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Regulation of DNA Repair Gene Expression in Human Cancer Cell Lines

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Abstract Although most advanced cancers are incurable, the majority of testicular germ cell tumors can be cured using cisplatin-based combination chemotherapy. The nucleotide excision repair (NER) pathway removes most DNA adducts produced by cisplatin, and the low levels of NER in testis tumor cells may explain why these cancers are curable. Three NER proteins: ERCC1, XPF, and XPA, are present at low levels in testis tumor cell lines, and addition of these proteins to protein extracts of testis tumor cells increases their in vitro DNA repair capacity to normal levels. The aim of this study was to identify the mechanism responsible for the low levels of these DNA repair proteins. The levels of the mRNA transcripts for *ERCC1, XPF*, and *XPA* were measured in a panel of 14 different human cancer cell lines, using real-time PCR. Three ERCC1 splice variants were identified and quantitated. Three alternative transcription start points (TSPs) were identified for ERCC1 but none were testis-specific. The significantly lower levels of ERCC1, XPF, and XPA protein in testis tumor cell lines cannot be explained solely by differences in transcriptional efficiency or mRNA stability. For *ERCC1*, post-transcriptional control by alternative splicing does not account for the testis-specific low levels of protein expression. Pulse-chase experiments showed that the half-life of ERCC1 protein in a testis tumor cell line was not significantly different to that in a prostate cancer cell line. Taken together, these results suggest that constitutive levels of these DNA repair proteins are controlled at the level of translation. J. Cell. Biochem. 97: 1121–1136, 2006.

Key words: ERCC1; XPF; XPA; testicular cancer; real-time PCR; nucleotide excision repair

The introduction of cisplatin to the treatment of metastatic testicular germ cell tumors resulted in the majority of patients being cured, and now over 85% of men with advanced disease can expect to live a normal life-span [Einhorn, 2002]. In stark contrast, almost all other cancers in adults are incurable once they have spread beyond the primary site. The long-term goal of our studies is to understand the molecular basis of chemosensitivity in testicular germ cell tumors, as it may then be possible to translate the successful treatment of metastatic disease to other types of cancer.

The inorganic metal co-ordination complex cisplatin [*cis*-diamminedichloroplatinum(II)] exerts toxicity by damaging DNA. DNA repair is an important mechanism determining sensitivity to chemotherapeutic drugs. The nucleotide excision repair (NER) pathway repairs the majority of lesions produced by chemotherapeutic agents. Testis tumor cell lines retain

Abbreviations used: NER, nucleotide excision repair; TSP, transcription start point; nt, nucleotides; FCS, fetal calf serum; UNG, uracil-*N*-glycosylase; UTR, untranslated region.

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their sensitivity to cisplatin in vitro [Walker et al., 1987] and the cells have low levels of NER capacity [Bedford et al., 1988; Köberle et al., 1996, 1997].

ERCC1 and XPF form a heterodimeric complex that functions as an endonuclease in the NER pathway. The NER pathway is essential for removal of cytotoxic DNA adducts such as those produced by cisplatin. XPA is a protein involved in the initial damage recognition and recruitment stage of this pathway. It has been shown that the protein levels of three essential NER subunits: ERCC1, XPF, and XPA are present at low levels in testis tumor cell lines relative to other more cisplatin resistant cell lines [Köberle et al., 1999; Welsh et al., 2004]. Addition of these proteins to protein extracts of testicular germ cell tumor cell lines increased their DNA repair capacity to normal levels [Köberle et al., 1999]. These findings may explain why testis tumor cells are more sensitive to cisplatin than most other types of cancer cell.

The aim of this study was to determine why testis tumor cells express low levels of ERCC1, XPA, and XPF repair proteins. Despite the critical role of ERCC1, XPF, and XPA, little is known about the mechanisms controlling their constitutive expression. In this study, we quantitated the mRNA levels of *ERCC1, XPF*, and *XPA* in a panel of human tumor cell lines from a variety of tissue types using real-time quantitative PCR. Despite considerable variation in mRNA levels between cell lines the results suggest that the low levels of ERCC1, XPF, and XPA protein expression in testis tumor cells could not be explained by differences in transcription.

A relationship between an ERCC1 splice variant lacking exon 8 and repair of cisplatin-DNA adducts was shown in human ovarian carcinoma cell lines and human T lymphocyte cell lines [Dabholkar et al., 1995; Yu et al., 1998]. Therefore, we investigated whether post-transcriptional control by alternative splicing could explain why testis tumor cells have significantly lower levels of ERCC1 protein. We identified three splice variants each lacking exons in the essential XPF binding region in the C-terminal area of the protein. All the cell lines examined expressed these alternative transcripts, but there was no evidence of testis-specific expression. In addition, three endogenous *ERCC1* transcripts with 5′-UTRs of 3 nt, 33 nt, and 74 nt were found in human testis and prostate cancer cell lines. The stability of ERCC1 protein was examined also, but the half-life of the protein was not significantly different between human testis and prostate cancer cell lines. Based on these observations, we conclude that constitutive ERCC1, XPF, and XPA expression may be regulated at the translational level.

MATERIALS AND METHODS

Cell Lines

The human cancer cell lines used were derived from testicular germ cell tumors: SuSa, 833K, GH, GCT27, 1618K, breast cancer: MCF7, prostate cancer: DU145, PC3, LNCaP, colon cancer: HT29, kidney cancer: OUR10, cervical cancer: HeLa, and bladder cancer: MGH-U1, RT112. All the cell lines were maintained in RPMI-1640 supplemented with 10% (v/v) fetal calf serum (FCS) and 2 mM L-glutamine (Invitrogen) at 36.5°C in 5% CO_2 in air.

Clonogenic Survival Assays

Cell survival following cisplatin treatment was assessed using clonogenic assay. Cells were seeded in 60 mm × 15 mm dishes in 5 ml medium and allowed to attach overnight. The cells were treated with a range of cisplatin doses for 1 h, with three replicates for each concentration. Controls were incubated in drug-free medium. Following cisplatin exposure, cells were washed three times with PBS and incubated in drug-free medium for 14 days. At least three independent experiments were performed. Colonies were fixed using methanol and stained with 1% methylene blue and colonies containing more than 50 cells were counted.

Total RNA Extraction and cDNA Synthesis for Taqman Analysis

Cells growing exponentially in 75-cm^2 tissue culture flasks were trypsinized and pelleted by centrifugation at 1,000*g* for 5 min at 4°C. RNA was isolated using the Clontech Nucleospin kit following manufacturer's instructions. The kit included a DNase treatment step. SuperScript II reverse transcriptase (Invitrogen) was used to prepare cDNA from total RNA. The reaction was carried out at 42°C for 50 min in a total volume of 19 μl containing: 2 μg RNA, 1 μl oligo $(dT)_{12-18}$ (500 μg/ml), 1 μl dNTPs (10 mM), 4 μl (5 × buffer, supplied with enzyme), and 2 μl DTT (0.1 M, supplied with enzyme). The reaction was terminated by incubation at 70°C for 10 min.

Primers and Probes for Taqman Analysis

The primer design software Primer Xpress™ (Applied Biosystems) was used to design primers and TaqMan probes for each gene. The probes were designed to span exon–exon boundaries. The TaqMan universal PCR master mix, primers, and probes and a pre-developed control primer-probe set for the β-actin gene were purchased from Perkin–Elmer Applied Biosystems. The test gene probes were 5′-labeled with FAM (6-carboxy-fluorescein), a reporter fluorescent dye and 3′-labeled with TAMRA (6-carboxy-tetramethyl-rhodamine), a quencher fluorescent dye. The β-actin TaqMan probe was 5′-labeled with VIC reporter fluorescent dye and 3′-labeled with TAMRA (6-carboxy-tetramethyl-rhodamine). The sequences of the TaqMan primers and probes used are shown in Table I.

