Comparison of the Global Genomic and Transcription-Coupled Repair Rates of Different Lesions in Human Cells

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There are two subclasses of nucleotide excision repair (NER). One is the global genomic repair (GGR) which removes lesions throughout the genome regardless of whether any specific sequence is transcribed or not. The other is the transcription-coupled repair (TCR), which removes lesions only from the transcribed DNA sequences. There are data that GGR rates depend on the chemical nature of the lesions in a manner that the lesions inflicting larger distortion on the DNA double helix are repaired at higher rate. It is not known whether the TCR repair rates depend on the type of lesions and in what way. To address this question human cells were transfected with pEGFP and pEYFP plasmids treated with UV light, *cis*-diamminedichloroplatinum(II) (cisplatin) and angelicin and 24 h later the restored fluorescence was measured and used to calculate the respective NER rates. In a parallel series of experiments the same plasmids were incubated in repair-competent protein extracts to determine GGR rates in the absence of transcription. From the two sets of data, the TCR rates were calculated. We found out that cisplatin, UV light and angelicin lesions were repaired by GGR with different efficiency, which corresponded to the degree of DNA helix distortion induced by these agents. On the other hand the three lesions were repaired by TCR at very similar rates which showed that TCR efficiency was not directly connected with the chemical nature of the lesions.

Key words: Transcription-Coupled Repair, Global Genomic Repair, Host-Cell Reactivation

Introduction

During evolution, cells have developed multiple mechanisms to repair damaged DNA. The most general DNA repair mode is if damaged or inappropriate bases are excised from the genome and replaced by the correct nucleotide sequence. This type of lesion removal is referred to as excision repair and includes mismatch repair, base excision repair, and nucleotide excision repair (NER). For historical reasons, the best-studied repair pathway is the human NER pathway. It includes 7 genes designated XP-A through XP-G (Friedberg *et al.*, 1995; Aboussekhra *et al.*, 1995; Sancar, 1996; Wood, 1996; Mu *et al.*, 1997). NER can remove a huge variety of lesions among them many DNA lesions caused by environmental agents and by anticancer drugs. There are two major NER subpathways, designated as transcription-coupled repair (TCR) and global genomic repair (GGR). TCR selectively removes transcription-blocking DNA damage from the transcribed strand of the genes, while GGR operates throughout the genome regardless of whether the genes are transcribed or not (Bohr *et al.*, 1985; Mellon *et al.*, 1987). It has been shown that some lesions such as cyclobutane pyrimidine dimers (CPD) and *cis*-diamminedichloroplatinum(II) (cisplatin) could be removed by both pathways (Venema *et al.*, 1992). Nevertheless, in these cases the sensitivity of the cells towards the genotoxic agents was determined by their TCR performance and not by their GGR performance (van Oosterwijk *et al.*, 1996; Furuta *et al.*, 2002). This makes it important to determine the TCR and GGR rates separately to be able to assess the effect of the different drugs on the organism.

Abbreviations: Angelicin, *2H*-furo[2,3-*h*]benzopyran-2-one; CFS, cell-free system; cisplatin, *cis*-diamminedichloroplatinum(II); CPD, cyclobutane pyrimidine dimer; CS, Cockayne syndrome; CSB, Cockayne sundrome complementation group B; GGR, global genomic repair; HCR, host cell reactivation; HEPES, *N*-(2-hydroxyethyl)piperizine-*N*′-ethanesulfonic acid; NER, nucleotide excision repair; TCR, transcription-coupled repair; TE buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 8; Tris, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; XP, xeroderma pigmentosum; XP-A, xeroderma pigmentosum complementation group A.

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However, this is not a trivial task using normal cells. On one hand, cells have different capacity to repair different lesions and the results obtained with one cell line could not automatically apply to other cell lines (Barret *et al.*, 1996; Atanassov *et al.*, 2003). On the other hand, the examination of cellular repair generally requires that the cells are damaged in some manner, which makes it difficult to determine the constitutive repair rates since both pathways could be induced by the damaging agent (McKay *et al.*, 1995; Francis and Rainbow, 1999; Cline and Hanawalt, 2003).

In an attempt to determine and compare the constitutive GGR and TCR rates in cell lines with unmutated NER pathways we developed the following approach. Total NER (TCR + GGR) rates of different DNA lesions were determined *in vivo* by host cell reactivation (HCR) assay and the respective GGR rates were determined *in vitro* by cell free system (CFS) assay. Experiments with TCR deficient CSB cells confirmed that the *in vitro* determined repair rates corresponded well to the *in vivo* determined GGR repair rates. By comparing the values for the total NER (GGR + TCR), obtained by HCR assay, with those for GGR obtained in protein extracts prepared from the same cells, the respective TCR rates were calculated. We found out that GGR rates differed significantly for the different lesions and roughly corresponded to the degree of helix distortion caused by the agents thus supporting the hypothesis that in GGR the damage recognition factors recognized topological changes in the DNA double helix. On the other hand, TCR rates of the different lesions did not differ significantly, which showed that the stalled RNA polymerase II molecules and not the lesions themselves are recognized in the TCR pathway.

