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Sequence-specific *p53* gene damage by chloroacetaldehyde and its repair kinetics in *Escherichia coli*

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Oxidative stress and certain environmental carcinogens, e.g. vinyl chloride and its metabolite chloroacetaldehyde (CAA), introduce promutagenic exocyclic adducts into DNA, among them 1,N^6-ethenoadenine (εA), 3,N^4-ethenocytosine (εC) and N^2,3-ethenoguanine (εG). We studied sequence-specific interaction of the vinyl-chloride metabolite CAA with human *p53* gene exons 5–8, using DNA Polymerase Fingerprint Analysis (DPFA), and identified sites of the highest sensitivity. CAA-induced DNA damage was more extensive in *p53* regions which revealed secondary structure perturbations, and were localized in regions of mutation hot-spots. These perturbations inhibited DNA synthesis on undamaged template. We also studied the repair kinetics of CAA-induced DNA lesions in *E. coli* at nucleotide resolution level. A plasmid bearing full length cDNA of human *p53* gene was modified *in vitro* with 360 mM CAA and transformed into *E. coli* DH5α strain, in which the adaptive response system had been induced by MMS treatment before the cells were made competent. Following transformation, plasmids were re-isolated from transformed cultures 35, 40, 50 min and 1–24 h after transformation, and further subjected to LM-PCR, using ANPG, MUG and Fpg glycosylases to identify the sites of DNA damage. In adaptive response-induced *E. coli* cells the majority of DNA lesions recognized by ANPG glycosylase were removed from plasmid DNA within 35 min, while MUG glycosylase excised base modifications only within 50 min, both in a sequence-dependent manner. In non-adapted cells resolution of plasmid topological forms was perturbed, suggesting inhibition of one or more bacterial topoisomerases by unrepaired ε-adducts. We also observed delayed consequences of DNA modification with CAA, manifesting as secondary DNA breaks, which appeared 3 h after transformation of damaged DNA into *E. coli*, and were repaired after 24 h.

Keywords: chloroacetaldehyde, vinyl chloride, sequence-specific DNA damage, exocyclic DNA adducts, DNA repair, *p53*, replication, LM-PCR

Vinyl chloride (VC) is an important precursor of chemicals used by the plastics industry for countless applications (Green & Hathway, 1978). VC has been shown to be mutagenic and carcinogenic (Bartsch *et al.*, 1994) inducing liver angiosarcomas, hepatocellular carcinomas, cholangiocarcinomas and other tumours in rats (Viola, 2001) and humans (Maltoni *et al.*, 1974). Three cases of VC-related liver angiosarcomas were reported among VC factory workers (Creech & Johnson, 1974).

In mammalian cells, vinyl chloride is activated to chloroethylene oxide (CEO) by cytochrome P450-dependent microsomal monooxygenases (Bartsch *et al.*, 1994). CEO binds directly to nitrogen atoms of DNA bases, or rearranges to form chloroacetaldehyde (CAA). CEO forms various linear and

Abbreviations: CAA, chloroacetaldehyde; CEO, chloroethylene oxide; DPFA, DNA polymerase fingerprint analysis; εA, 1,N^6-ethenoadenine; εC, 3,N^4-ethenocytosine; N^2,3-εG, N^2,3-ethenoguanine; 1,N^2,εG, 1,N^2-ethenoguanine; LM-PCR, ligation-mediated polymerase chain reaction; LPO, lipid peroxidation; M1dG, (pirymido[1,2α]purin-10(3*H*)-one; MMS, methyl methanesulfonate; ROS, reactive oxygen species; VC, vinyl chloride.

exocyclic adducts in the following quantitative order: N^7-(2-oxoethyl)guanine >>> 1,N^6-ethenoadenine (εA) > hydroxyethanoguanine > N^2,3-ethenoguanine (N^2,3-εG) > 3,N^4-ethenocytosine (εC) > 1,N^2-ethenoguanine (1,N^2-εG) (Guengerich *et al.*, 1993; Muller *et al.*, 1997). Hydroxyethanoguanine undergoes further rearrangement to N^2-(2-oxoethyl)guanine (Langouët *et al.*, 1997). CAA binds to adenine and cytosine in DNA, forming mainly hydroxyethano derivatives, which subsequently dehydrate to εA and εC (Fig. 1). The hydroxyethano-precursors of εA and εC in cell-free systems inhibit DNA synthesis more strongly than εA and εC themselves (Tudek *et al.*, 1999; Hang *et al.*, 2003). Reaction of CAA with guanine in DNA results in the formation of N^2,3-εG, and with at least 100-fold lower efficiency, 1,N^2-εG (Kuśmierek & Singer, 1992). The quantitative relationship among the etheno-adducts induced by CAA in double-stranded DNA is different than that of CEO and is as follows: εC ≥ εA > N^2,3-εG>>>1,N^2-εG (Kuśmierek & Singer, 1992). Etheno-DNA adducts are relatively unstable in DNA. N^2,3-εG depurinates to form apurinic sites (Kuśmierek *et al.*, 1989). To a lesser extent also εA can spontaneously depurinate leaving behind AP sites and DNA strand breaks (Speina *et al.*, 2001). In neutral pH εA also undergoes pyrimidine ring opening and deformylation to form 4-amino-5-(imidazol-2-yl)imidazole (compound β), although with an extremely low rate; the reaction is accelerated in alkali (Speina *et al.*, 2001; Basu *et al.*, 1993). Compound β is a strong inhibitor of DNA synthesis and induces a complex pattern of mutations, which involve single, double and triple base substitutions as well as frameshifts (Basu *et al.*, 1993).

In bacteria and mammals etheno-DNA adducts induce base substitutions, frameshift mutations, sister chromatid exchanges and chromosomal aberrations (Bartsch *et al.*, 1994). The mutagenic efficiency of these cyclic lesions is strongly affected by the nature of DNA polymerase (Moriya *et al.*, 1994; Langouët *et al.*, 1997) and by the sequence context (Litiński *et al.*, 1997). In bacteria 1,N^6-ethenoadenine gives rise mainly to the A:T → T:A transversions, albeit with a low frequency (0.1%) (Basu *et al.*, 1993). In contrast, in mammalian cells 70% of εA residues in DNA are replicated erroneously, with the most frequent mutation being the A:T → G:C transition (Pandya & Moriya, 1996), while on the leading strand the prevalent mutations observed were the A:T → T:A transversions (Levine *et al.*, 2000).