Quantitation of mRNA Using Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR was used to measure mRNA expression using the TaqMan chemistry. Gene-specific PCR primer pairs were generated and a TaqMan probe was produced which anneals within the amplicon as described above. The reporter dye emission is quenched by the TAMRA dye at the 3′-end of the probe. The AmpliTaq Gold polymerase used in the amplification reaction has a 5′-exonuclease activity and can cleave the reporter resulting in an increase in reporter dye emission over time.

Single reporter TaqMan assays were performed in an ABI Prism 7700 sequence detector (Applied Biosystems). Amplification was carried out in 96-well plates on cDNA produced from 20 ng of total RNA. Test gene PCRs consisted of a total volume of 25 μl containing: 100 nM forward primer, 300 nM reverse primer, 100 nM probe, 1 × TaqMan buffer [200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 5 mM $MgCl_2$, 1.25 unit of AmpliTaqGold, 0.5 unit of Amperase uracil-*N*-glycosylase (UNG)]. For β-actin, the reaction was as recommended by the manufacturers. The real-time PCR conditions were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was measured in triplicate and each set of samples was independently generated three times. The results are therefore based on three separate analyses, with three repeats for every measurement.

TABLE I. Oligonucleotide Primers and Probes Used in Real-Time PCR

Names	Forward primer	TaqMan probe	Reverse primer
ERCC1	5′-ATACCCCTCGACGAGGATGAG-3′	5′-CGGAATAAGGGCTTGGCCACTCCA-3′	5′-ACAGTGGGAAGGCTCTGTGTAGA-3′
XPF	5′-TGTTTCCACTGACACTCGGAAA-3′	5′-ACCATTCTGTTCCTGGCCACCGG-3′	5′-CGAAATTCACGCATATCCACAA-3′
XPA	5′-GCTGCCCGGCCCTACTC-3′	5′-ATTAGCCATGCCTCCAGTAGCCGCA-3′	5′-TGAAGCCTCCTCCTGTGTCAAT-3′
ERCC1 (exon 8)	5′-CCAGCGGACCTCCTGATG-3′	5′-AGGACTTCGTCTCCCGGTCTCTGGAAC-3′	5′-CTCTTGATGCGGCGATGAG-3′
ERCC1 (exon 9a)	5′-TCTTTCCCAGAGCTCTTACTTTCTG-3′	5′-CATCAAGAGAAGATCTGGCCTTATGCCCA-3′	5′-ACCACATTTGGATCTCTGGAACA-3′
ERCC1 (exons 7–9)	5′-GCCGACTGCACATTGATCCT-3′	5′-AACAGCCTCCGGGCCTCCAGG-3′	5′-GGGTCATCAGGGTACTTTCAAGA-3′

TaqMan Sequence Detector software was used to calculate a threshold cycle (Ct) value from the amplification plots for each reaction. The comparative Ct method [Fink et al., 1998] was used for relative quantitation of gene expression. Target gene expression was normalized to an endogenous control gene (β-actin) to correct for differences in cDNA quantity between samples. A normalized Ct value (ΔCt) was generated by the following equation: target gene Ct−control gene Ct = ΔCt. To further normalize across samples, the highest ΔCt value was subtracted from each ΔCt value to give ΔΔCt values. These ΔΔCt values were converted to relative expression levels by the following formula: $2^{-\Delta\Delta Ct}$.

Northern Blotting

Total RNA (10 μg) was resolved on formaldehyde-containing agarose gels and blotted onto nylon membranes (Hybond-XL, Amersham Pharmacia Biotech). The blots were probed with ^{32}P-labeled, full-length *ERCC1* cDNA according to the manufacturer's instructions.

RT-PCR of ERCC1

The cDNA generated from mRNA isolated from 1618K, LNCaP, MGH-U1, and 833K cells was used as a template for PCR using a forward primer spanning the exon 5/6 boundary of *ERCC1* (5′-TGTCCAGGTGGATGTGAAAG-ATC-3′) and reverse primers located at positions downstream of the stop codon. The reverse primers were located at nt 189 (3′UTR189): (5′-GCAGCAGCAGCCTGTGTAGTC-3′), nt 412 (3′UTR412): (5′-GTCACACAGTGACTGA-GCCAATTC-3′), nt 707 (3′UTR707): (5′-GA-AAAGGGACAGATGGCAATGAT-3′) where nt 1 denotes the first nucleotide after the stop codon. The amplification conditions using Advantage 2 DNA polymerase mix (Clontech) were: 94°C for 5 min then 40 cycles of: 94°C 1 min, 62°C 1 min, and 72°C 2 min. Amplifications were also carried out on control mRNA samples which were subject to the same conditions except that Superscript II was omitted from the cDNA generation reaction mixture. PCR products were gel purified using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and sequenced using the ABI Prism 377 DNA sequencer.

Rapid Amplification of cDNA Ends (RACE)

5′-RACE and 3′-RACE reactions were performed using the SMART RACE system (Clontech) according to the manufacturer's instructions. Total RNA was isolated as described. RNA was reverse transcribed into cDNA using the supplied SMART II oligonucleotide and 5′-RACE cDNA synthesis primer (for 5′-RACE) and the supplied 3′-RACE cDNA synthesis primer (for 3′-RACE). RACE−PCR amplifications were carried out with the supplied universal primer mix and *ERCC1*-specific primers for 5′-RACE: (5′-CCACAGTGGGAAGGCTCTGTGTAGATCG-3′) and 3′-RACE: (5′-GCAATCCCGTACTGAA-GTTCGTGCG-3′) respectively. Nested RACE−PCR was carried out with the supplied nested universal primer and nested *ERCC1*-specific primers for 5′-RACE: (5′-CAGGAGGGACCT-CATCCTCGTCGAG-3′) and 3′-RACE: (5′-GCC-CTGTTCCTGAGCCTCCGCTA-3′) respectively. For analysis of luciferase 3′ transcripts primers LUC1: (5′-GGTGTCGCAGGTCTTCCCGACGA-3′) and LUC2: (5′-C-GCAGTCAAGTAACAACC-GCGAAAA-3′) were used for the first round of amplification. Nested luciferase primers were NLUC1: (5′-CGGAAAAGAGATCGTGGATTA-CGTCGC-3′) and NLUC2: (5′-CCGAAAGGTC-TTACCGGAAAA-CTCGACG-3′). RACE−PCR products were separated on 1% agarose gels based on the expected size of the product. The sequences of all the products were confirmed by direct sequencing.

Pulse-Chase Assay

PC3 and 833K cells were seeded in 6-well plates at approximately 50% confluency. The following day, the cells were washed twice with methionine-free RPMI and then incubated overnight in methionine-free RPMI (1 ml/well) containing FCS and 100 μCi [^{35}S]-L-methionine. The medium was then replaced with chase medium (RPMI containing FCS and 10-fold excess of cold methionine, 1.01 mM). Protein extracts were prepared at 0, 2, 4, 8, and 24 h post-chase in RIPA buffer and the protein concentration of each extract was normalized prior to immunoprecipitation. Immunoprecipitation was carried out using sheep anti-mouse IgG dynabeads (Dynal), coated with anti-ERCC1 mouse monoclonal antibody (Ab 8F1, AbCAM). A negative control immunoprecipitation using uncoated beads was also carried out. Immunoprecipitated proteins were resolved by SDS−PAGE. Gels were fixed in 10% acetic acid, 25% isopropanol, then soaked in Amplify solution (Amersham), before being dried and autoradiographed. ERCC1 half-lives were calculated

from densitometry readings of the autoradiograph.