Materials and Methods

Cell culture

HEK293 and K562 cells were purchased from ATCC and CSB (AG 07075) cells were from Coriell Cell Repositories. HEK293 were grown in complete DMEM medium supplemented with 10% fetal calf serum in 5% CO_2 atmosphere. XP-A cells (XP25RO) were cultured in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum and antibiotics in 2.5% CO_2 atmosphere (van Hoffen *et al.*, 1999) and K562 and CSB were cultured in RPMI 1640 medium with 10% fetal calf serum in 5% CO_2.

Plasmid treatment with UV light

Plasmids pDsRed1-N1, pEGFP-N1 and pEYFP-N1 (Clontech) were propagated in *E. coli* XL1-Blue (Inoue *et al.*, 1990). pEGFP-N1 and pEYFP-N1 were dissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8 (TE buffer) to a final concentration of 100 μg/ml and poured in a Petri dish to form 1–2 mm thick layer. The Petri dish was placed on ice and irradiated with a germicidal mercury lamp with an emission maximum at 254 nm from 10-cm distance for different times. The approximate exposure rate was 8.3 $Jm^{-2}s^{-1}$ as measured with an ultraviolet power energy meter Scientech 362.

Cisplatin treatment

1-μl aliquots of a stock solution of 1 mg/ml cisplatin (Sopharma, Bulgaria) in TE were added to 10 μg plasmid DNA dissolved in 200 μl TE buffer and the samples were incubated at 37 °C for the desired periods (Fichtinger-Schepman *et al.*, 1985). At the end of the incubation period, 1 M NaCl was added to a final concentration of 0.2 M NaCl, plasmid DNA was precipitated with 2 volumes of ethanol, extensively washed with 70% ethanol and dissolved in TE buffer.

Angelicin treatment

To 10 μg plasmid DNA dissolved in 200 μl TE buffer 40 μl angelicin (*2H*-furo[2,3-*h*]benzopyran-2-one, Sigma) were added from stock solutions of 1 mg/ml in dimethylsulfoxide. Plates were kept on ice in the dark for 10 min and were then irradiated with long wave UV light at 365 nm for the desired time interval (Gunz *et al.*, 1996). 1 M NaCl was added to a final concentration of 0.2 M NaCl and plasmid DNA was precipitated with 2 volumes of ethanol and dissolved in TE buffer.

Transfection

Monolayer HEK293 cells were grown in 5-cm Petri dishes to about 30% confluence and transfected by the $Ca_3(PO_4)_2$ method as described by Chen and Okayama (1987). 10 μg plasmid DNA in TE buffer was mixed with 1/20 volumes of 0.25 M $CaCl_2$, and then 1/20 volumes of 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH 6.95, were added under intense stirring. The mixture was

poured on top of the cells and the transfection was carried out for 24 h at 37 °C in an humidified CO_2 incubator. Transfection time was recorded from the moment of pouring the transfection solution on top of the monolayer HEK293 culture. The suspension cultures of K562, XP-A and CSA cells were transfected with the liposome Gene Porter 2 Transfection Reagent (Gene Therapy Systems Inc., San Diego, CA) as recommended by the manufacturer. After 24 h 2×10^6 cells were washed with ice-cold PBS (twice), resuspended in 0.5 ml 200 mM NaCl, 50 mM Na_2HPO_4, 10 mM Tris-HCl, pH 8, 10 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF and sonicated in an ice-bath with a 4710 series Ultrasonic Homogenizer for 1 min. The homogenates were clarified by centrifugation at $12000 \times g$ at 4 °C for 10 min, diluted with sonication buffer and measured with a spectrofluorimeter.

Protein extract

Protein extracts were prepared as described by Wood *et al.* (1988) with modifications. Cells were rinsed twice in ice-cold PBS, resuspended in hypotonic buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and a tablet of Complete™ protease inhibitor cocktail per 50 ml solution (Roche Diagnostics GmbH, Graz, Austria) was added and the mixture was left on ice for 20 min to swallow. Cells were then homogenized by 20–30 strokes in a Dounce homogenizer. To each cell lysate 4 ml of an ice-cold solution containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, 25% sucrose and 50% glycerol were added slowly under stirring. 1 ml of neutralized saturated ammonium sulfate was then added under gentle mixing. The mixture was kept on ice under occasional stirring for 30 min and then centrifuged at 25000 rpm in a Beckman's SW 41 rotor at 2 °C for 3 h. The upper two thirds of the supernatant were withdrawn and the protein precipitated by addition of 0.33 g/ml neutralized ammonium sulfate. The precipitate was collected by centrifugation, resuspended in a minimum volume of 25 mM HEPES-KOH, pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol and 17% glycerol. The extract was desalted using a Sephadex G-25 coarse column and the total protein concentration determined by the Bradford reaction.