3,N^4-Ethenocytosine both in bacteria and mammalian cells induces the C:G → A:T transversions and C:G → T:A transitions, in mammalian cells being one of the most potent mutagenic lesions (Palejwala *et al.*, 1991; Basu *et al.*, 1993; Moriya *et al.*, 1994).

N^2,3-Ethenoguanine induces mainly the G:C → A:T transitions while 1,N^2-ethenoguanine causes several types of base substitutions as well as frameshifts (Cheng *et al.*, 1991; Singer *et al.*, 1991; Langouët *et al.*, 1998).

Ethenobases are eliminated from DNA by the base excision repair system. In bacteria εA is excised by 3-methyladenine DNA-glycosylase II (AlkA protein), although with a low efficiency, and in humans by alkylpurine-DNA-*N*-glycosylase (ANPG) (Saparbaev *et al.*, 1995). AlkA glycosylase additionally excises N^2,3-εG (Matjasevic *et al.*, 1992). εC is repaired by bacterial mismatch-specific uracil DNA-glycosylase (MUG) (Jurado *et al.*, 2004) and in humans by thymine-DNA-glycosylase (TDG) (Saparbaev & Laval, 1998). Both MUG and ANPG proteins additionally recognize 1,N^2-εG (Saparbaev *et al.*, 2002). These enzymes are monofunctional DNA-glycosylases and require AP-endonuclease to incise DNA at the site of the removed base. Recently, repair of εA and εC has also been demonstrated for the purified AlkB protein (Mishina *et al.*, 2005), as well as in *E. coli*, where AlkB-dependent removal of the etheno-bridge and restoration of unmodified parent bases was observed (Delaney *et al.*, 2005). AlkA and AlkB are part of *E. coli* adaptive response system to alkylating agents (Volkert, 1988), and have analogs in mammalian cells, for AlkB numerous ones (Lee *et al.*, 2005).

The pattern of mutations observed in different types of tumors depends on the cancer initiation factor. In cases of human and rat liver angiosarcomas associated with expo-

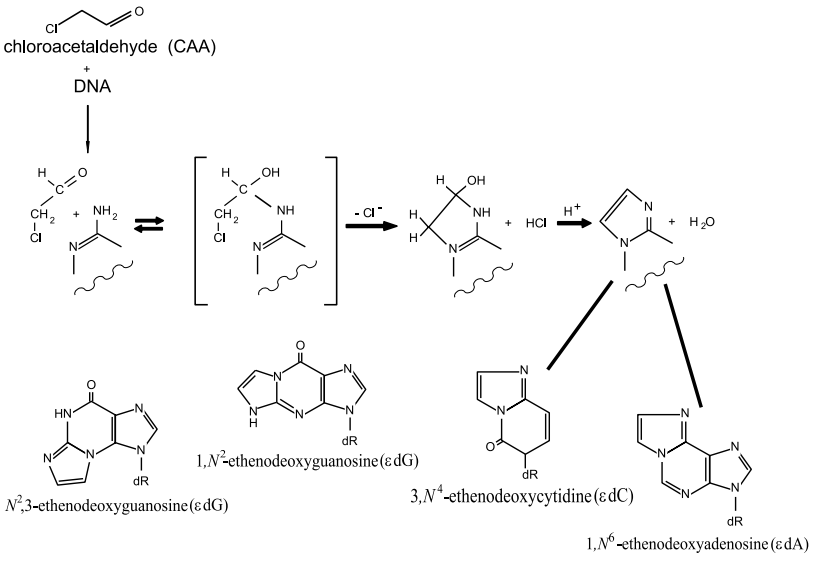

Figure 1. Formation of hydroxyethano- and ethenoadducts to DNA bases by chloroacetaldehyde.

sure to vinyl chloride, characteristic A:T → T:A transversions were found in codons 179, 249 and 255 of the *p53* gene (Hollstein *et al.,* 1994a; Barbin *et al.,* 1997).

We addressed the question about sequence specificity of *p53* gene damage by CAA, and its repair kinetics at nucleotide resolution level. We show here that CAA interacts preferentially with those human *p53* gene sequences which are difficult to bypass by DNA polymerase even when DNA is not damaged, probably due to structure abnormalities, e.g. supercoiled DNA, cruciform DNA structures, or Z-DNA. These sequences contain mutation hotspots for human *p53* gene. We show that in adaptive response-induced *E. coli* repair of CAA-triggered exocyclic DNA adducts was accomplished within 50 min, with sequence-specific differences in the repair rate. We also observed delayed consequences of DNA modification with CAA, manifesting as secondary DNA breaks, which appeared 3 h after transformation of damaged DNA into *E. coli* and were repaired after 24 h.

MATERIALS AND METHODS

Materials. Chloroacetaldehyde was from Fluka. DNA sequencing kit (T7 Sequencing Kit), [α-^{35}S]dATP (1000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Amersham-Pharmacia Biotech or ICN.

All the oligonucleotides were synthesized according to standard procedures using an Applied Biosystems synthesizer (Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics PAS, Warsaw, Poland).

In DPFA the following primers were used: (F-forward, complementary to sequences of the non-transcribed strand and R-reverse, complementary to the sequences of the transcribed strand of exons 5–8 in the *p53* gene):

p53-R3 5′-CTG GAG TCT TCC AGT GTG AT-3′
p53-R10 5′-AAA TAT TCT CCA TCC AGT GG-3′
p53-R11 5′-AAA TTT CCT TCC ACT CGG-3′
p53-R13 5′-TTC CGT CCC AGT AGA TTA CC-3′
p53-F3 5′-GTT GGC TCT GAC TGT ACC AC-3′
p53-F10 5′-TCA TCT TCT GTC CCT TCC C-3′
p53-F12 5′-ACT CCC CTG CCC TCA ACA AG-3′
p53-F11 5′-GTT GAT TCC ACA CCC CCG-3′

The following gene-specific primers were used for primer extension and PCR amplification in the LM-PCR method:

primer LK1-23 5′-GAG CGA CTG CGA TAA CAC ATA AC-3′
primer LK23-6 5′-GTT ATG TGT TAT CGC AGT-3′
primer 841SEQ 5′-TTC CTC TGT GCG CCG GTC-3′
primer 820PCR 5′-CTC CCA GGA CAG GCA CAA AC-3′
primer 811LAB 5′-CAG GAC AGG CAC AAA CAC GCA CCT C-3′
primer 827EXT 5′-GTC TCT CCC AGG ACA GG-3′

T7 polymerase (Sequenase version 2.0) was obtained from Amersham, *Taq* polymerase and ligase were from Promega. T4 polynucleotide kinase was from TaKaRa.

