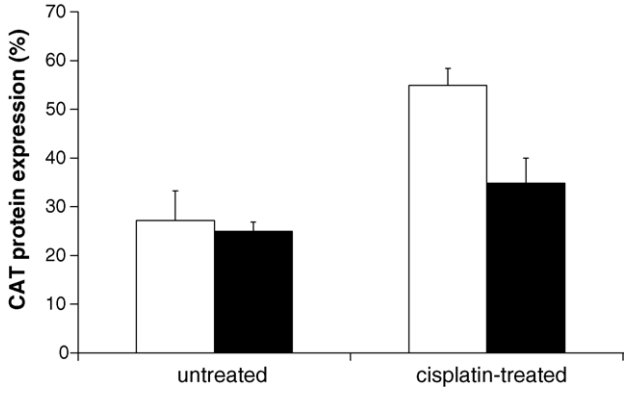

Fig. 8 – The effects of MZF1 overexpression on the transcription activity of *ERCC1* determined by CAT assay. MZF1 expression vectors (black bars) or blank vectors (white bars) were co-transfected into A2780/CP70 cells with pCAT410 reporter plasmids and then CAT assay was performed. A2780/CP70 cells were treated with IC_{50} dose of cisplatin (40 μM) for 1 h and then cultured for 24 h prior to CAT assay.

3.6. *Overexpression of MZF1 represses ERCC1 transcription*

To further confirm that MZF1 repressively regulates *ERCC1* transcription, we performed CAT assay with pCAT410 reporter plasmid, plus either blank CMV plasmid or MZF1/CMV expression plasmid (co-transfection). MZF1/CMV plasmid was generated by cloning *MZF1* gene into CMV plasmid. As shown in Fig. 8, the luciferase expression through *ERCC1* promoter was significantly repressed by the co-expressed MZF1 in the cisplatin treated cells. This result further confirmed that MZF1 protein may play a negative role in transcription regulation of the *ERCC1* gene upon cisplatin exposure.

4. Discussion

In our present investigation, a 448-bp sequence upstream of exon I of the *ERCC1* gene, including the putative promoter region, was analyzed with MatInspector program and TRANSFAC transcription factor database. Binding sites of classic transcription factors USF, AP1 and c-Ets1 were identified in the upstream region of the examined sequence. The c-Ets1 was reported to co-operate with AP1 as a transcription activator [33]. Within the *ERCC1* promoter region, binding sites of these two activators were located closely to each other (AP1 −370 to −360 and c-Ets1 −332 to −318). More binding matrices were found in the upstream region, most of them were transcription activators, and only MZF1 located at −320 to −313 is a potential transcription repressor [26]. Two other putative repressors, NRSF and CDP, located at the downstream region of the *ERCC1* promoter, were identified [34]. Regulatory function of these transcription factors and their interactions with *ERCC1* promoter are under investigation.

A previous study by Van Duin and colleagues showed that the 448-bp region upstream of the transcription start site could drive the transcription of *ERCC1* gene [20]. This suggested that the promoter region (448-bp, extending from −415 to +32 nucleotides) is required for full expression of *ERCC1* gene and

may contain functionally important *cis*-DNA elements. The results of our CAT assay suggest that the sequence between −220 and −110 of *ERCC1* promoter is required for fundamental promoter activity under normal cellular conditions. Deletion of this fragment resulted in dramatic decrease of *ERCC1* mRNA transcription. In addition, the sequence of −415 to −220 is critical to cisplatin-induced overexpression of the *ERCC1* mRNA. Deletion or mutation of this region eliminated *ERCC1* mRNA expression in response to cisplatin exposure, whereas, the constitutive expression (without drug stimulation) was slightly affected. Thus, the entire 448-bp fragment within the 5′-region of the *ERCC1* is necessary for the biologic function of this gene.

The results of cisplatin-induced *ERCC1* transcription imply that the upstream promoter region contains functionally important *cis*-DNA elements. Computer analysis suggests that this region contains several binding sites of putative transcription factors such as USF, AP1, Ets1 and MZF1. We analyzed the functional importance of these *cis*-DNA elements using electrophoretic mobility shift assay in human ovarian cancer A2780/CP70 cells in the presence or absence of cisplatin. The transcription activator AP1 at binding site of −375 to −355, and the transcription repressor MZF1 at binding site of −324 to −298, significantly shifted the binding complexes in EMSA. Notably, the close location of these two binding sites within *ERCC1* promoter made it possible for interaction between AP1 and MZF1 trans-factors.