DNA repair reactions

50 μl reaction solution contained 300 ng pEGFP to serve as repair substrate, 45 mM HEPES-KOH, pH 7.8, 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 25 μM each of dGTP, dATP, dTTP and dCTP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 18 μg bovine serum albumin and 100 μg cell-extract protein. The solution wase incubated at 30 °C for 3 h and finally equal volumes of 40 mM EDTA, 100 mM Tris-HCl, pH 8.0 were added. DNA was subjected to electrophoresis and densitometered to adjust concentration.

PCR

pEGFP-N1 was linearized by *EcoRI* digestion and used as a template to amplify a 0.495 kb fragment (nt 797–1292) using the primers: 5′-ACGGCAAGCTGACCCTGAA-3′ and 5′-GGGTGCTCAGGTAGTGGTT-3′. 12 PCR cycles were run using the following parameters: denaturation at 95 °C for 60 s, annealing at 59 °C for 45 s and extension at 72 °C for 60 s. Amplified products were run on 1% agarose gel, stained with ethidium bromide and quantified with Gel Pro Analyzer v.3 software for Windows (Media Cybernetics, Auburn, CA). Alternatively, reactions were stained with SYBR Green and the amount of the generated PCR product was measured with a spectrofluorimeter.

DNA lesion calculation

The number of DNA lesions/kb was determined using the Poisson formula $N = e^{-LF}$, where N is the portion of DNA fragments or segments without lesions, L is the lesion concentration in lesions/kb and F is the length of the DNA fragment or segment in which the number of lesions is determined. F was different in the different experiments. In the HCR assay we accepted $F = 0.85$ kb, which represents the length of the transcription unit along with about 130 bp upstream including the TATA box. In the PCR experiments, F was 0.495 kb, which was the length of the amplified DNA fragment.

Results

NER rates determined by HCR

To measure and compare the rates at which DNA lesions inducing different structural changes in the double helix are repaired by NER (GGR + TCR), we applied the HCR assay. In this assay, cells are transfected with plasmids damaged

in vitro, and the repair of the lesions is measured by monitoring the restoration of the transcription activity of the reporter genes *in vivo*. We used the following three plasmids that have their respective fluorescent reporter genes optimized for high expression in mammalian cells. pDsRed1 contains the gene for the red fluorescent protein with an excitation maximum at 558 nm and an emission maximum at 583 nm. pEYFP contains the gene for the enhanced yellow fluorescent protein with an excitation maximum at 513 nm and an emission maximum at 527 nm and pEGFP contains the gene for the enhanced green fluorescent protein with an excitation maximum at 488 nm and an emission maximum at 507 nm.

As damaging agents we used cisplatin, which formed intrastrand purine-purine bridges, UV light, which caused the formation of intrastrand pyrimidine-pyrimidine crosslinks of which about 80% represented CPD and about 20% pyrimidin(4–6)pyrimidone photoproducts (Douki *et al.*, 2000), and angelicin, which formed bulky monoadducts in the DNA molecules. These lesions inflict different topological changes in the DNA double helix. Cisplatin distorts the helix most severely, a single 1,2-d(GpG) bridge inducing a 30–35° rigid helical kink in DNA (Sherman and Lippard, 1987; Takahara *et al.*, 1995). In comparison, CPD induces only a 7–9° kink (Wang and Taylor, 1991), while angelicin adducts do not induce any significant kink or bend in the helix axis (Spielmann *et al.*, 1995). pEGFP and pEYFP were treated with the three agents *in vitro* to obtain different numbers of lesions in the reporter genes. In each case, we calculated the average number of lesions/kb from the percentage of lesion-free DNA, using the Poisson formula. Since there is strong evidence that cisplatin, UV light and psoralen products are effective blocks of DNA polymerase *in vitro* (Ploskonosova *et al.*, 1999; Komura *et al.*, 2002; Fernando *et al.*, 2002), the percentage of lesion-free DNA fragments was determined by PCR.