The Fpg and AlkA proteins were purified as previously described (Tudek *et al.,* 1998) from an overproducing strain *JM 105* bearing the plasmid pFPG230 or pAlkA, respectively (a kind gift of Drs. S. Boiteux and J. Laval; Boiteux *et al.,* 1987). ANPG, HAP1 and MUG proteins were purified according to Saparbaev and Laval (1994).

Modification of plasmid DNA by chloroacetaldehyde (CAA). Plasmid pSP65 (a kind gift of Dr. Pierre Hainaut, International Agency for Research on Cancer, Lyon, France) bearing full-length cDNA of the human *p53* gene was reacted with 0.5 mM, 50 mM and 360 mM CAA in 0.3 mM cacodylate buffer, pH 7.6, at 37°C for 3 h. After the reaction, DNA was ethanol-precipitated, washed and resuspended in 10 mM cacodylate buffer, pH 6.5, for dehydration of hydroxyethano into etheno adducts, which was performed for 72 h at 37°C. After dehydration, the DNA was ethanol-precipitated, dissolved in 10 mM Tris/HCl buffer, pH 8.0, with 1 mM EDTA and stored at –20°C.

Induction of the adaptive response and preparation of competent cells. In order to induce the adaptive response *E. coli* DH5α strain grown in LB medium were treated with 20 mM MMS for 15 min at 37°C, centrifuged, washed twice and made competent by the $CaCl_2$ method, as described (Sambrook *et al.*,1989).

Repair kinetics in *E. coli* of DNA lesions induced by chloroacetaldehyde. Plasmid pSP65 DNA modified by 360 mM CAA was transformed into competent DH5α *E. coli* cells preincubated (or not) with 20 mM MMS for 15 min in order to induce the adaptive response system. Transformation mixtures were subsequently incubated at 37°C in LB medium for 30 min, centrifuged, washed twice and resuspended in a medium containing 100 mM $MgSO_4$, 0.5% glucose and 50 μg/ml ampicillin. Plasmid DNA was isolated from 100 ml aliquots of cultures after 35, 40, 50 min or 3, 6, 9, 15 and 24 h by alkaline lysis with final purification by CsCl gradient according to (Sambrook *et al.*,1989). After purification, DNA was digested with the ANPG, MUG, and Fpg DNA glycosylases individually and in various combinations.

Selective excision of modified bases induced by CAA. Since some exocyclic adducts may not block DNA synthesis, they were converted by repair enzymes into DNA strand breaks. εA and N^2,3-εG residues in the template were converted into DNA strand breaks by digestion of the plasmid with AlkA protein

(in DPFA experiments), and subsequently with the Fpg protein. In LM-PCR experiments ANPG80 was used to excise εA. The standard reaction mixture (20 µl final volume) for the ANPG protein contained 10 µg plasmid DNA, 0.09 µg ANPG, 70 mM Hepes/KOH, pH 7.8, 1 mM EDTA, 5 mM β-mercaptoethanol, 100 mM KCl, 100 µg/ml BSA (bovine serum albumin) and 5% glycerol. Incubation was carried out at 37°C for 30 min. The AP-sites were cleaved by the Fpg protein (0.09 µg/sample) in a further 30 min incubation at 37°C in a reaction mixture containing 100 mM Hepes/KOH, pH 7.6, 10 mM EDTA and 150 mM KCl. εC and 1,N^2-εG residues in the template were converted into DNA strand breaks by digestion of the modified plasmid with MUG and Fpg or MUG alone followed by human AP-endonuclease. The standard reaction mixture (20 µl final volume) for the MUG protein contained 10 µg plasmid DNA, 0.06 µg of MUG, 70 mM Hepes/KOH, pH 7.8, 1 mM EDTA, 5 mM β-mercaptoethanol, 100 mM KCl, 100 µg/ml BSA and 5% glycerol. The mixtures were incubated for 30 min at 37°C. The AP-sites were further digested by the Fpg protein (0.09 µg/sample) for 30 min at 37°C. The standard reaction mixture (20 µl final volume) for the HAP1 protein contained 10 µg plasmid DNA, 0.06 µg of HAP1, 20 mM Hepes/KOH, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 100 µg/ml BSA and incubation was for 30 min at 37°C. After cleavage of the plasmid DNA with DNA glycosylases and AP-endonuclease, the enzymes were removed by chloroform extraction. The DNA was further precipitated with 4 volumes of cold 96% ethanol with 0.1 volume of 3 M sodium acetate, pH 5.2, kept at –20°C overnight or at –80°C for 2 h in order to sediment all small DNA fragments, and subsequently centrifuged at 12000 r.p.m. for 15 min. The DNA pellet was dissolved in water and DNA concentration was determined spectrophotometrically. To ensure complete removal of protein the A_{260}/A_{280} ratio was kept at 1.8 to 2.0. The DNA solution was stored at –20°C until further use. The quality of the reaction products was analyzed by electrophoresis on 1% agarose gel.

DNA polymerase fingerprint analysis of templates modified by CAA. The plasmid DNA was denatured with 0.2 M NaOH for 30 min at 37°C, as described by (Sambrok *et al.*,1989) and annealed with one of the primers complementary to exons 5–8. The primers were labeled for 5 min at 17–20°C by addition of an excess (1–2 µl) of [α-^{35}S]dATP (1000 Ci/mmol) to a sequencing reaction mixture containing T7 DNA polymerase (4 units per sample). Subsequently, four dNTPs, each at a final concentration of 80 µM, were added, and the samples were incubated for 10 min at 37°C for chain elongation. The reaction was terminated by formamide dye addition, and the products were analyzed by electrophoresis on 8% sequencing gel. Unmodified plasmid DNA used as a reference ladder was sequenced by the Sanger method (Sambrook *et al.*, 1989) using a T7 Sequencing Kit.