Chimeric UTR-Luciferase Reporter Constructs

833K DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega) and used as a template for PCR. PCR primers were designed for amplification of *ERCC1* 5′-UTR and 3′-UTR fragments based on the published nucleotide sequence of the human *ERCC1* gene (GenBank: AF512555). Forward *ERCC1* 5′-UTR: (5′-TTAGGAGGAAATGTTTGC-3′) and reverse *ERCC1* 5′-UTR: (5′-CTGGAGCCTGAAAGGG-3′) primers were used to amplify the ERCC1 promoter, exon 1, intron 1, and the portion of exon 2 located before the translation start site. This 1,392-bp fragment was gel purified and PCR was subsequently carried out using a forward primer containing a *Kpn*I sequence: (5′-ATAGGTACCTTAGGAGGAAATGTTTGC-3′) and a reverse primer containing an *Xho*I sequence: (5′-ATACTCGAGCTGGAGCCTGAAAGGG-3′). The product was digested with *Kpn*I and *Xho*I and cloned upstream of the firefly luciferase (*luc*) gene in the pGL3 basic eukaryotic expression vector (Promega) to create the *ERCC1-luc* vector. All constructs are shown in Figure 9. Forward *ERCC1* 3′-UTR: (5′-TGATGACCCCAGCTGCCA-3′) (located at the end of the *ERCC1* coding region) and reverse *ERCC1* 3′-UTR: (5′-CAGTCACTCAGGAGGCAGTGAATG-3′) primers were used to amplify the *ERCC1* 3′-UTR from the stop codon. This fragment was gel purified and PCR was subsequently carried out using forward (5′-ATATCTAGATGATGACCCCAGCTGCC-3′) and reverse (5′-ATATCTAGACAGTCACTCAGGAGGCAGTG-3′) primers containing an *Xba*I sequence. The product was digested with *Xba*I to generate an 873-bp product. Shorter 3′-UTR sequences were generated with reverse primers located at nt 189 (3′UTR189): (5′-ATATCTAGAGCAGCAGCAGCCTGTGTAGTC-3′), nt 412 (3′UTR412): (5′-ATATCTAGAGTCACACAGTGACTGAGCCAATTC-3′), nt 707 (3′UTR707): (5′-ATATCTAGAGAAAAGGGACAGATGGCAATGAT-3′) where nt 1 denotes the first nucleotide after the stop codon. The 3′-UTR sequences shorter than 100 bp were prepared by synthesis (Invitrogen). These products were digested with *Xba*I and cloned downstream of the firefly luciferase (*luc*) gene in the pGL3 control eukaryotic expression vector (Promega) and *ERCC1-luc* vector. The sequences of all the constructs were confirmed by direct sequencing.

Transient Transfections of Chimeric UTR-Luciferase Reporter Constructs

Cells were seeded into 96-well plates, allowed to attach overnight and were transfected with reporter vectors using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions. Cells in eight wells were transfected with 0.2 μg of each firefly luciferase vector and co-transfected with 0.2 μg of pRL-TK renilla luciferase vector (Promega) to control for transfection efficiency.

Dual Luciferase Assays

One day after transfection, firefly luciferase and renilla luciferase activities were measured using the Dual-Glo Luciferase Reporter Assay System (Promega) following the manufacturer's instructions. Relative light units (RLU) were calculated as the ratio of firefly luciferase activity: renilla luciferase activity to normalize for transfection efficiency and cell number.

RT-PCR of Luciferase Transcripts

PC3 cells in 25-cm^2 flasks were transfected with 10 μg of each of the firefly luciferase constructs. After 24 h total mRNA was isolated and cDNA was generated as described. For semi-quantitative analysis of luciferase mRNA levels, primers LUCF1: (5′- ATTGCTTTTACAGATGCACATATCGAG-3′) and LUCR1: (5′-AGGTAGATGAGATGTGACGAACGTG-3′) were used to generate a 401-bp product. LUCF2: (5′-GAAAACTCTCTTCAATTCTTTATGCCG-3′) and LUCR2: (5′-CTCGAAATCCACATATCAAATATCCG-3′) were used to generate a 537 bp-product. The control gene GAPDH was also analyzed using primers GAPDHF1: (5′-CCACCCATGGCAAATTCCATGGCA-3′) and GAPDHR1: (5′-TCTAGACGGCAGGTCAGGTCCAC-3′) to generate a 597-bp product. The amplification conditions using Advantage 2 DNA polymerase mix (Clontech) were: 94°C for 5 min then 21 cycles of: 94°C 1 min, 58°C 1 min, and 72°C 1 min. Amplification products were electrophoresed on 1% agarose gels. PCR products were quantitated using a digital imaging system (GeneGenius, Syngene).

RESULTS

Cisplatin Sensitivity of Cancer Cell Lines

Clonogenic assays were carried out to determine the IC_{50}s of 14 human cancer cell lines following a 1 h exposure to cisplatin. The numbers of colonies were expressed as a percentage of the untreated controls and concentrations resulting in IC_{50} were calculated (Table II). The testis tumor cell lines were on average 2–3 times more sensitive to cisplatin than the other cell lines examined here. A Mann–Whitney *U*-test was carried out and a statistically significant difference was found between the median of the testis tumor cell line's IC_{50} and the other cancer cell lines ($U = 5$; $P < 0.05$).

Quantitation of Relative *ERCC1, XPF,* and *XPA* Transcript Expression in Cancer Cell Lines

Real-time PCR quantitation was used to measure mRNA transcript levels of *ERCC1, XPF*, and *XPA* mRNA in six cell lines: 833K, MCF7, HeLa, OUR10, DU145, and HT29. The relative expression levels of β-actin were the most consistent amongst 11 candidate control genes tested between the cell lines, and so β-actin was included in a human endogenous control plate (Applied Biosystems).

The comparative Ct method for quantifying mRNA levels from TaqMan data requires that the control and target genes be amplified with equal efficiencies across a range of template dilutions. Log ng of template cDNA was plotted against ΔCt values for each target gene. The calibration curve was generated with serial dilutions of cDNA from 120 ng to 15 ng and 75 ng to 9.375 ng. The slopes of the lines were less than 0.1 for each target gene (*ERCC1* = 0.026, *XPF* = 0.099, *XPA* = 0.096).

Steady-state *ERCC1*, *XPF*, and *XPA* transcript levels were examined in the 14 cancer cell lines. TaqMan probes were designed to span the *ERCC1* exon 2/3 boundary, *XPF* exon 10/11, and *XPA* exon 1/2 boundary. The expression levels of *ERCC1*, *XPF*, and *XPA* transcripts in the cell lines is shown in Figure 1. With some exceptions (see Fig. 1), the cell lines expressed mRNA from each gene at a similar level.

TABLE II. IC_{50} Values Determined by Clonogenic Assay Following a 1 h Cisplatin Treatment

Tumor type	Cell line	IC_{50} (μM)
NSTGCT	833K	4.3 ± 0.4 [5]
NSTGCT	1618K	5.3 ± 0.2
NSTGCT	SuSa	5.6 ± 0.3 [5]
NSTGCT	GCT27	6.3 ± 0.9 [5]
NSTGCT	GH	2.8 ± 0.1
Prostate	DU145	14.2 ± 0.3
Prostate	PC3	6.8 ± 0.2
Prostate	LNCaP	18.3 ± 3.5
TCC Bladder	RT112	16.1 ± 2.9 [5]
TCC Bladder	MGH-U1	14.2 ± 1.7 [5]
TCC Colon	HT29	8.7 ± 0.1
Kidney	OUR10	7.3 ± 1.2
Glandular ca of cervix	HeLa	2.6 ± 0.1
Breast	MCF7	10.6 ± 1.7

Each value represents the mean ± SE.
NSGCT, non-seminomatous testicular germ cell tumor; TCC, transitional cell cancer.

