

Nucleotide excision repair by extracts of human fetal hepatocytes

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Abstract Human hepatocytes are particularly exposed to genotoxins, and nucleotide excision repair (NER) in these cells is essential for the maintenance of genome integrity. To characterize NER under conditions that closely resemble the pathway *in vivo*, we report the preparation and use of primary human fetal liver extracts to define the repair of a 1,3-intrastrand d(GpTpG)-cisplatin DNA lesion. Endonucleolytic cleavage at unique sites on either side of the adduct occurs at similar positions to the dominant NER incisions that have been reported for HeLa extracts. However, incisions effected by primary hepatocyte extracts are more precise as no secondary cleavage sites are detected 5' and 3' to the cisplatin lesion. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Nucleotide excision repair; Cisplatin adduct; Fetal hepatocyte extract

1. Introduction

There are several DNA repair mechanisms that counteract the potentially cytotoxic and carcinogenic effects of mutagens. These include base excision repair, transcription-coupled repair and nucleotide excision repair (NER). NER is responsible for the removal of bulky helix-distorting lesions in an intricate mechanism that involves several protein–DNA and protein–protein interactions [1]. The pathway is initiated by factors that recognize damaged DNA and recruit TFIIH, the multi-unit transcription factor [2]. XPB and XPD, which are helicase components of TFIIH, unwind DNA around the lesion. An open complex is formed to allow XPF-ERCC1 and XPG endonucleases access to incise the damaged DNA strand at sites 5' and 3' to the lesion [3]. A single-stranded oligomer of 25–32 nucleotides, which includes the damaged site, is then removed. Finally, using the undamaged strand as template, NER is completed by the formation of a repair patch, which is carried out by PCNA-dependent DNA polymerases and a DNA ligase (reviewed in [4–7]). In humans, NER is required to neutralize the potentially carcinogenic DNA lesions that are caused by chemical mutagens and ultraviolet (UV) irradiation. Mutagens that are absorbed from the entire gastrointestinal tract are conveyed to the liver via the portal circulation. Hepatocytes are thus particularly exposed to ingested mutagens [8]. An example is aflatoxin, which is also a powerful hepatocarcinogen. Another common cause of liver cancer,

persistent hepatitis B virus (HBV) infection, is thought to inhibit NER by a mechanism that underlies hepatocarcinogenesis [9–12]. Thus, defining hepatic NER is central to understanding the maintenance of hepatocyte genome integrity and the etiology of liver cancer. To analyze NER under conditions that closely resemble hepatocyte DNA repair *in situ*, we used primary extracts from human fetal liver to effect repair of a defined 1,3-intrastrand d(GpTpG)-cisplatin DNA adduct. The data indicate that incision steps of NER are more precisely regulated in primary human liver extracts and the pathway *in vivo* may involve an accessory function that is absent from cells that are selected in culture.

2. Materials and methods

2.1. Generation and analysis of circular duplex DNA containing a site-specific intrastrand cisplatin crosslink

Preparation of duplex modified M13 DNA containing a unique 1,3-intrastrand cisplatin adduct was according to the methods of Moggs et al. with minor modifications [13]. Briefly, a synthetic 24-mer oligonucleotide (5'-TCTTCTTCTGTGCACTCTTCTTCT-3', Integrated DNA Technologies Inc.) was platinated at the unique GTG sequence by incubation for 16 h at 37°C in a buffer containing 500 µM Na₂HPO₄, 500 µM NaH₂PO₄, 3 mM NaCl with or without (control) a two-fold molar excess of *cis*-platinum(II)-diamine dichloride (Sigma). The platination was stopped by the addition of NaCl to a final concentration of 500 mM. Products were purified by exclusion chromatography (Sephadex G-25) and the efficiency of each reaction was confirmed using denaturing polyacrylamide gel electrophoresis (PAGE) with autoradiography. After 5' phosphorylation, control and platinated oligonucleotides were purified using preparative PAGE.