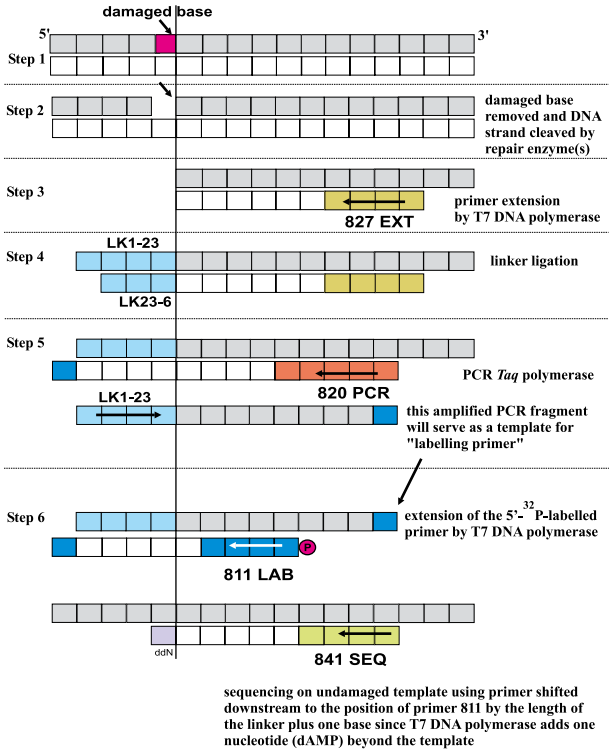

Figure 2. A schematic presentation of the ligation-mediated-PCR method.

Ligation-mediated PCR (LM-PCR). LM-PCR was conducted according to the protocol proposed by (Pfeifer & Dammann, 1999). For the recognition of DNA damage by CAA, modified templates were digested with ANPG, MUG and Fpg proteins according to the procedures described above. Primer selection is presented in Fig. 2. Three gene-specific primers were used: Primer 1 (827 EXT) was used for the primer extension by T7 DNA polymerase to the site of DNA damage, primer 2 (820 PCR) and the linker for the PCR amplification of DNA fragments marked by DNA damage, and primer 3 (811 LAB) for radiolabelling of DNA fragments. The three primers slightly overlapped each other, and had the following melting temperatures: 52°C for primer 1, 56°C for primer 2, 63°C for primer 3.

RESULTS

Sequence-specific interaction of chloroacetaldehyde with *p53* gene

A plasmid bearing full length cDNA of human *p53* gene was reacted *in vitro* with various

CAA concentrations (0.5–360 mM), hydroxyethano adducts were dehydrated into etheno adducts and they were identified either by DPFA or LM-PCR. *E. coli* AlkA and Fpg DNA glycosylases were used to identify DNA lesions in both methods (Figs. 3, 4, 5).

The DPFA method is based on the monitoring of the sites of premature chain terminations by DNA polymerase caused by unmodified sequences difficult to bypass by DNA polymerase, modified bases or their derivatives (apurinic sites or chain breaks caused, e.g., by digestion with DNA glycosylases and AP-lyases or AP-endonucleases, as well as by alkali-accelerated degradation of ethenoadducts causing DNA strand breaks, which might occur during plasmid denaturation for sequencing). The LM-PCR method detects sites of DNA cleavage by repair enzymes (Pfeifer & Dammann, 1999). A typical DPFA analysis is shown in Fig. 3. Two types of DNA polymerase premature terminations were observed. The first type were the stops on unmodified template (Fig. 3, arrow A) caused by template structures difficult to bypass by DNA polymerase, which will further be referred to as "structural stops". They were visible on CAA-modified DNA as well. Their constant presence made it difficult to find CAA modifications in these sites. This was possible only if the

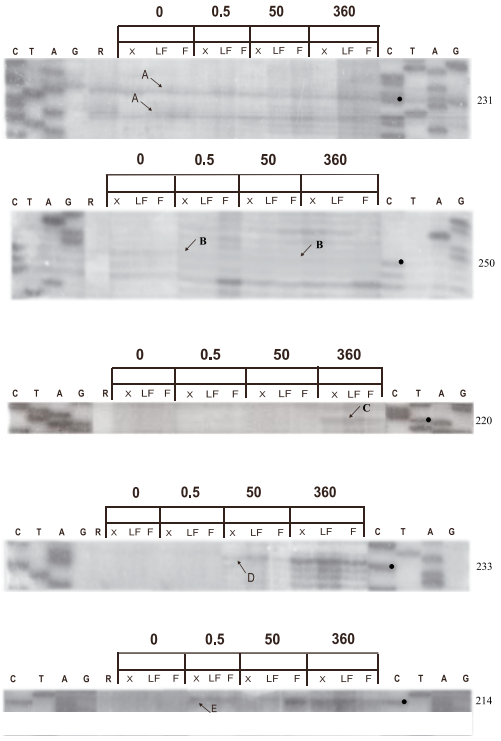

Figure 3. DNA polymerase fingerprint analysis of CAA-induced modifications within cDNA of human *p53* gene, an example of analysis.

Lanes: CTAG, sequence of the template is presented; R, primer elongation on unmodified plasmid which was not subjected to modification procedure; 0, primer elongation on templates which were subjected to modification procedure in the absence of CAA; X, primer elongation on template untreated with DNA repair enzymes; LF, primer elongation on template digested with *E. coli* AlkA and Fpg proteins; F, primer elongation on template digested with *E. coli* Fpg protein; 0, 0.5, 50, 360, *p53* cDNA unmodified or modified *in vitro* with 0.5, 50 or 360 mM CAA; dehydration procedure was performed (the template contains predominantly εC, εA and εG). **Arrows**: A, indicates non-specific structural stops on unmodified and modified template; B, sites where "structural stops" disappeared after CAA modification; C, premature chain termination observed only on template modified at 360 mM CAA; D, premature chain termination observed only on template modified at 50 or 360 mM CAA; E, premature chain termination observed on template modified at 0.5, 50 or 360 mM CAA. Dots and numbers indicate the first nucleotide of a codon.