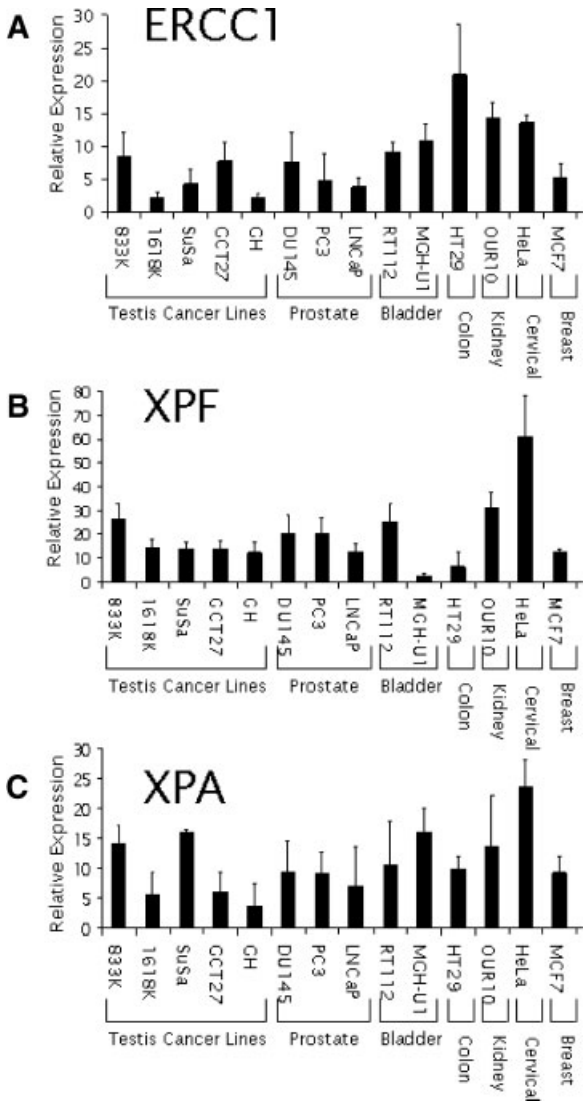

Fig. 1. Relative expression levels of (**A**) ERCC1, (**B**) XPF, and (**C**) XPA mRNA as determined by real-time PCR. Results are the mean of three independent experiments ± SD.

For *ERCC1*, the highest expression was found in HeLa (cervix), HT29 (colon), and OUR10 (kidney). The lowest expressers of *ERCC1* were 1618K and GH (both testis) and LNCaP (prostate). HeLa (cervix), 833K (testis), OUR10 (kidney), and RT112 (bladder) had the highest levels of *XPF* transcripts. The lowest amounts of *XPF* transcripts were detected in MGH-U1 (bladder), MCF7 (breast), and HT29 (colon) cells. For *XPA*, the highest expression was found in HeLa (cervix), MGH-U1 (bladder), and SuSa (testis). The lowest expressers of *XPA* were all testis: 1618 K, GCT27, and GH. Using the TaqMan RT-PCR data and the IC_{50} values, we applied the Spearman's rank correlation to identify r_S values for *ERCC1*, *XPF*, and *XPA* mRNA levels. There is no significant correlation between the mRNA levels of *ERCC1* ($r_S = 0.15$), *XPF* ($r_S = -0.28$), and *XPA* ($r_S = 0.36$) and the cancer cell line IC_{50}s ($P =$ not significant for *ERCC1, XPF*, and *XPA*).

A Mann–Whitney *U*-test was carried out and no statistically significant difference was found between the median of the testis tumor cell line *ERCC1, XPF*, and *XPA* mRNA levels and the other cancer cell lines (*ERCC1*: $U = 9$; $P < 0.05$, *XPF*: $U = 21$; $P < 0.05$, *XPA*: $U = 14$; $P < 0.05$). Thus, these data show that the levels of *ERCC1, XPF*, and *XPA* mRNA are not lower in testis tumor cell lines as a group relative to the other cancer cell lines.

Estimation of *ERCC1* mRNA Expression by Northern Blotting

As an independent measure of *ERCC1* expression, a Northern blot of total RNA from HeLa (cervical), PC3 (prostate), and 833K (testis) tumor cells was probed with the human *ERCC1* cDNA (data not shown). HeLa cells have the highest levels of *ERCC1* mRNA expression, whereas PC3 and 833K cells are moderate expressers. These data are in agreement with the TaqMan quantitation.

Identification of *ERCC1* Splice Variants

No alternative splicing of either *XPA* or *XPF* genes has been described. An ERCC1 splice variant lacking exon 8 has been identified in human tumor cell lines [Dabholkar et al., 1995]. Therefore, we examined *ERCC1* to see if any testis-specific splicing could be identified. Expression of an alternative transcript that is not translated into functional protein in testis tumor cell lines could explain why testis tumor cells have similar levels of mRNA, but significantly lower levels of ERCC1 protein [Welsh et al., 2004]. RNA from 1618K, SuSa, and 833K (testis) tumor cell lines was compared with RNA from DU145, LNCaP, and PC3 (prostate) tumor cell lines. 3′-RACE experiments were performed on total RNA using an *ERCC1*-specific primer and four products were identified (Fig. 2a).

Sequence analysis of the four RACE–PCR products revealed that they were (1) the full-length sequence, (2) a previously identified splice variant which lacks exon 8 [van Duin et al., 1986; Dabholkar et al., 1995; Yu et al., 1998], (3) a splice variant lacking exons 7–9, and (4) a splice variant which lacks exon 10 but contains the 230 nt 5′ of intron 9 (exon 9a) in addition to exon 9 (Fig. 2b). Thus, these results indicate that there are three potential splice variants that could produce alternative non-functional protein isoforms. These splice variants would have been measured in the total ERCC1 quantitation (Fig. 1a) and, if expressed at higher levels in testis tumor cell lines, could explain why ERCC1 mRNA levels are similar to

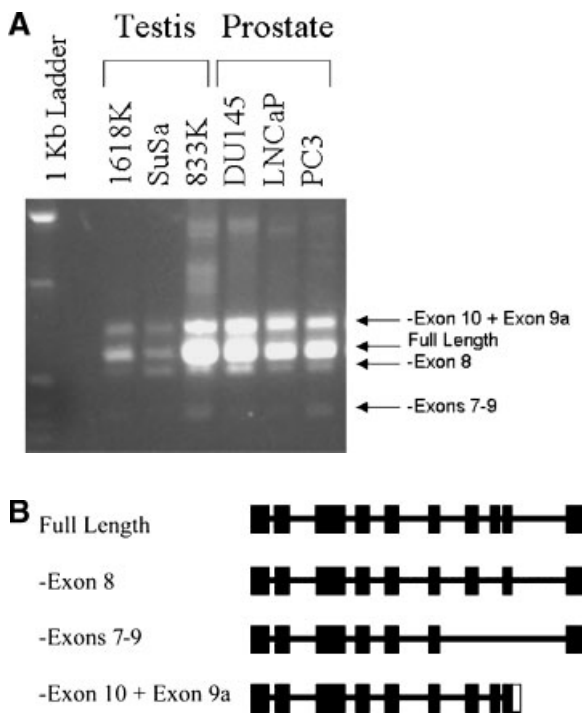

Fig. 2. Alternative splicing of *ERCC1* gene transcripts. **A**: RACE–PCR amplification of *ERCC1* mRNA in the 3′ direction in testis and prostate cancer cell lines. **B**: Diagrammatic representation of the alternative *ERCC1* mRNA transcripts identified in testis and prostate cancer cell lines. Filled boxes indicate exons and the shaded box indicates an alternative exon comprising of intronic sequence.

other tumor cell lines (Fig. 1a) despite the lower levels of protein relative to other cell lines [Welsh et al., 2004]. These alternative splice variants may cause inhibition of the full-length protein.

Identification of Transcription Start Points (TSPs) or 5′ End(s) of *ERCC1* mRNA

Different 5′ untranslated region (UTR) sequences in testis tumor cell lines could also result in tissue-specific differences in translation efficiency and therefore protein levels. We searched for TSPs by mapping the 5′-end(s) of *ERCC1* mRNA. We performed 5′-RACE experiments on total RNA using nested *ERCC1*-specific primers. RNA from 1618K, SuSa, and 833K testis tumor cells were compared with RNA from DU145, LNCaP, and PC3 prostate tumor cells to see if any testis-specific splicing could be identified (Fig. 3a).