HEK293, K562, XP-A and CSB cells were cotransfected with pEYFP and pEGFP. pEYFP was undamaged and served as internal control, while pEGFP was damaged with different agents. 24 h after transfection clear cell homogenates were prepared and the fluorescent signals were measured with a spectrofluorimeter. The EGFP signals were normalized against the EYFP signal and expressed as fraction of 1.0, assuming the normalized EGFP signal from undamaged pEGFP as 1.0. These signals were directly proportional to the fractions of the lesion-free reporter genes and using again the Poisson formula we calculated the average number of lesions in the damaged plasmids that remained unrepaired 24 h after transfection of the host cells. By subtracting these figures from the number of lesions before transfection, the NER rates at which the cells repaired the three lesions were calculated as previously described (Roguev and Russev, 2000). It should be noted that the number of lesions in the reporter genes prior to transfection is of major importance for the sensitivity of the HCR assay and should be chosen to match the ability of the host cells to repair the respective lesions. If, for instance, the damage is too heavy, the cells will not be able to remove all lesions from the reporter gene and as a result the signal will be week and will not reflect correctly the different repair rates for the different lesions. The same holds true when the damage is too light. In this case cells will be able to remove all, or most of the lesions despite differences in their repair rates. We used pEGFP carrying 5.8 cisplatin lesions/kb, 2.2 or 1.6 UV lesions/kb, and 1.4 angelicin adducts/kb, on average. These lesions were in the range to give between 25 and 60% recovery of the fluorescent signal in the HCR assay (Fig. 1). Our results

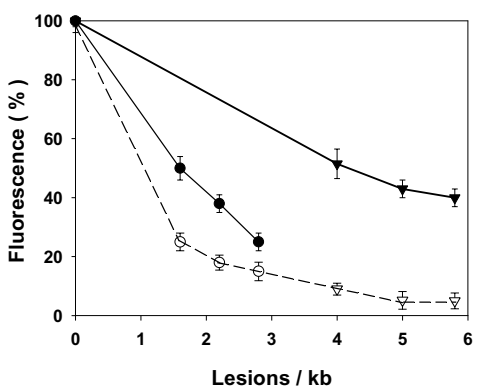

Fig. 1. Recovery of the fluorescent signal as a function of the damage. HEK293 (filled symbols, solid lines) and XP25RO (XP-A) cells (open symbols, dashed line) were cotransfected with control pEYFP and pEGFP damaged with cisplatin (triangles) and UV light (circles). The average number of lesions per kb was determined in each case by PCR as described in Materials and Methods. 24 h after the transfection clear supernatants were prepared and the EGFP signal was determined relative to the EYFP signal as described by Roguev and Russev (2000). The results are means of three independent experiments and standard deviations are shown.

Table I. NER rates as determined by HCR assay.

Agent	Cisplatin (5.8 l/kb[a])			UV light (2.2 l/kb)			Angelicin (1.4 l/kb)		
Cell line	Fluorescence (%)	Unrepaired [l/kb]	Repaired [l/kb]	Fluorescence (%)	Unrepaired [l/kb]	Repaired [l/kb]	Fluorescence (%)	Unrepaired [l/kb]	Repaired [l/kb]
HEK	40 ± 5	1.1 ± 0.1	4.7 ± 0.1	40 ± 7	1.1 ± 0.1	1.1 ± 0.1	57 ± 8	0.7 ± 0.1	0.7 ± 0.1
K562	46 ± 7	0.9 ± 0.1	4.9 ± 0.1	55 ± 6	0.7 ± 0.1	1.5 ± 0.1	47 ± 6	0.7 ± 0.1	0.7 ± 0.1
CSB	21 ± 4	1.8 ± 0.1	4.0 ± 0.1	23 ± 3	1.4 ± 0.1	0.8 ± 0.1	25 ± 4	1.3 ± 0.1	0.1 ± 0.1
XP-A	Bkg[b]	5.8	0	15 ± 4	2.2 ± 0.2	0	25 ± 4	1.4 ± 0.1	0

[a] lesions/kb.
[b] background.

showed that both HEK293 and K562 cells repaired cisplatin bridges most efficiently, followed by the UV lesions, which were repaired about four times less efficiently and the angelicin adducts, which were repaired even less efficiently. As expected, XP-A cells did not repair any of the lesions above the background levels. On the other hand TCR deficient CSB cells repaired cisplatin very efficiently, UV lesions with moderate efficiency and did not practically repair the angelicin adducts (Table I). These results showed that angelicin adducts were repaired predominantly by TCR, cisplatin predominantly by GGR and UV lesions by both NER pathways. This last result is in good agreement with earlier reports showing that CBS and XP-C cells each can repair about half of the UV lesions repaired by unmutated cells (Francis and Rainbow, 1999).

In the course of these experiments we noticed that the expression of the control undamaged reporter gene decreased when cotransfected with damaged gene and the decrease was directly proportional to the amount of damage (Fig. 2). This was observed regardless of whether we used as control pDsRed1, pEGFP, or pEYFP and also regardless of the type of damage. We interpreted this result that the damaged genes permanently retain part of the transcription complex, which led to decreased transcription rates of the control reporter gene. This result is in agreement with a recent report showing that cisplatin- and UV-damaged DNA lure the basal transcription factor TFIIH/TBP and lead to inhibition of transcription from independent and transcriptionally viable templates *in vitro* (Vichi *et al.*, 1997).