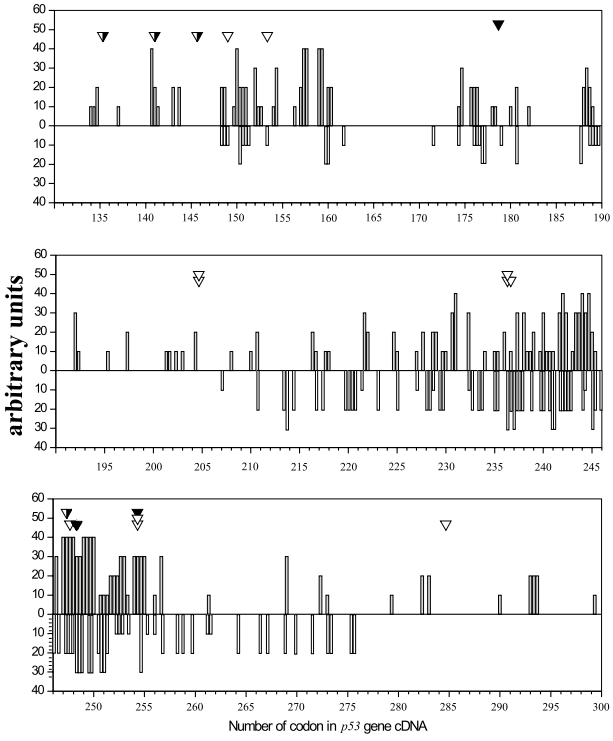

Figure 4. Distribution of CAA-induced DNA damage within exons 5–8 of *p53* gene, as analyzed by DPFA method, and spectrum of mutations in liver angiosarcomas related to vinyl chloride exposure.

CAA *in vitro* modifications (white bars below the axis) and structural stops (grey bars above the axis) at different CAA concentrations 0.5, 50 and 360 mM CAA. Inhibition of DNA synthesis by the tertiary structure of *p53* gene sequence (grey bars above the axis) was estimated in arbitrary units on a scale 0–40, the most intense bands given the value of 40. Assessment of the degree of *p53* gene modification by CAA (white bars below the axis) is done by the CAA concentration at which modifications were identified at given sites. The shortest bars (value 10) represent modifications identified only at 360 mM CAA, longer (value 20) at 50 and 360 mM CAA, the longest bars (value 30) at 0.5, 50 and 360 mM CAA. ▼ Mutations in human angiosarcoma **not** associated with vinyl chloride exposure; ▼ Mutations in human angiosarcoma associated with vinyl chloride exposure (all AT→TA); ▽ Mutations in rat angiosarcoma associated with vinyl chloride exposure (codon 147 CG→TA, codon 152 GC→AT, codons 160, 235, 253, 283 AT→TA, codon 203 AT→GC, codon 235 AT→CG, TA→GC, codon 246 GC→AT).

intensity of bands was increased after digestion with enzymes recognizing CAA-induced adducts, or if they disappeared after modification (Fig. 3 arrow B), which might be explained by the relaxation of the template structure by ε-adducts or their possible derivatives formed in alkali during DNA denaturation. The second type were premature chain terminations observed only after modification of DNA with CAA and digestion with repair enzymes. The number of identified lesion sites increased with increasing CAA concentration (Fig. 3). At the highest CAA concentration the total number of sites which reacted with CAA within exons 5–8 of *p53* gene was about 20% of the investigated *p53* region, which suggests that not all bases are equally susceptible to modification. CAA adducts were localized at adenine (39% of all modifications), guanine (30%), cytosine (28%), with only 3% of the stop bands observed at thymine sites in the template since thymine lacks an exocyclic nitrogen atom and does not react with CAA (Bartsch *et al*., 1994).

Using different CAA concentrations (Fig. 3, arrows C, D and E) we attempted to identify the sites particularly sensitive to CAA modification, and assumed that at these sites the "stop" bands should be identified at all CAA concentrations (0.5, 50 and 360 mM). These sensitive sites were found only in 14 out of 102 sites of DNA damage within the 510 nucleotide fragment, among others in codons 237, 249 and 255, which are mutational hot-spots in liver angiosarcomas related to vinyl chloride exposure. They were also found in other sites, in which no mutations related to VC were located. Thirteen of these sensitive sites were localized within codons 235–255, the region of both increased mutation rate and structural perturbations. Since the sites of CAA modification could be masked in the DPFA method by "structural stops", abundant in regions of mutational hot-spots (Fig. 4), we examined the frequently mutated *p53* gene region (within codons 190–275) also by LM-PCR, for damage recognition using *E. coli* AlkA glycosylase and Fpg glycosylase/AP-lyase, which cleaves DNA at abasic (AP) sites. Many sites within regions of structural stops were recognized by AlkA and Fpg proteins suggesting that they were the sites of CAA-adducts formation (Fig. 5). The sites of modifications detected using LM-PCR were similar to these obtained by the DPFA method (Fig. 5). However, using LM-PCR additional, to those detected by the DPFA method, sites of CAA modifications were identified, e.g. at codons 231, 241, 244, 245, 252 and 255. These modifications were located between codons 214–221, 226–230 and 232–256. Other regions were cleaved by the repair enzymes less densely, e.g., codons 130–213 and 257–300.

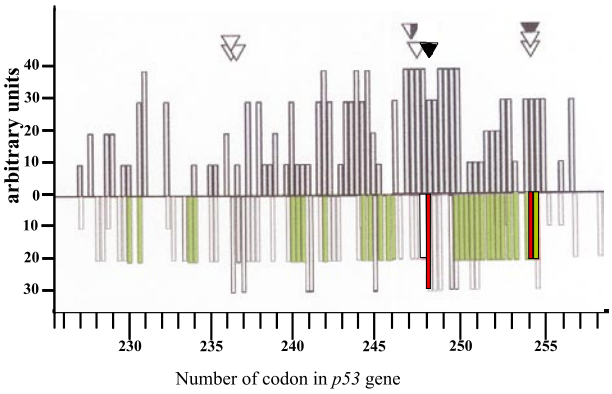

Figure 5. Comparison of CAA-induced DNA damage within exons 7–8 of *p53* gene analyzed by two methods, LM-PCR and DPFA, with the spectrum of *p53* mutations in liver angiosarcomas related to vinyl chloride exposure.
Modifications induced by CAA (white bars below the axis) were identified by DPFA method at different CAA concentrations 0.5, 50 and 360 mM CAA and LM-PCR at 360 mM CAA concentration (green bars below the axis). ▽ Mutations in human angiosarcoma **not** associated with vinyl chloride exposure; ▼ Mutations in human angiosarcoma associated with vinyl chloride exposure; ▽ Mutations in rat angiosarcoma associated with vinyl chloride exposure. Structural stops (above the axis); Glycosylase recognition sites at mutation sites, Glycosylase recognition sites identified by LM-PCR. For other explanations see Fig. 4.