We obtained multiple fragments that corresponded to three TSPs. Sequence analysis indicated that the obtained sequences were exon 1a (lacking nt 1–68 of exon 1), exon 2a (alternative TSP within intron 1 so that exon 1 is completely excluded and the last 30 nt at the 3′-end of intron 1 are included in the mRNA transcript), and a splice variant which completely lacks exon 1 (Fig. 3b). As exon 1 is untranslated, these variants give 5′-UTRs of 74 nt (exon 1a), 33 nt (exon 2a), and 3 nt (exon 1). None of the cancer cell lines examined expressed mRNAs containing the full exon 1 sequence. 833K (testis tumor cells) only expressed mRNA containing exon 2a. All the other cell lines examined expressed exon 1a instead of exon 1 and PC3 (prostate tumor cells) expressed mRNAs with exon 1a in addition to mRNAs that completely lacked exon 1. Thus, although no testis-specific 5′-UTRs were found, the variability amongst TSPs suggests that control of translation may be an important mode of regulation of *ERCC1* in both testis and prostate tumor cell lines.

Quantitation of *ERCC1* Splice Variants

As RACE–PCR is not a quantitative technique, we used the TaqMan technique to quantify the relative levels of *ERCC1* splice variants in the panel of 14 tumor cell lines. We quantitated the splice variants that could give rise to alternative protein isoforms (i.e., not involving UTRs). Calibration experiments were carried out as previously described for *ERCC1*, *XPF*, and *XPA*. The slopes of the lines were exon 8 = 0.08, exon 9a = 0.00, and exons 7–9 = 0.07. Three independent total RNA extractions were carried out from each cell line. TaqMan probes were designed to span the *ERCC1* exon 7/9 boundary to examine levels of the exon 8 splice variant and *ERCC1* exon 6/10 boundary to examine levels of the exon 7–9 splice variant. For the exon 9a variant, a probe was designed to span the exon 8/9 boundary, a forward primer designed to span the exon 9/9a boundary, and a reverse primer spanning the exon 8/9 boundary (to exclude genomic DNA). The relative

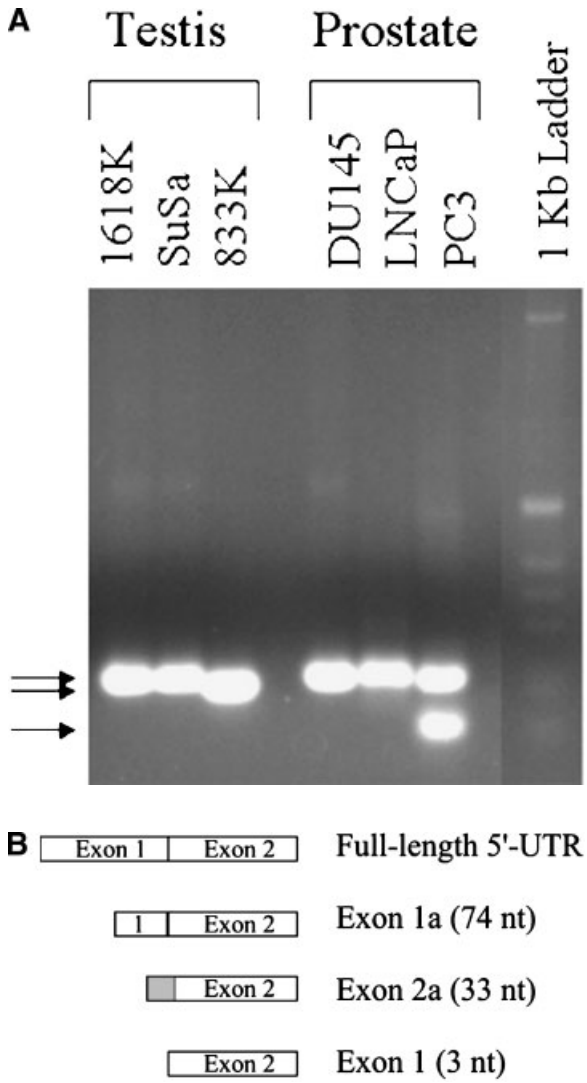

Fig. 3. Alternative 5′ ends of *ERCC1* gene transcripts. **A**: RACE–PCR amplification of *ERCC1* mRNA in the 5′ direction in testis and prostate cancer cell lines. **B**: Diagrammatic representation of the alternative *ERCC1* transcription start points (TSPs) identified in testis and prostate cancer cell lines. The shaded box represents an alternative exon comprising of intronic sequence.

expression of *ERCC1* splice variant transcripts in the cell lines is shown in Figure 4.

For the *ERCC1* exon 8 splice variant, the highest expression was found in MGH-U1 (bladder), MCF7 (breast), and HT29 (colon). The lowest expressers of *ERCC1* exon 8 splice variant were 1618K, GCT27, and GH (all testis). MGH-U1 and RT112 (both bladder) and DU145 (prostate) had the highest levels of *ERCC1* exon 9a transcripts. The lowest amounts of *ERCC1* exon 9a transcripts were detected in OUR10 (kidney), GCT27, and GH (both testis). For the *ERCC1* exons 7–9 splice variant, the highest expression was found in MCF7 (breast), 833K, and SuSa (both testis). The lowest expressers of *XPA* were HeLa (cervix), DU145, and LNCaP (both prostate). Thus, as differential expression of *ERCC1* splice variants was not observed in the testis tumor cell lines, these results cannot explain the significantly lower levels of ERCC1 protein.

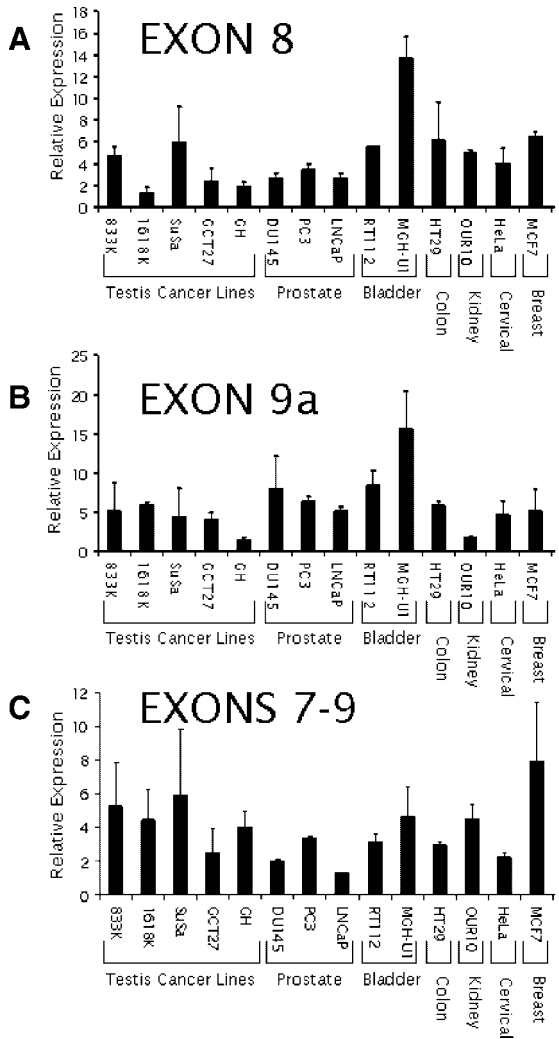

Fig. 4. Relative expression levels of *ERCC1* mRNA splice variants as determined by real-time PCR. **A**: *ERCC1* mRNA lacking exon 8 (**B**) *ERCC1* mRNA lacking exon 10 but containing sequence from intron 9 (exon 9a), and (**C**) *ERCC1* mRNA lacking exons 7–9. Results are the mean of three experiments ± SD.

The Rate of ERCC1 Protein Turnover

Post-translational control mechanisms could maintain low levels of ERCC1 protein in testis tumor cells. We therefore estimated the half-life of ERCC1 protein in prostate (PC3) and testis (833K) tumor cells using pulse-chase. The estimated half-life of ERCC1 protein in 833K cells was very similar to that in PC3 cells (being 6.5 h and 6.9 h, respectively; Fig. 5). Therefore, the low levels of ERCC1 protein in testis tumor cells do not appear to be a result of faster protein turnover.