Different DNA lesions are repaired by GGR with different, and by TCR with similar efficiency

The comparison of the ratio at which CSB cells repaired cisplatin, UV and angelicin lesions (4:0.8:0.1) with the same ratio for the unmutated HEK293 (4.7:1.1:0.7) and K562 cells (4.9:1.5:0.7) (Table I) suggested that the three types of lesions were repaired by GGR at different, and by TCR at similar rates. To determine TCR and GGR in the same unmutated cells we decided to use the following approach. HCR assay was applied to measure the total NER (GGR + TCR), and a protein extract from the same cells was prepared in which GGR was measured. Then, by comparison of the two sets of data it would be possible to cal-

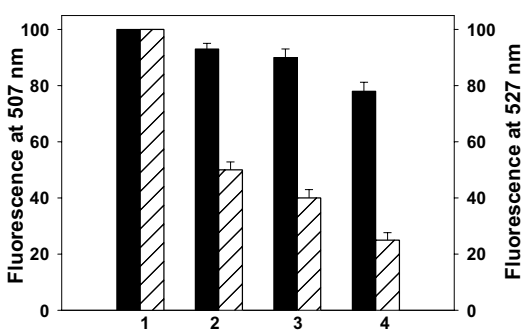

Fig. 2. Simultaneous expression of the undamaged pEYFP (filled columns) and UV irradiated pEGFP (hatched columns). HEK293 cells were cotransfected with a mixture of control undamaged pEYFP and undamaged pEGFP (1), or pEGFP treated with UV light to obtain 1.6 (2), 2.2 (3) and 2.8 (4) lesions/kb. 24 h after the transfection cells were harvested, clear supernatants were prepared, and the yellow and green fluorescence were measured at 527 and 507 nm, respectively. The results are means of three independent experiments and standard deviations are shown.

culate the respective TCR rates. To measure the GGR rates at which HEK293 cells remove different DNA lesions we prepared repair competent protein extracts and pEGFP plasmids treated *in vitro* with the genotoxic agents were incubated in them for 3 h as previously described (Gospodinov *et al.*, 2003). Since the presence of HMGB proteins inhibits the repair efficiency of protein extracts in respect to cisplatin adducts (Huang *et al.*, 1994b), we made sure that our extracts did not contain HMGB proteins by Western blot analysis with anti-HMGB antibody (not shown). After completion of the incubation, plasmid DNA was isolated and amplified by PCR. The PCR products obtained prior and after incubation were normalized against the respective controls (Fig. 3) and were used to calculate the average number of lesions removed by the extract (Table II). These results showed that the protein extracts repaired cisplatin intrastrand crosslinks most efficiency, UV generated lesions with lower efficiently and were practically not able to remove any angelicin adducts. The ratio between the repair efficiency of the three types of lesions was 4:0.5:0.1, which was very similar to that obtained with CSB cells *in vivo*

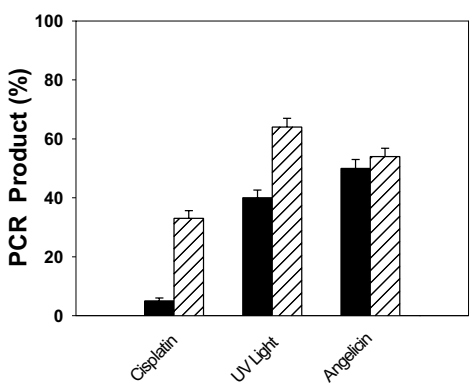

Fig. 3. DNA repair in protein extracts. pEGFP was treated with cisplatin, UV light and angelicin to obtain 5.8, 1.6 and 1.4 lesions/kb, respectively. The damaged plasmids were incubated either in repair deficient XP25RO, or repair competent HEK293 protein extracts for 3 h. Undamaged pEGFP was incubated in parallel as control. After incubation, plasmid DNA was isolated, linearized by digestion with *EcoRI* and 1 ng was used as template for PCR. The amount of PCR product generated by the plasmids incubated in XP25RO extract (filled columns) and in HEK293 extract (hatched columns) is expressed as percentage of the PCR product of the control plasmid DNA. Results are means of three independent experiments and standard deviations are shown.

Table II. Repair rates of HEK293 cells expressed in repaired lesions/kb.