LM-PCR showed formation of ethenoadenine at two mutation hot-spots, namely at codon 249, AGG, and 255, ATC (Fig. 5).

Repair kinetics of CAA-induced DNA lesions in *E. coli*

Since mutations are often formed at sites of persistent DNA damage arising due to inefficient repair we also studied the rate of repair of ethenoadducts in the human *p53* gene by the *E. coli* repair machinery.

The plasmid modified *in vitro* by 360 mM CAA (3 h, 37°C) and dehydrated to form ethenoadducts as described (see Materials and Methods) was transformed into *E. coli* DH5α cells with induced or un-induced adaptive response system, and re-isolated after different time intervals to monitor the disappearance of DNA damage with time. The plasmid was severely damaged by CAA modification; only the *oc* and linear forms were preserved, and severe disintegration, seen as a smear on agarose gel was observed. Fifty minutes after transformation into bacteria with induced AlkA glycosylase and AlkB protein all three topological forms of the plasmid were reconstituted. After a longer time (3–9 h) the ratio of the *ccc* to *oc* plasmid form as well as its overall quantity were decreased, which might suggest the existence of unrepaired DNA strand breaks

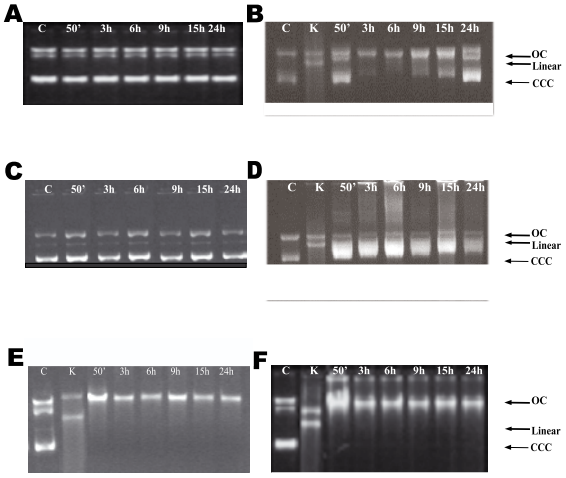

Figure 6. Kinetics of DNA repair in *E. coli* DH5α cells.
(A) Unmodified plasmid transformed into bacteria adapted by 20 mM MMS. (B) CAA-modified plasmid transformed into bacteria adapted by 20 mM MMS. (C) Unmodified plasmid transformed into non-adapted bacteria. (D) CAA-modified plasmid transformed into non-adapted bacteria. (E) CAA-modified plasmid transformed into topoisomerase I *E. coli* mutant with induced adaptive response system. (F) CAA-modified plasmid transformed into topoisomerase II *E. coli* mutant with induced adaptive response system. Lanes: C, unmodified plasmid; K, plasmid modified *in vitro* by 360 mM CAA used for transformation 50′, 3 h, 6 h, 9 h, 15 h, 24 h, time after plasmid transformation to bacteria.

(Fig. 6B). All three plasmid forms in the same ratio as 50 min after transformation were again found 24 h after transformation. This suggests that during the first hour the majority, but not all lesions were repaired, and that "a second wave" of repair took place several hours after damaged DNA had been introduced into the bacteria.

When unmodified plasmid was transformed into adapted or uninduced *E. coli* DH5α strain no major changes in the ratio of *ccc* to *oc* and linear forms of the plasmid were found (Fig. 6A and C).

Interestingly, in uninduced bacteria CAA-modified plasmid seemed to be also reconstituted 50 min after transformation (Fig. 6D). However, it was possible to distinguish only the *oc* form, and a broad band migrating on agarose gel between the *oc* and *ccc* forms, which might contain linear plasmid form (Fig. 6D). No differences in the ratio of the two plasmid forms and in its quantity were observed in all time intervals after transformation.

When CAA-modified plasmid was transformed into topoisomerase I and II deficient mutants no resolution of topological plasmid forms was observed (Fig. 6E and F), however, in these mutants the plasmid remained only in the *oc* form.

In order to further investigate the kinetics of ε-adducts repair, the plasmid isolated up to 50 min following transformation was digested with ANPG

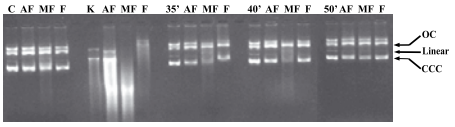

Figure 7. Repair kinetics of εA and εC in *E. coli* after induction of adaptive response system.
Lanes: C, unmodified plasmid; K, plasmid modified *in vitro* by 360 mM CAA; 35, 40 and 50 min, plasmid re-isolated from bacteria after defined time, not cleaved with repair enzymes; AF, MF, F, plasmid digested with ANPG (A) and MUG (M) and Fpg (F) proteins.

and MUG glycosylases which excise εA and εC, respectively, and with the Fpg protein cleaving DNA at abasic (AP) sites (Fig. 7), and was subjected to further examination by LM-PCR (Fig. 8).

Repair of εA in adapted bacteria was more efficient than that of εC, since no cleavage of the plasmid by ANPG/Fpg proteins was observed already 40 min after transformation. Thirty five minutes after transformation the ratio of the *ccc* to *oc* plasmid forms cleaved with ANPG/Fpg proteins was similar as in the control, but several short fragments seen as a smear were still visible on the agarose gel. Repair of εC, recognized by MUG glycosylase, was completed only 50 min after transformation (Fig. 7).