Identification of *ERCC1* Transcripts With Longer 3′-UTR Sequences in Human Cancer Cell Lines

Longer ERCC1 mRNA transcripts of 3.0 Kb, 3.4 Kb, and 3.8 Kb have been identified previously in cancer samples and cancer cell lines by Northern blotting [van Duin et al., 1986; Geleziunas et al., 1991]. Tissue-specific mRNA transcripts with different 3′-UTR sequences may result in tissue-specific differences in protein levels. As conditions using RACE–PCR and Northern blotting techniques may favor the shorter mRNA transcripts we looked for longer *ERCC1* 3′-UTR sequences using RT-PCR. A forward primer was designed to span *ERCC1* exons 5/6 and reverse primers were designed to bind sequences downstream of the 1.1 Kb GenBank mRNA reference sequence (NM_001983). RT-PCR products were generated with reverse primers at nt 189, nt 412, and nt 707, where nt 1 is the first nt downstream of the stop codon. These longer *ERCC1* 3′-UTR products were identified in all four cell lines examined: 1618K and 833K (testis), MGH-U1 (bladder), and LNCaP (prostate) but no tissue-specific differences were observed (data not shown).

The ERCC1 5′-UTR and Promoter Sequences are Not Responsible for Low Levels of ERCC1 Protein in Testis Tumor Cells

Based on the fact that the cancer cell lines examined have various TSPs generating alternative 5′-UTR transcripts, we investigated

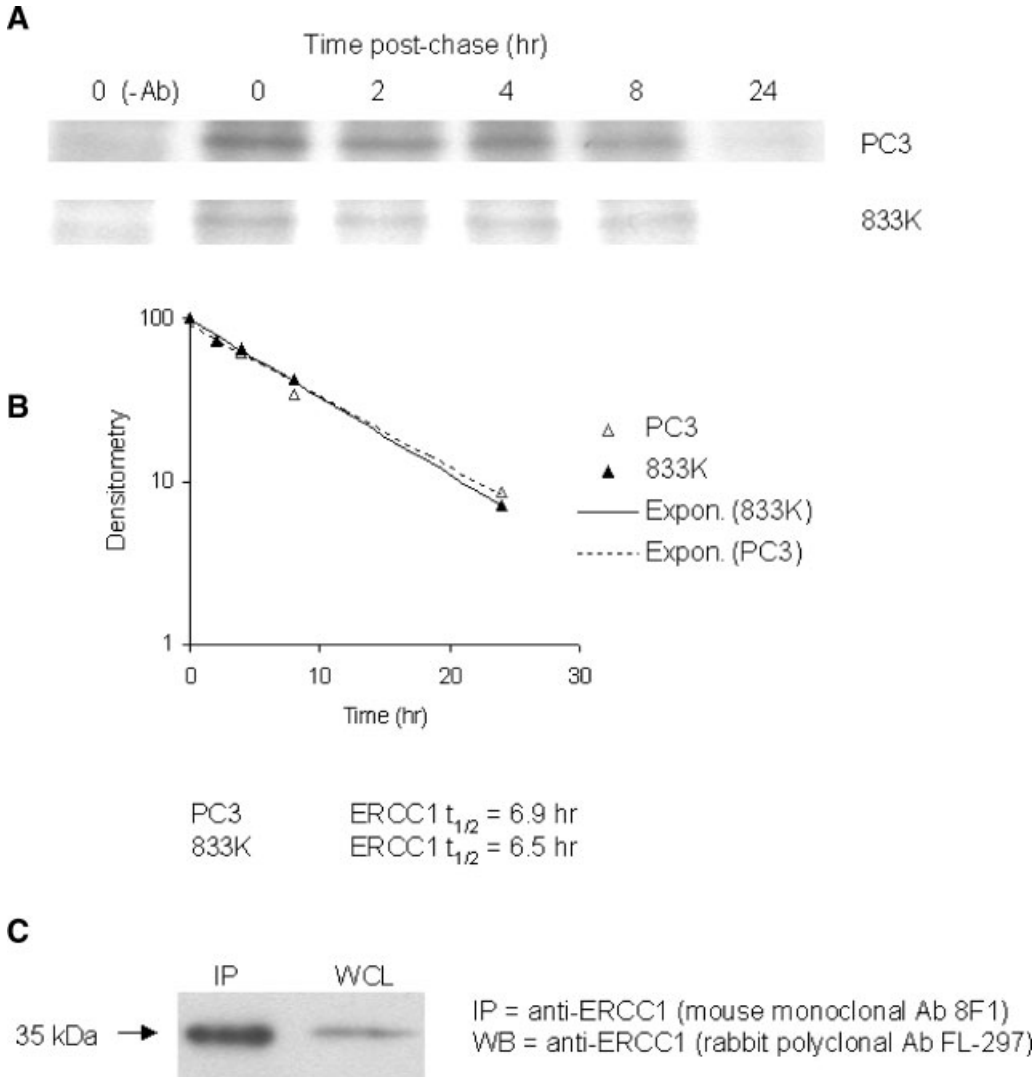

Fig. 5. Pulse chase studies on ERCC1 in prostate (PC3) and testis (833K) tumor cell lines. **A**: Autoradiograph of immunoprecipitated ERCC1 at various time points post-chase. A parallel immunoprecipitation without anti-ERCC1 antibody was performed as a negative control (see Materials and Methods). A 35 kDa band is immunoprecipitated in both cell lines but not in the negative control lanes. **B**: Logarithmic plot of densitometry readings from (A), expressed as percentage density at time zero. Exponential trendlines are shown and are used to calculate the half-life of ERCC1 in each cell line. **C**: Confirmation of specificity for the antibody used in the immunoprecipitation experiments: ERCC1 was immunoprecipitated from non-radioactive PC3 cell extracts following the same protocol used for the pulse-chase experiments. A Western of immunoprecipitated (ERCC1) and PC3 whole cell lysate (WCL) was probed with a rabbit polyclonal ERCC1 antibody (FL-297, Santa Cruz Biotechnology).

the role of the 5′-UTR sequences on gene expression. We used the pGL3 firefly luciferase reporter system to determine if upstream regulatory sequences were responsible for the low levels of ERCC1 protein in testis cancer cell lines. As we were unsure which of the multiple 5′-UTR sequences would be important, we cloned in upstream of the firefly luciferase gene the entire *ERCC1* promoter and untranslated sequences from −1392 to −1 (if the first nt of the start codon is +1). This *ERCC1* sequence included exon 1 (−546 to −407), intron 1, and the first seven untranslated nts of exon 2. In this way, we could transfect the same construct (*ERCC1-luc*) into different cell types and if different TSPs and 5′-UTR sequences are utilized then these would be cell-type specific and this would better reproduce the de novo regulation of the native gene transcript.

We carried out transient transfection assays with 833K (testis), PC3 (prostate), and MGH-U1 (bladder) cancer cell lines and compared the

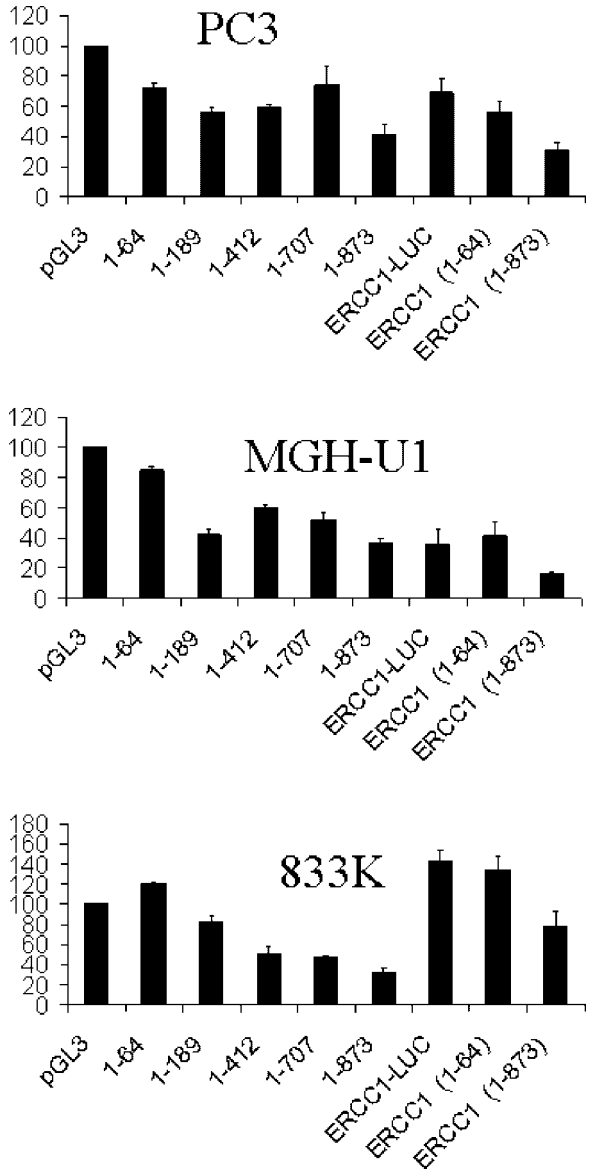

Fig. 6. Expression levels of ERCC1-luc constructs relative to control luciferase reporter in PC3 (prostate), MGH-U1 (bladder), and 833 K (testis) cancer cell lines.