Agent	Repaired by HCR [l/kb]	Repaired by CFS (GGR) [l/kb]	Repaired by TCR (HCR-CFS) [l/kb]
Cisplatin	4.7 ± 0.1	4 ± 0.2	0.7 ± 0.2
UV light	1.1 ± 0.1	0.5 ± 0.2	0.6 ± 0.2
Angelicin	0.7 ± 0.1	0.1 ± 0.1	0.6 ± 0.2

and showed that the three lesions were repaired by the constitutive GGR pathway *in vitro* at similar rates as *in vivo*. It should be also noted that this ratio corresponds well with the ratio of helix distortion inflicted on DNA by the three agents (4:0.8:0.1), which strongly supports the evidence that GGR damage recognition factors recognize conformational changes in DNA (Hess *et al.*, 1997a, b; Osman *et al.*, 2000). Finally this result is in good agreement with the reports that human CFS removes cisplatin lesions about 10 times more efficiently than psoralen monoadducts, which were excised very poorly (Huang *et al.*, 1994a). Essentially no repair was recorded when the incubation was carried out in protein extracts of repair deficient human XP-A cells, which showed that the decrease of the number of lesions in the course of incubation was a result of NER.

When the GGR rates were compared with the respective NER rates the conclusion was drawn that cisplatin bridges, CPD and angelicin adducts were repaired by TCR *in vivo* at similar rates (Table II). This result supported the hypothesis that DNA lesions were recognized by TCR independently of the chemical nature of the damaging agents.

Different lesions do not interfere with one another in TCR

To investigate further this possibility we monitored the simultaneous repair of two lesions. Competition experiments in protein extracts have been successfully used to study the efficiency of the damage-recognition events in GGR. Thus it has been demonstrated that the GGR recognition factors have 2 to 3 orders of magnitude higher affinity to lesions that distort the DNA helix than to lesions that have no major effect on it (Gunz *et al.*, 1996; Hess *et al.*, 1997a, b). In order to carry out similar competition experiments concerning TCR

we applied a multiplex fluorescent HCR assay. In this assay, HEK293 cells were cotransfected with a mixture of three plasmids. pDsRed1 was undamaged and served as an internal control to normalize the signals of the other two plasmids. The other two plasmids – pEGFP and pEYFP – were treated with different agents. The fluorescent signal of the control DsRed stood apart from the other two on the wavelength band and was measured directly. The other two signals partially overlapped and were determined as previously described (Roguev and Russev, 2000). We performed two sets of experiments in which HEK293 cells were cotransfected with pairs of plasmids damaged with cisplatin and angelicin and with UV light and angelicin. Since angelicin adducts were not repaired by GGR (Table II) we assumed that the lesions would not compete for the GGR recognition factors, but only for the TCR recognition factors which would permit to determine the relative efficiency of the recognition step for the three agents in TCR. In each case we cotransfected the cells with plasmids with a known average number of lesions and 24 h later harvested the cells and determined the number of repaired lesions from the respective fluorescent signals. In both cases we found out that the introduction of the second lesions did not interfere with the repair of the first lesions, *i.e.* they were repaired simultaneously and independently regardless of their chemical nature (Fig. 4).

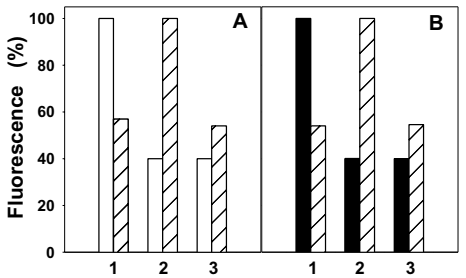

Fig. 4. Competitive fluorescent HCR assay. HEK293 cells were cotransfected with a mixture of pDsRed1, pEGFP and pEYFP. pEGFP and pEYFP were treated with cisplatin (empty columns), angelicin (hatched columns) and UV light (filled columns) as follows: (A) pEGFP was treated with cisplatin and pEYFP was treated with angelicin. The cells were cotransfected with the following combinations: (1) cisplatin lesions – 0/kb, angelicin adducts – 1.4/kb; (2) cisplatin lesions – 5.8/kb, angelicin adducts – 0/kb; (3) cisplatin lesions – 5.8/kb, angelicin adducts – 1.4/kb. (B) pEGFP was treated with UV light and pEYFP was treated with angelicin. The cells were cotransfected with the following combinations: (1) UV lesions – 0/kb, angelicin adducts – 1.4/kb; (2) UV lesions – 2.2/kb, angelicin adducts – 0/kb; (3) UV lesions – 2.2/kb, angelicin adducts – 1.4/kb. pDsRed1 was in all cases undamaged and served as internal control. 24 h after transfection clear supernatants were prepared from the transfected cells and the three fluorescent signals were read. The red fluorescent signal was taken as 100% and the other two fluorescent signals were normalized against it. All figures are means of three to five independent experiments and the standard deviations are shown.

Discussion

Our results indicate that structurally diverse lesions, such as cisplatin, CPD, and angelicin adducts, may be repaired by TCR in human cells with similar efficiency, which could not be related to their DNA helix destabilization potential. This result suggests, that in the case of TCR the damage recognition factors recognize not the damaged DNA directly, but the stalled transcription complex which is permanently blocked at the site of damage. To confirm this conclusion we applied HCR assay to TCR deficient CSB. The results with these cells showed that GGR rates in CSB cells depended on the chemical nature of the lesion in the same way as in CFS assay and suggested that the NER rates of unmutated cells *in vivo* were formed by superimposing a common TCR component on their different GGR rates.