We further mapped the sites of DNA damage with the LM-PCR method using the ANPG, MUG and Fpg glycosylases for damage recognition. Different fragments of *p53* gene were repaired at different rates, which suggests that the repair was sequence-dependent. For example CAA-induced C and G adducts at codon 257 (C*TG*) that were recognized in the control CAA-treated plasmid, disappeared after 35 min repair in adapted bacteria (Fig. 8). The dam-

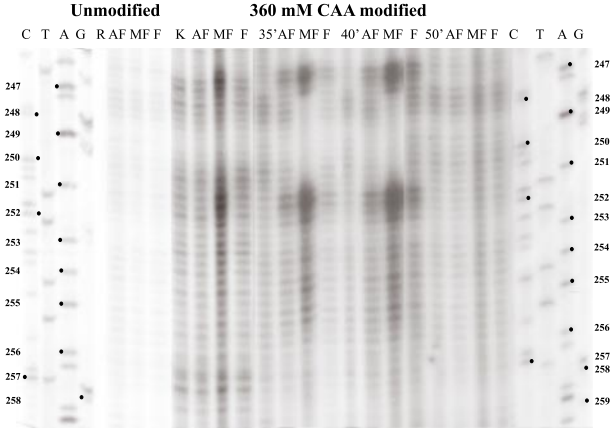

Figure 8. Kinetics of CAA-induced DNA damage repair in *E. coli* investigated by LM-PCR.
Analysis was performed on DNA modified with 360 mM CAA. Lanes: CTAG, reference sequence; R, products of DNA synthesis performed on an unmodified plasmid and not digested with repair enzymes; K, plasmid modified *in vitro* by 360 mM CAA; 35′, 40′, 50′, plasmid isolated 35, 40 and 50 min following transformation into *E. coli*; AF, MF, F, plasmid digested with ANPG (A), MUG (M) and Fpg (F) proteins. Numbering of codons is given along the sequence.

age at two adenine residues at codon 247 (A^*A^*C), which seemed to be very heavy, as judged on the basis of the intensity of bands in LM-PCR, was efficiently repaired in bacteria within 50 min. However, the G and A damage at codons 248 (CG^*G^*) and 249 (A^*GG), which were moderately modified by CAA, could still be identified 50 min after transformation. Two mutation hot-spots, codons 249 (A^*GG) and 255 (A^*TC), were recognized by the ANPG glycosylase 50 min after transformation. Although the bands were very week, their presence suggested incomplete repair of εA at these mutation hot-spot sites. In contrast, εA at codon 258, in which no mutations were found in VC-related liver angiosarcomas, was repaired very efficiently within 35 min.

DISCUSSION

Sequence specific interaction of CAA with *p53* gene

We used a vinyl chloride metabolite, chloroacetaldehyde (CAA), to investigate sequence-specificity of *p53* gene damage. DNA damage was detected as premature chain terminations of T7 DNA polymerase at adenine, guanine and cytosine sites of CAA-modified plasmid DNA containing full length cDNA of human *p53* gene, and only sparingly they were found at thymine sites. In the conditions used, ε-adducts (Fig. 4) very efficiently inhibited DNA synthesis by T7 DNA polymerase. In another *in vitro* study, in which several DNA polymerases, namely T7 exo^-, *Tag*, Pol I Klenow, Tli, and calf pol β were used to replicate DNA containing specific lesions, εA was found as a strong inhibitor of DNA synthesis as an abasic site (Speina *et al.*, 2003). εA was also an efficient inhibitor of DNA synthesis by damage-specific human DNA polymerases η and ι, as well as for pol α and pol β (Hang *et al.*, 2003). However, we cannot exclude that in the alkaline conditions applied for DNA denaturation during primer annealing a fraction of ε-adducts were rearranged into derivatives that more efficiently inhibited DNA synthesis than the ε-adducts themselves. εdA rearranges in alkali to pyrimidine ring-opened derivatives, which are characterized by stronger inhibition of DNA synthesis than εA itself, and depurinate at the final stage of rearrangement, initiating DNA strand breakage (Basu *et al.*, 1993; Speina *et al.*, 2001; 2003). εdC also initiates DNA strand breakage in alkali (Borys-Brzywczy *et al.*, 2005).

Using the DPFA and LM-PCR methods, as well as applying different concentrations of CAA we found that the interaction of CAA with *p53* gene was sequence-dependent. Sites of CAA-adducts formation within the *p53* gene were grouped in several clusters, namely between codons 214–221, 226–230 and 232–256. Other regions were modified less densely, e.g., codons 130–213 and 257–300 (Figs. 4 and 5). Marked differences in the mode of interaction with DNA between different classes of chemical carcinogens have been observed. While methylating agents, like dimethyl sulfate, caused a relatively random alkylation pattern *in vitro* (O'Connor *et al.*, 1988), as subsequently also demonstrated in tissue cultures (Bouziane *et al.*, 1998), compounds that form bulky adducts, like aflatoxin B_1, benzo[*a*]pyrene and products of lipids peroxidation such as hydroxynonenal, show a tendency to bind to specific sequences (Puisieux *et al.*, 1991, Chung *et al.*, 1996; Chen *et al.*, 1999). Also, a similar sequence-specific reaction of CAA with single-stranded DNA was suggested (Premaratne *et al.*, 1993). Some *p53* gene sites appeared to be particularly sensitive to CAA modification, since they were identified even at a very low CAA concentration (0.5 mM). They were found only in 14 out of 102 sites of DNA damage within the 510 nucleotide fragment analyzed. These particularly sensitive sites were identified in three codons (237, 249 and 255) that are mutation hot-spots in liver angiosarcomas related to vinyl chloride exposure. They were also found in other sites, in which no mutations related to VC were located. Thirteen of these sensitive sites were localized within codons 235–255, a region of both increased mutation rates and structural perturbations. Structural perturbations in supercoiled DNA, cruciform DNA structures, Z-DNA zones and misaligned purines have been shown to be preferential sites for the interaction of haloaldehydes with DNA (Lilley, 1986; Bartsch *et al.*, 1994). Several inverted repeats within the *p53* gene sequence, which were potentially capable of forming cruciform structures, were found in this region by *in silico* search (Tudek *et al.*, 1999), suggesting that these sequences may preferentially interact with CAA due to the local specific DNA conformation.