levels of luciferase expression from the *ERCC1-luc* construct with the pGL3 control luciferase reporter vector. Figure 6 shows that expression from the *ERCC1-luc* construct was equal to or higher than the control vector in testis cancer cells (833K). In contrast, expression was lower from the ERCC1 construct relative to the control pGL3 vector in prostate (PC3) or bladder (MGH-U1) cancer cells. Therefore, the *ERCC1* 5′-UTR and promoter sequences are not responsible for the low levels of ERCC1 protein in testis cancer cells.

The *ERCC1* 3′-UTR Functions as a *cis*-Acting Translational Repressor

As *ERCC1* 3′-UTR sequences may regulate gene expression we created a series of chimeric luciferase reporter constructs which have different lengths of *ERCC1* 3′-UTR sequences fused downstream of the luciferase gene. We studied the effect of 3′-UTR sequences independently of other *ERCC1* regulatory sequences by cloning them downstream of the pGL3 control luciferase vector. As there is evidence to suggest that the ends of mRNA transcripts interact [for review see Mazumder et al., 2003], we also cloned *ERCC1* 3′-UTR sequences into the *ERCC1-luc* construct containing the *ERCC1* promoter and 5′-UTR sequences upstream of the luciferase gene. The constructs are depicted in Figure 7.

ERCC1 3′-UTR sequences from 1 to 64 (where +1 denotes the first nt following the stop codon) slightly reduced the levels of luciferase expression from either *ERCC1-luc* or control pGL3 constructs. *ERCC1* 3′-UTR sequences: 1–189, 1–412, 1–707, and 1–873 were all able to repress luciferase expression by approximately 50% when located downstream from both the *ERCC1-luc* and control pGL3 constructs (Fig. 6). *ERCC1* 3′-UTR sequences 1–189, 1–412, 1–707, and 1–873 repressed luciferase expression to comparable amounts suggesting that this regulation is not sequence length dependent.

We confirmed that the *ERCC1* 3′-UTR sequences were not destablilizing the luciferase

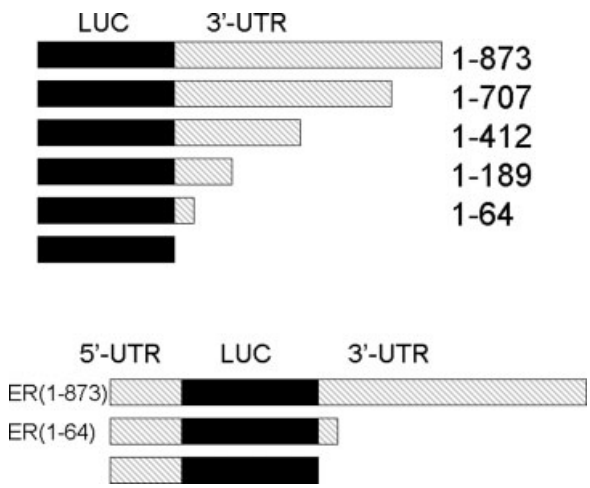

Fig. 7. Structures of the ERCC1 3 and 5 prime sequences cloned into the ERCC1-luc construct up and downstream of the luciferase gene.

mRNA by measuring the levels of mRNA produced by PC3 cells transiently transfected with each of the constructs in Figure 7. Levels of mRNA were not significantly different (data not shown).

To confirm that the 3′-UTR sequences were being transcribed and were not being processed post-transcriptionally, we carried out 3′-RACE–PCR with primers specific to the luciferase gene. We transiently transfected the construct with the longest 3′-UTR sequence (1–873) and used two different nested primer sets for 3′-RACE–PCR. Figure 8 shows that the *ERCC1* 3′-UTR sequences are present on the luciferase transcripts from the transfected cells.

DISCUSSION

ERCC1, XPF, and XPA are NER proteins that are important in the removal of lesions produced by chemotherapeutic drugs on DNA, and consequently response to chemotherapy. Elucidation of the mechanisms responsible for DNA repair gene expression could provide insight into strategies to control DNA repair gene expression in human cancers. Many studies have demonstrated an increased repair rate in resistant tumor cells. In addition, overexpression of NER genes has been correlated with repair and resistance [for review see Reed, 1998].

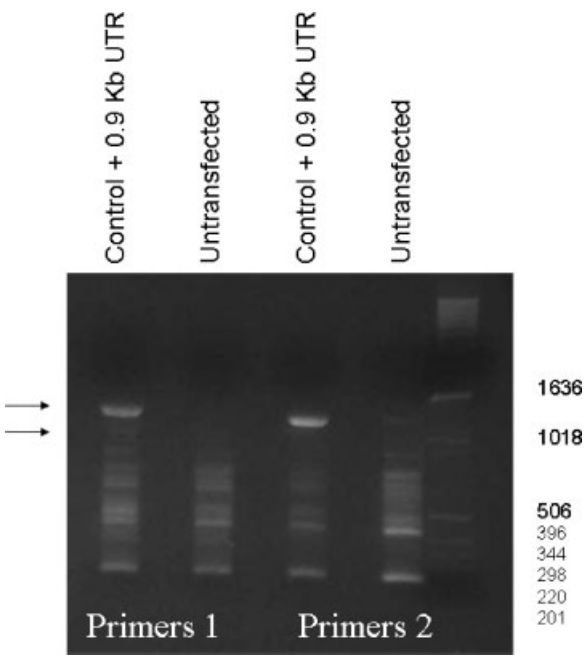

Fig. 8. 3′-RACE–PCR products produced from the longest 3′-UTR sequence following transient transfection.

The protein levels of ERCC1, XPF, and XPA in the five human testis tumor cells lines examined here are significantly lower than in cell lines derived from prostate, bladder, breast, lung, oral, cervical, and ovarian cancers [Welsh et al., 2004]. In the present study, real-time quantitative RT-PCR (TaqMan) was used to quantitate mRNA expression of three genes important in NER. TaqMan is an accurate, robust, and sensitive method to detect gene expression. In particular, for low-copy number transcripts such as *XPA*, this is the only fully quantitative method available as the sensitivity of Northern blotting is too low to detect *XPA* mRNA. In addition, for TaqMan analysis relatively little RNA is required and therefore is an ideal method for analyzing gene expression in clinical samples. This is the first study to examine *XPA* or *XPF* expression using real-time quantitative PCR. *ERCC1* expression has recently been examined in colorectal tumors [Shirota et al., 2001] non-small cell lung tumors [Lord et al., 2002], breast tumors [Juhasz et al., 2003], and a human leukemia cell line [Galm et al., 2002] by real-time quantitative PCR.