Little is known about the detailed mechanism of the recognition step in TCR. Transcription initiation requires the establishment of the initiation complex TFIIH that contains RNA polymerase II. After the transcription has begun, during the transcription elongation phase, TFIIH is released from the transcription machinery and is available for other initiation events. However, since TFIIH takes also part in TCR repair it has to be recruited back by the stalled RNA polymerase II in the cases when it encounters an insurmountable lesion on DNA (Batty and Wood, 2000). This would suggest that a massive and permanent damage in any transcribed DNA would inhibit the transcription rate of all other transcribed sequences because TFIIH would be permanently sequestered at the damaged sites. Our results support this suggestion. We have observed that if a mixture of damaged and control plasmids were transfected in HEK293 cells, the transcription rate of the control plasmid decreased (Fig. 2). This confirms the suggestion

that the stalled RNA polymerase II binds the TFIIH complex thus depleting the pool of the nuclear TFIIH, which is normally available for initiation of transcription. How the stalled RNA polymerase II lures the available TFIIH is not clear, but one have to keep in mind that a similar event takes place at the initiation stage of transcription. At this stage, RNA polymerase II binds to the initiation site but transcription does not begin until TFIIH attaches to the quiescent RNA polymerase II thus setting it in motion.

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Aboussekhra A., Biggerstaff M., Shivji M., Vilpo J., Moncollin V., Podust V., Protic M., Hubscher U., Egly J.-M., and Wood R. D. (1995), Mammalian DNA nucleotide excision repair reconstituted with purified protein components. Cell **80**, 859–868.

Atanassov B. S., Ninova P. D., Anachkova B., and Russev G. (2003), Relationship between DNA repair capacity and resistance to genotoxins in four human cell lines. Cancer Detect. Prevent. **27**, 24–29.

Barret J.-M., Calsou P., Laurent G., and Salles B. (1996), DNA repair activity in protein extracts of fresh human malignant lymphoid cells. Mol. Pharmacol. **49**, 766–771.

Batty D. P. and Wood R. D. (2000), Damage recognition in nucleotide excision repair of DNA. Gene **241**, 193–204.

Bohr V. A., Smith C. A., Okumoto D. S., and Hanawalt P. C. (1985), DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell **40**, 359–369.

Chen C. and Okayama H. (1987), High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7**, 2745–2752.

Cline S. and Hanawalt P. C. (2003), Who's on first in the cellular response to DNA damage? Nature Rev. Mol. Cell Biol. **4**, 361–372.

Douki T., Court M., Sauvaigo S., Odin, F., and Cadet J. (2000), Formation of the main UV-induced thymine dimeric lesions within isolated and cellular DNA as measured by high performance liquid chromatography-tandem mass spectrometry. J. Biol. Chem. **275**, 11678–11685.

Fernando L. P., Kurian P. J., Fidan M., and Fernandes D. J. (2002), Quantitation of gene-specific DNA damage by competitive PCR. Analyt. Biochem. **306**, 212–221.

Fichtinger-Schepman A. M. J., van der Veer J. L., den Hartog J. H. J., Lohman P. H. M., and Reedijk J. (1985), Adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA: formation, identification, and quantitation. Biochemistry **24**, 707–713.

Francis M. A. and Rainbow A. J. (1999), UV-enhanced reactivation of a UV-damaged reporter gene suggests transcription-coupled repair is UV-inducible in human cells. Carcinogenesis **20**, 19–26.

Friedberg E., Graham W., and Siede W. (1995), In: DNA Repair and Mutagenesis. ASM Press, Washington, pp. 91–399.

Furuta T., Ueda T., Aune G., Sarasin A., Kraemer K. H., and Pommier Y. (2002), Transcription-coupled nucleotide excision repair as a determinant of cisplatin sensitivity of human cells. Cancer Res. **62**, 4899–4902.

Gospodinov A., Ivanov R., Anachkova B., and Russev G. (2003), Nucleotide excision repair rates in rat tissues. Eur. J. Biochem. **270**, 1000–1005.

Gunz D., Hess M., and Naegeli H. (1996), Recognition of DNA adducts by human nucleotide excision repair. J. Biol. Chem. **271**, 25089–25098.

Hess M., Gunz D., Luneva N., Geacintov N., and Naegeli K. (1997a), Base-pair conformation-dependent excision of benzo[a]pyrene diol epoxide-guanine adducts by human nucleotide excision repair enzymes. Mol. Cell. Biol. **17**, 7069–7076.