The activator protein 1 (AP1) family is a group of transcription factors responsible for activation of a wide variety of genes in different cell types and tissues [35,36]. The AP1 transcription factor consists of either Jun/Jun homodimers, or Fos/Jun heterodimers, which bind to the palindromic sequence TGA(C/G)TCA [37]. AP1 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) [38] and therefore AP1 binding sites function as TPA-responsive elements (TREs) [38]. As reported by Li et al., AP1 played an important role in the up-regulation of *ERCC1* overexpression upon cisplatin stimulation, and de novo synthesis of Jun and/or Fos and a post-translational activation of Jun were involved in this regulation [22]. The current studies confirmed the biological function of AP1 in *ERCC1* overexpression upon cisplatin exposure.

The myeloid zinc finger gene 1 (*MZF1*), encodes a transcription factor, which is expressed preferentially, but not restrictedly, in myeloid cells [24,39]. MZF1 protein contains 13 zinc fingers arranged in two distinct sets: the first set contains zinc fingers 1–4, and the second set, in the carboxyl terminus, contains zinc fingers 5–13 [39]. Both sets of zinc fingers are able to bind to DNA, and DNA consensus binding sites have been identified for each of these DNA-binding domains [39] (Table 1). MZF1 is a bi-functional transcription regulator, repressing transcription in non-hematopoietic cells, and activating transcription in cells of hematopoietic origins [26–28]. *MZF1* expression is necessary for hematopoietic cell differentiation [28] and critical to the regulation of cell proliferation and apoptosis [27]. As shown in our EMSA results, protein-binding activity of the MZF1-like site was decreased after cisplatin treatment, whereas the *ERCC1* mRNA was increased at the same time. It implies that the interaction of MZF1-like binding site and its corresponding trans-factor, MZF1, may function as a transcription repressor of *ERCC1*. Furthermore, overexpression of MZF1 repressed *ERCC1* promoter activities, which suggested that the corresponding trans-factor of this functional cis-element is MZF1. Moreover, the ZN1–4 domain of *MZF1* is essential in this DNA–protein interaction, as shown in our data of competition EMSA. In a RT-PCR experiment, we found that *MZF1* mRNA constitutively expressed in untreated human ovarian cancer A2780/CP70 cells (data not shown). Our quantitative PCR in the same cells demonstrated that *MZF1* mRNA was decreased upon cisplatin exposure. These results further confirmed that the transcription factor MZF1 functions as a transcription repressor on *ERCC1* expression in human ovarian cancer cells, and that cisplatin-evoked *MZF1* repression may be involved in overexpression of *ERCC1* during chemotherapeutic process. Hoffman et al. reported that *MZF1* is a telomeric gene and located at the extreme end of the q arm of human chromosome 19. *MZF1* promoter region lies less than 10 kb from the telomeric repeats [40]. Thus, it is possible that the regulatory region of *MZF1* or the transcribed sequence of the gene is disrupted by cisplatin, resulting in decreased expression of *MZF1* upon drug exposure. These mechanisms are under investigation in our lab.

Li et al. previously reported that cisplatin could induce *ERCC1* overexpression through transient induction of Fos/Jun [22]. Our current data illustrated that reduced mRNA expression of *MZF1* may also contribute to the overexpression of *ERCC1* upon cisplatin stimulation. In addition, the binding sites of AP1 and MZF1 are located very closely within the *ERCC1* promoter, thus enhanced AP1 binding activity might alleviate the binding activity of MZF1. Also, reduced MZF1 binding activity might boost the binding activity of AP1, resulting in the synergistic effects between the enhanced AP1 and reduced MZF1 in up-regulation of the *ERCC1* expression.