To synthesize covalently closed circular DNA (cccDNA) with the site-specific platinum adduct, a 5–10 times molar excess of control or platinated oligonucleotide (70 ng) was annealed to complementary sequences on 10 µg of modified single-stranded M13 DNA (M13mp18GTG). Hybridized oligonucleotides were then extended by T4 DNA polymerase and formation of the covalently closed circular duplex DNA was completed by T4 DNA ligase. To remove single-stranded, linear and nicked M13mp18GTG-derived DNAs, each reaction mixture was treated with T5 exonuclease (0.5 µg per reaction) [14]. After heat inactivation of the T5 exonuclease, cccDNA was purified by exclusion chromatography (Sephacryl S-400) and effective purification of cccDNA was confirmed using agarose gel electrophoresis.

To confirm the 1,3-intrastrand d(GpTpG)-cisplatin crosslink, control (Con-GTG) and platinated (Pt-GTG) cccDNA samples were digested with *Apa*LI and *Hind*III (Amersham Life Science), and products analyzed by agarose gel electrophoresis. The platination target site is located at a unique *Apa*LI recognition sequence, and resistance to digestion with this enzyme is diagnostic for correct platination. The precise location of the cisplatin adduct was determined by primer extension analysis. 20 pmol of a synthetic 17-mer oligonucleotide (5'-CGCCCTCGAGCAGCAA-3', Pt-GTG(+38)), which is complementary to a sequence 38 nucleotides downstream of the GTG platination target, was used to prime 50 fmol of *Apa*LI-digested Con-GTG or Pt-GTG. The oligonucleotide was extended by polymerization with

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Abbreviations: NER, nucleotide excision repair

thermal cycling (Sequitum II cycle-sequencing kit, Epicentre Technologies Inc.).

2.2. Preparation of nuclear extracts from human fetal hepatic tissue

Ethical approval for acquiring fetal liver tissue was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (Protocol Number M990519). Prior to elective termination of pregnancy, voluntary informed consent was given by patients to allow the use of fetal tissue. The procedure for the preparation of nuclear protein extracts included several modifications to the procedure described by Gorski et al. [15]. Complete livers from fetuses of 14 weeks gestational age (approximately 10 g in each case) were removed immediately post abortion. They were submerged in a solution containing 0.9% NaCl and all subsequent procedures were carried out on ice. The livers were minced, washed three times in 20 ml of 0.9% NaCl, then resuspended in 25 ml of buffer H (25 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 1.0 mM PMSF and 10 mM HEPES, pH 7.9) and homogenized thoroughly with a blender. To isolate nuclei, the homogenate was layered on a cushion of the sucrose-containing buffer H then centrifuged for 30 min at 80 000×g. Pelleted nuclei were removed and incompletely homogenized tissue at the interface of the sucrose cushion was subjected to re-isolation of the nuclei as described above. Pooled nuclei were lysed by gentle suspension in 10 ml of buffer L (100 mM KCl, 3 mM MgCl₂, 0.1 mM EDTA, 10% glycerol, 1.0 mM PMSF, and 10 mM HEPES, pH 7.9). 1 ml of a saturated solution of ammonium sulfate (approximately 4 M) was added. Samples were incubated on ice with gentle agitation for a further 30 min then centrifuged at 90 000×g to remove precipitated chromatin. Solid ammonium sulfate (0.35 g per ml of recovered supernatant) was added slowly over a period of 30 min, then incubated with shaking for a further 2 h. Nuclear proteins were recovered by centrifugation (95 000×g for 30 min), pooled and then resuspended in 750 µl of dialysis buffer (40 mM KCl, 0.1 mM EDTA, 10% glycerol and 25 mM HEPES, pH 7.9). After extensive dialysis, the nuclear protein preparation was aliquoted in 20 µl volumes, snap-frozen in liquid nitrogen and stored at −80°C for no more than 6 months. Using this procedure, the yield of nuclear protein was routinely 300–400 µg per gram of liver tissue and at a concentration of approximately 6 µg/µl in the final extract.