Hess M., Schwitter U., Petretta M., Giese B., and Naegeli H. (1997b), Bipartate substrate discrimination by human nucleotide excision repair. Proc. Natl. Acad. Sci. USA **94**, 6664–6669.

Huang J. C., Hsu D., Kazantsev A., and Sancar A. (1994a), Substrate spectrum of human excinuclease: repair of abasic sites, methylated bases, mismatches and bulky adducts. Proc. Natl. Acad. Sci. USA **91**, 12213–12217.

Huang J. C., Zamble D. B., Reardon J. T., Lippard S. J., and Sancar A. (1994b), HMG-domain proteins specifically inhibit the repair of the major DNA adduct of the anticancer drug cisplatin by human excision nuclease. Proc. Natl. Acad. Sci. USA **91**, 10394–10398.

Inoue H., Nojima H., and Okayama H. (1990), High efficiency transformation of *Escherichia coli* with plasmids. Gene **96**, 23–28.

Komura J., Ikehata H., Hosoi Y., Riggs A. D., and Ono T. (2002), Mapping psoralen cross-links at the nucleotide level in mammalian cells: suppression of cross-linking at transcription factor- or nucleosome-binding sites. Biochemistry **40**, 4096–4105.

McKay B. C., Francis M. A., and Rainbow A. J. (1995), Wild type p53 is required for heat shock and ultraviolet light enhanced repair of UV-damaged reporter gene. Carcinogenesis **18**, 245–249.

Mellon I., Spivak G., and Hanawalt P. C. (1987), Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell **51**, 241–249.

Mu D., Wakasugi M., Hsu D., and Sancar A. (1997), Characterization of reaction intermediates of human excision repair nuclease. J. Biol. Chem. **272**, 28791–28979.

Osman R., Fuxreiter M., and Luo N. (2000), Specificity of damage recognition and catalysis of DNA repair. Comput. Chem. **24**, 331–339.

Ploskonosova I. I., Baranov V. I., and Gaziev A. I. (1999), PCR assay of DNA damage and repair at the gene level in brain and spleen of gamma-irradiated young and old rats. Mutat. Res. **434**, 109–117.

Roguev A. and Russev G. (2000), Two-wavelength fluorescent assay for DNA repair. Analyt. Biochem. **287**, 313–318.

Sancar A. (1996), DNA excision repair. Annu. Rev. Biochem. **65**, 6543–6581.

Sherman S. and Lippard S. J. (1987), Structural aspects of platinum anticancer drugs interaction with DNA. Chem. Rev. **87**, 1153–1181.

Spielmann H. P., Dwyer T. J., Hearst J. E., and Wemmer D. E. (1995), Solution structures of psoralen monoadducted and cross-linked DNA oligomers by NMR spectroscopy and restrained molecular dynamics. Biochemistry **34**, 12937–12953.

Takahara P. M., Rosenzweig A. C., Frederik C. A., and Lippard S. J. (1995) Crystal structure of double-stranded DNA containing the major adduct of the anticancer drug cisplatin. Nature **377**, 649–652.

van Hoffen A., Kalle V. H., de Jong-Versteed A., Lehmann A. R., van Zeeland A. A., and Mullenders L. H. (1999), Cells from XP-D and XP-D-CS patients exhibit equally inefficient repair of UV-induced damage in transcribed genes but different capacity to recover UV-inhibited transcription. Nucl. Acids. Res. **27**, 2898–2904.

van Oosterwijk M. F., Filon R., Kalle W. H., Mullenders L. H., and van Zeeland A. A. (1996), The sensitivity of human fibroblasts to *N*-acetoxy-2-acetylaminofluorene is determined by the extent of the transcription-coupled repair, and/or their capability to counteract RNA synthesis inhibition. Nucl. Acids. Res. **24**, 4653–4659.

Venema J., Bartoshova Z., Natarajan A. T., van Zeeland A. A., and Mullenders L. H. (1992), Transcription affects the rate but not the extent of repair of cyclobutane pyrimidine dimers in the human adenosinedeaminase gene. J. Biol. Chem. **267**, 8852–8856.

Vichi P., Coin F., Renaud J.-P., Vermeulen W., Hoeijmakers J. H. J., Moras D., and Egly J.-M. (1997), Cisplatin- and UV-damaged DNA lure the basal transcription factor TFIIH/TBP. EMBO J. **16**, 7444–7456.

Wang C. I. and Taylor J. S. (1991), Site-specific effect of thymine dimer formation on dAn.dTn tract binding and its biological implication. Proc. Natl. Acad. Sci. USA **88**, 9072–9076.

Wood R. D. (1996), DNA repair in eukaryotes. Annu. Rev. Biochem. **65**, 135–167.

Wood R. D., Robins P., and Lindahl T. (1988), Complementation of the Xeroderma Pigmentosum DNA repair defect by cell-free extracts. Cell **53**, 97–108.