In summary, our studies indicate that the core promoter region (∼220 bp) is required in the constitutive expression of the *ERCC1* gene during homeostasis maintenance, and that the upstream region (∼410 bp) and its corresponding trans-factors are responsible for cisplatin-induced transcription activation of the *ERCC1* gene. Our findings also suggest that MZF1 possesses repressively regulatory function in *ERCC1* expression, and that cisplatin reduces this repressive activity, which may be relevant to the mechanism of cisplatin resistance in the treatment of human ovarian cancer. Further investigations are warranted to reveal the molecular mechanism through which AP1 versus MZF1 mediates *ERCC1* expression. Such studies may enable the development of novel anticancer strategies that prevent and/or reverse the platinum-resistant phenotype by inhibiting or controlling *ERCC1* expression in human ovarian cancer.

Acknowledgments

We would like to thank Michael D. Mueller for editorial consultation. Supported by a post-doctoral intramural research training award (IRTA) from the National Institutes of Health.

REFERENCES

[1] Reed E, Dabholkar M, Chabner BA. Platinum analogues. In: Chabner BA, Longo DL, editors. Cancer chemotherapy. 2nd ed., Philadelphia: Lippincott–Raven Publishers; 1996. p. 357.
[2] Loehrer PJ, Einhorn LH. Drugs 5 years later. Cisplatin. Ann Intern Med 1984;100:704.
[3] Timmer-Bosscha H, Mulder NH, de Vries EGE. Modulation of *cis*-diamminedichloroplatinum(II) resistance: a review. Br J Cancer 1992;66:227.
[4] Masuda H, Ozols RF, Lai G-M, Fojo A, Rothenberg M, Hamilton TC. Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. Cancer Res 1988;48:5713–6.
[5] Dabholkar M, Vionnet JA, Bostick-Bruton F, Yu JJ, Reed E. mRNA levels of XPAC and ERCC1 in ovarian tumor tissue correlates with response to platinum containing chemotherapy. J Clin Invest 1994;94:703–8.
[6] Behrens BC, Hamilton TC, Masuda H. Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. Cancer Res 1987;47:414–8.
[7] Eastman A. Characterization of the adducts produced in DNA by *cis*-diamminedichloroplatinum(II) and *cis*-dichloro(ethylenediamine)platinum(II). Biochemistry 1983;22:3927–33.
[8] Szymkowski DE, Yarema K, Essigmann JM, Lippard SJ, Wood RD. An intrastrand d(GpG) platinum cross-link in duplex M13 DNA is refractory to repair by human cell extracts. Proc Natl Acad Sci USA 1992;89:10772–6.
[9] Jones JC, Zhen W, Reed E, Parker RJ, Sancar A, Bohr VA. Gene-specific formation and repair of cisplatin intrastrand adducts and interstrand cross-links in Chinese hamster ovary cells. J Biol Chem 1991;266:7101–7.
[10] Masuda M, Tanaka T, Matsuda H, Kusaba I. Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to *cis*-diamminedichloroplatinum(II). Cancer Res 1990;50:1863–6.
[11] Parker RJ, Eastman A, Bostick-Bruton F, Reed E. Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin–DNA lesions and reduced drug accumulation. J Clin Invest 1991;87:772–7.
[12] Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF, et al. Role of platinum–DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. Biochem Pharmacol 1994;47:689–97.
[13] Katz E, Andrews P, Howell S. The effect of DNA polymerase inhibitors on the cytotoxicity of cisplatin in human ovarian carcinoma cells. Cancer Commun 1990;2:159–64.
[14] Sancar A. Mechanisms of DNA excision repair. Science 1994;266:1954–6.
[15] Moggs JG, Yarema KJ, Essigmann JM, Wood RD. Analysis of incision sites produced by human cell extracts and purified proteins during nucleotide excision repair of a 1,3-intrastrand d(GpTpG)-cisplatin adduct. J Biol Chem 1996;271:7177–86.
[16] Park CH, Sancar A. Formation of a ternary complex by human XPA, ERCC1, and ERCC4(XPF) excision repair proteins. Proc Natl Acad Sci USA 1994;91:5017–21.
[17] van Vuuren AJ, Appeldoorn E, Odijk H, Humbert S, Moncollin V, Eker AP, et al. Partial characterization of the DNA repair protein complex containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities. Mutat Res 1995;337:25–39.