Kinetics of repair in *E. coli* of DNA lesions induced by CAA

Mutations often arise at sites of persistent DNA damage caused by delayed repair. We studied the repair kinetics of CAA-induced DNA lesions within the *p53* gene at nucleotide resolution level using the LM-PCR method.

In MMS-treated bacteria, in which the adaptive response system was induced, the majority of εA residues were repaired within 40 min, while εC only after 50 min, as judged on the basis of the cleavage of CAA-modified plasmid with ANPG, MUG and Fpg glycosylases. Repair of εA, which in the BER system is initiated by AlkA glycosylase, seems to be more rapid than the repair of 7-methyl-

guanine, which was not accomplished within 60 min following *E. coli* treatment with MMS (Grzesiuk *et al*., 2001). Since εA is very inefficiently excised from DNA by *E. coli* AlkA glycosylase, and cell extracts from adapted bacteria do not cleave oligonucleotides containing εA (not shown), one can not exclude that AlkB oxygenase may be an important player in the repair of ε-adducts in *E. coli*. The lack of plasmid cleavage by ANPG, MUG and Fpg glycosylases after 50 min of repair in bacteria does not exclude the existence of unrepaired DNA damages, which are not recognized by these proteins. Between 3 and 15 h after transformation a change of the *ccc* to *oc* ratio of the plasmid was observed as well as a decline in its quantity, suggesting formation of secondary DNA lesions, processed by the cellular repair machinery (Fig. 6B). No such phenomenon was observed when unmodified plasmid was transformed into bacteria (Fig. 6A). The identity of the lesions repaired with delay is not clear, however, exocyclic DNA adducts are relatively unstable and with time may give rise to secondary lesions which will be not recognized by DNA glycosylases, but will initiate formation of DNA strand breaks. εG and εA undergo spontaneous depurination (Kuśmierek *et al.,* 1989; Speina *et al.,* 2001) generating AP sites. εA also sequentially rearranges to pyrimidine ring-opened derivatives, which finally depurinate, and initiate DNA strand breakage (Speina *et al.,* 2001). Although the rate of this rearrangement is low, the formation of secondary lesions might, at least partially, explain the observed delayed effects of DNA damage by CAA. A similar phenomenon of delayed genome instability, which included mutations, chromosomal aberrations and reproductive cell death was found after treatment of mammalian cells with ionizing radiation and UV (for a review see Coates *et al.,* 2004). The nature of these delayed effects is not clear, but several mechanisms have been proposed, e.g. oxidative stress, affected cell repair capacity and epigenetic alterations, such as changes in methylation, acetylation and phosphorylation patterns (for a review see Kadhim *et al.,* 2004).

In uninduced bacteria no difference in the ratio of plasmid forms, and in the plasmid quantity was observed in all time intervals after transformation. However, only two plasmid forms were distinguished, with no *ccc* form visible (Fig. 6). Since the endogenous level of AlkA glycosylase in non-adapted bacteria is very low, and also the alternative AlkB catalyzed repair does not operate, it is possible that εA residues were not repaired in plasmid DNA and they could affect one or more bacterial topoisomerases. The presence of εA in DNA stimulates DNA cleavage by mammalian topoisomerases I and IIα, and inhibits the religation step by topoisomerase I, or stabilizes the topo II–DNA cleavage complex (Pourquier *et al.,* 1998; Sabourin & Osheroff, 2000). εC and M1dG exerted similar effects on mammalian topoisomerase IIα (Velez-Cruz *et al.,* 2005). The broad band of CAA-modified plasmid DNA isolated from non-adapted bacteria (Fig. 6C) might represent such cleaved, but not religated DNA, or stabilized cleavage complex.

Analyzing CAA-adducts repair at nucleotide resolution level, we noticed that the rate of repair was sequence-dependent. The very heavy damage at some codons, e.g., AA at codon 247 (AAC) or G at codon 258 (GAA) was efficiently repaired in bacteria within 35 or 50 min. However, after 50 min repair, some regions still seemed to contain DNA lesions, which produced bands during LM-PCR. Among them, adenine residues at two mutation hot-spots in human liver angiosarcomas related to VC exposure, codons 249 (AGG) and 255 (ATC), were recognized by the ANPG glycosylase 50 min after transformation, which suggested incomplete repair of εA at these sites. Also in a *p53* hot-spot of rat liver angiosarcomas, codon 248, repair of all lesions, including εA was slow. Similarly, up to 17-fold differences in the efficiency of hypoxanthine repair by the ANPG protein was observed in different sequence contexts (Xia *et al.,* 2005). Also, slower repair of UV damage was observed in *p53* mutational hot-spots in skin cancer (Tornaletti & Pfeifer, 1994).

Mutations induced by vinyl chloride in human and rodent cancers are targeted mainly at A:T base pairs (Hollstein *et al.,* 1994b; Barbin *et al.,* 1997). Although the direct VC metabolite chloroethylene oxide reacts primarily with guanine yielding N^7-(2-oxoethyl)G, this derivative is not mutagenic (Barbin *et al.,* 1985), and the next most abundant modification is εA. The amount of εA in chloroethylene oxide-modified DNA is about one order of magnitude higher than that of both isomers of εG (Cheng *et al.,* 1991; Langouët *et al.,* 1997) and about two orders of magnitude higher than that of εC (Muller *et al.,* 1997). In CAA-modified DNA, the amount of εA is lower or equal to that of εC and is twice as high as that of εG (Kuśmierek & Singer, 1992). In cells, where there may be a limited degree of chloroethylene oxide rearrangement to CAA, εA might be the quantitatively predominant lesion, while in this *in vitro* study almost equal numbers of A, C and G sites within the *p53* gene reacted with CAA. Although we have demonstrated that modification as well as repair of CAA damaged DNA is sequence-specific, only partial correlation with the spectrum of mutations induced by vinyl chloride in the *p53* gene was found. This may be due to the differences in the reactivity of CEO, the primary VC metabolite, and CAA, as well as to the different bacterial and mammalian chromosome structure and methylation, and finally to selective expansion of mutated clones

in mammalian organisms. However, this study shows the importance of the sequence-dependent DNA conformation in susceptibility to modification by CAA, and indicates that some CAA-modification-derived lesions may give rise to secondary DNA damage which demands a long time for repair and causes genome instability.

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