All the cell lines examined express the mRNA for *ERCC1, XPF*, and *XPA*. However, unlike the protein expression, mRNA expression is highly heterogeneous between cell lines and does not display a tissue-specific pattern. The mRNA levels of these three NER genes in testis tumor cells are not significantly lower overall than the other cell lines examined. One exception is HeLa cells, which show very high levels of *ERCC1, XPF*, and *XPA* mRNA. For *ERCC1*, 1618K and GH (testis tumor cell lines) also expressed the lowest levels of mRNA expression among the cell lines examined while some other cell lines such as PC3 and LNCaP (prostate) or MCF7 (breast) also expressed *ERCC1* mRNA at a relatively low level. Testis tumor cells also had the lowest levels of *XPA* expression although two testis cell lines, 833K and SuSa, expressed *XPA* mRNA at moderate levels. A large variability in expression is seen between different RNA extractions. This variability is highest for *XPA* and may be a sign of a relatively low copy number of *XPA* transcripts per cell of only 5–8 [Layher and Cleaver, 1997]. Overall, these results suggest that the regulation of steady-state levels of ERCC1, XPF, and XPA protein in testis tumor cells is not modulated by transcriptional efficiency or mRNA stability.

Previously, an in silico study of the human EST database found *ERCC1* splice variants involving exons 1, 2, 3, 7, 8, and 9 [Wilson et al., 2001]. An *ERCC1* splice variant lacking exon 8 has been observed in HeLa and the K562 human leukemic cell line [van Duin et al., 1986], human ovarian cancer cells, and tissues and human T cell lines [Dabholkar et al., 1995; Yu et al., 1998]. An inverse correlation between amount of *ERCC1* exon 8 splice variant and cisplatin-DNA adduct repair was found in human ovarian cancer and T cell lines [Yu et al., 1998]. In this study, we have identified two additional splice variants: one that excludes 240 nt of three internal exons (7–9) and another which transcribes into intron 9 and uses an alternative polyadenylation site 230 nt within intron 9 (exon 9a), thereby excluding exon 10 (111 nt). Each of the ERCC1 splice variants identified could produce proteins lacking the essential XPF binding region. These splice variants could regulate NER by binding to XPA as a competitor to the full-length ERCC1 as the XPA binding region is located in a central region of the protein. We, therefore, quantitated the levels of these two (exons 7–9 and exon 9a) and the exon 8 ERCC1 splice variant in 14 cancer cell lines. The variability in expression between RNA extractions is relatively large for the *ERCC1* exon 7–9 splice variant, which like *XPA* may reflect a relatively low copy number within the cell. We find all three splice variants expressed in all the cancer cell lines but no significant testis-specific differences that could account for the lower protein levels relative to the other tumor cell lines were found. A study of human ovarian cancer cells and tissues identified a splice variant lacking nt 94–135 of exon 1 that is associated with increased expression of ERCC1 [Yu et al., 2001]. However, this variant was not observed here. In this study, we have found three alternative 5′-UTR sequences for *ERCC1*, all variants involving untranslated exon 1. However, a comparison of the 5′-UTR sequences in testis and prostate tumor cell lines revealed no testis-specific splicing.

Both *ERCC1* and *XPA* mRNA expression levels have been correlated to DNA repair capacity in primary lymphocytes [Vogel et al., 2000]. In ovarian cancer cell lines, *ERCC1* mRNA and protein levels have been shown to correlate with DNA repair [Ferry et al., 2000; Li et al., 2000]. In human cervical cancer cells, *ERCC1* mRNA but not protein levels were shown to correlate with cisplatin resistance [Britten et al., 2000]. In contrast, no correlation was found between *ERCC1*, *XPA*, or *XPF* mRNA and DNA repair in eight different human cancer cell lines [Damia et al., 1998]. Likewise, no correlation between ERCC1 or XPA protein levels and anticancer drug resistance was observed in the National Cancer Institute panel of 60 human tumor cell lines [Xu et al., 2002].

Cisplatin-resistant human squamous carcinoma cells have higher levels of *ERCC1* mRNA than parental cells [Fujishima et al., 1997]. Similarly, high levels of *ERCC1* mRNA were found in samples from patients with resistant CLL compared to untreated patients [Geleziunas et al., 1991]. In resistant ovarian cancer cells *ERCC1* but not *XPA* mRNA was slightly higher than parental cells [Hector et al., 2001]. In contrast, drug-resistant breast cancer cells did not have increased *ERCC1* mRNA levels relative to parental cell line although an increased repair capacity was shown [Yen et al., 1995]. Likewise, in drug-resistant human melanoma cells repair was more efficient yet the *ERCC1* mRNA levels were found to be the same as parental cells [Zhao et al., 1995].

Low *ERCC1* and *XPA* mRNA expression was associated with a good response to chemotherapy in ovarian cancer [Dabholkar et al., 1992, 1994], gastric adenocarcinoma [Metzger et al., 1998], metastatic colorectal cancer [Shirota et al., 2001], and non-small cell lung cancer [Lord et al., 2002]. Whereas no correlation was shown between *ERCC1* mRNA expression and response to chemotherapy in ovarian cancer [Codegoni et al., 1997] nor B-cell chronic lymphocytic leukemia [Bramson et al., 1995]. Low *ERCC1* mRNA expression was associated with an increased risk of squamous cell carcinoma of the head and neck [Cheng et al., 2002].

There are numerous examples of genes regulated post-transcriptionally by UTR sequences [for review see Mazumder et al., 2003]. 3′-UTR mediated regulation often results in translational repression and activation of these silenced transcripts is found in response to environmental stress [Fu and Benchimol, 1997; Spicher et al., 1998]. In addition, there are many testis-specific proteins that bind specifically to 3′-UTR sequences [Leatherman and Jongens, 2003]. We have confirmed previous studies identifying *ERCC1* transcripts with longer 3′-UTRs. However, we do not know what proportion of the total *ERCC1* transcripts possess

these additional sequences. We have investigated translational control by generating chimeric reporter constructs with various lengths of *ERCC1* 3′-UTR fused downstream of a luciferase gene. We have shown that sequences between 65 and 189 in the *ERCC1* 3′-UTR are involved in translational repression of *ERCC1* mRNA. Further studies are required to determine if there are RNA-binding proteins that bind to this mRNA region to mediate this repression.

The *ERCC1* 3′-UTR region that we have found to regulate reporter gene expression in this study has also been found previously to contain a polymorphism, *C8092A*. This polymorphism is associated with overall survival in non-small cell lung cancer patients treated with cisplatin [Zhou et al., 2004], increased risk of brain tumors [Chen et al., 2000], and increased risk of squamous cell carcinoma of the head and neck in combination with a XPD polymorphism [Sturgis et al., 2002]. In this study, we have presented data that shows a biological function for this 3′-UTR region of *ERCC1*. Here, we have studied the *C*/*C* genotype using DNA from 833K (testis) cancer cells, however, it is possible that transcripts from the *A*/*C* or *A*/*A* genotypes may not result in reduced levels of expression. Further studies are required to test this hypothesis.

Post-translational regulation mechanisms could maintain a low protein level in testis tumor cells. However, we have shown that the half-life of ERCC1 protein was not different in testis tumor cells than in prostate tumor cells which exhibit a significantly higher level of protein expression. The relative abundance of *ERCC1, XPF*, and *XPA* mRNA does not correspond with the protein level in human testis relative to other human tumor cell lines, suggesting that translational control may play an important role in regulating *ERCC1, XPF*, and *XPA* gene expression. The variable but rather short 5′-UTR sequences (<100 bases) of the *ERCC1* gene identified here are indicative of efficiently translated mRNAs [for review see Meijer and Thomas, 2002]. In addition, genes such as *XPA* that have a low mRNA copy number are often regulated at the translational level. Moreover, proteins that are required under conditions of cellular stress are frequently regulated by translational mechanisms. Taken together, these results suggest that the significantly lower levels of ERCC1, XPF, and XPA protein found in testis tumor cells may be regulated via a translational repression mechanism.

DNA repair proteins are accepted as good targets for the development of new therapies to circumvent resistance. However, further studies to elucidate the mechanisms by which these genes are regulated are required. In addition, these proteins may prove to be more appropriate targets in some types of cancer than others in which alternative resistance mechanisms may predominate. Therefore, accurate comparisons between different cell lines and patient samples are necessary.

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