[18] Lee KB, Parker RJ, Bohr VA, Cornelison TC, Reed E. Cisplatin sensitivity/resistance in UV-repair deficient Chinese hamster ovary cells of complementation groups 1 and 3. Carcinogenesis 1993;14:2177–80.
[19] Zhen W, Link CJ, O'Connor PM, Reed E, Parker RJ, Bohr VA. Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. Mol Cell Biol 1992;12:3689–98.
[20] van Duin M, de Wit J, Odijk H, Westerveld A, Yasui A, Koken HM, et al. Molecular characterization of the human excision repair gene ERCC-1: cDNA cloning and amino acid homology with the yeast DNA repair gene RAD10. Cell 1986;44:913–23.
[21] Taverna P, Hansson J, Scanlon KJ, Hill BT. Gene expression in X-irradiated human tumour cell lines expressing cisplatin resistance and altered DNA repair capacity. Carcinogenesis 1994;15:2053–6.
[22] Li Q, Gardner K, Zhang L, Tsang B, Bostick-Bruton F, Reed E. Cisplatin induction of ERCC-1 mRNA expression in A2780/CP70 human ovarian cancer cells. J Biol Chem 1998;273:23419–25.
[23] Morris JF, Hromas R, Rauscher FJ. Characterization of the DNA-binding properties of the myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two DNA consensus sequences with a common G-rich core. Mol Cell Biol 1994;14:1786–95.
[24] Hromas R, Collins SJ, Hickstein D, Raskind W, Deaven LL, O'Hara P, et al. A retinoic acid-responsive human zinc finger gene, MZF-1, preferentially expressed in myeloid cells. J Biol Chem 1991;266:14183–7.
[25] Morris JF, Rauscher FJ, Davis B, Klemsz M, Xu D, Tenen D, et al. The myeloid zinc finger gene, MZF-1, regulates the CD34 promoter in vitro. Blood 1995;86:3640–7.
[26] Perrotti D, Melotti P, Skorski T, Casella I, Peschle C, Calabretta B. Overexpression of the zinc finger protein MZF1 inhibits hematopoietic development from embryonic stem cells: correlation with negative regulation of CD34 and c-Myc promoter activity. Mol Cell Biol 1995;15:6075–87.
[27] Hromas R, Boswell S, Shen RN, Burgess G, Davidson A, Cornetta K, et al. Forced overexpression of the myeloid zinc finger gene MZF-1 inhibits apoptosis and promotes oncogenesis in interleukin-3-dependent FDCP.1 cells. Leukemia 1996;10:1049–50.
[28] Hromas R, Davis B, Rauscher FJ, Klemsz M, Tenen D, Hoffman S, et al. Hematopoietic transcriptional regulation by the myeloid zinc finger gene, MZF-1. Curr Top Microbiol Immunol 1996;211:159–64.
[29] Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucl Acids Res 1995;23:4878–84.
[30] Wingender E, Chen X, Hehl R, Karas H, Liebich I, Matys V, et al. TRANSFAC: an integrated system for gene expression regulation. Nucl Acids Res 2000;28:316–9.
[31] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
[32] Dignam JP, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl Acids Res 1983;11:1475–89.
[33] Wasylyk B, Wasylyk C, Flores P, Begue A, Leprince D, Stehelin D. The c-Ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature 1990;346:191–3.
[34] Schoenherr CJ, Anderson DJ. The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. Science 1995;267:1360–3.
[35] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 1995;270:16483–6.

[36] Gottlicher M, Rahmsdorf HJ, Herrlich P. The regulation of AP-1 activity by mitogen-activated protein kinases. In: Papavassiliou AG, editor. Transcription factors in eukaryotes. Austin, TX: Landes Bioscience; 1997. p. 67.
[37] Abate C, Luk D, Curran T. Transcriptional regulation by Fos and Jun in vitro: interaction among multiple activator and regulatory domains. Mol Cell Biol 1991;11:3624–32.
[38] Angel P, Imagawa M, Chiu R, Stein B, Imbra RJ, Rahmsdorf HJ, et al. Phorbol ester-inducible genes contain a common cis-element recognized by a TPA-modulated trans-acting factor. Cell 1987;49:729–39.
[39] Hui P, Guo X, Bradford PG. Isolation and functional characterization of the human gene encoding the myeloid zinc finger protein MZF-1. Biochemistry 1995;34:16493–502.
[40] Hoffman SM, Hromas R, Amemiya C, Mohrenweiser HW. The location of MZF-1 at the telomere of human chromosome 19q makes it vulnerable to degeneration in aging cells. Leuk Res 1996;20:281–3.