2.3. DNA repair analysis

To measure DNA repair patch synthesis, mixtures (10 µl) were set up that contained 25 µg of nuclear protein extract, 5 µCi of [α -³²P]dCTP, 20 µM of each dNTP, 70 mM KCl, 7.5 mM MgCl₂, 0.9 mM dithiothreitol (DTT), 0.4 mM EDTA, 40 mM creatine phosphate, 0.5 µg creatine phosphokinase, 4% glycerol, 0.35 mg/ml of bovine serum albumin, 45 mM HEPES (pH 7.9) and 50 ng Con-GTG or Pt-GTG DNA. Following incubation at 30°C for 30 min, purified DNA was digested with *Hind*III and *Eco*RI. The products were resolved using denaturing PAGE, and labeled restriction fragments were detected autoradiographically.

Detection of excised cisplatin-containing DNA fragments and mapping of 5' incision sites were based on the method described by Shivji et al. [14]. A 10 µl reaction, which contained 25 µg of nuclear protein extract, 70 mM KCl, 7.5 mM MgCl₂, 0.9 mM DTT, 0.4 mM EDTA, 40 mM creatine phosphate, 0.5 µg creatine phosphokinase, 4% glycerol, 0.35 mg/ml of bovine serum albumin and 45 mM HEPES (pH 7.9), was preincubated at 30°C for 5 min. The repair reaction was initiated by adding Con-GTG or Pt-GTG and allowed to proceed for a further 30 min. To map the position of 3' incisions, extension reactions using the Pt-GTG(+38) oligonucleotide as primer were performed on purified incised DNA intermediates. To detect excised platinated fragments and 5' incisions, 12 ng (2 µl) of a synthetic 34-mer oligonucleotide (anti-PtGTG, 5'-GGGGGAAGAGTGCACAG-AAGAAGAGGCTGGTCC-3') was added to the repair reaction and then heated to 95°C for 5 min. After cooling to room temperature, insoluble material was removed by centrifugation. A cocktail containing 0.13 U modified T7 DNA polymerase (Sequenase, Amersham Life Science) and 2.0 µCi [α -³²P]dCTP, 5 mM DTT, 10 mM Tris-HCl (pH 7.5) in 1 µl was added to each sample and the tubes were incubated at 37°C for 5 min. The final dNTP concentration was then adjusted to 1 µM for dATP, dGTP, dTTP and 0.5 µM for dCTP by adding 1.5 µl of an appropriate dNTP mix, and the reaction kept at 37°C for a further 10 min. The samples were resolved on a 14%

denaturing polyacrylamide gel alongside a sequencing ladder that served as molecular weight marker. Radioactive DNA fragments were detected using autoradiography.

3. Results

3.1. Analysis of cccDNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink

To prepare the Pt-GTG substrate, a 1,3-intrastrand cisplatin crosslink was generated in a 24-mer oligonucleotide at its single d(GpTpG) triplet and formation of the desired 1,3-intrastrand d(GpTpG)-cisplatin adduct was verified using denaturing PAGE analysis. Compared to the mock-platinated oligonucleotide, the 1,3-d(GpTpG) adduct retards electrophoretic mobility of the oligonucleotide by the equivalent of approximately one nucleotide (Fig. 1A). The increase is due to structural distortion of the DNA backbone, and addition of +227 Da by the cisplatin adduct [16]. Overplatination with formation of additional adducts is characterized by a larger molecular weight smear on PAGE analysis ([14] and data not shown). Incorporation of the platinated and mock-treated oligonucleotides into Pt-GTG and Con-GTG cccDNA initially involves their hybridization to a complementary sequence of a single-stranded M13 derivative (M13mp18GTG). Thereafter, DNA polymerase extends the oligonucleotides and the synthetically circularized strand is ligated to complete formation of the cccDNA. At completion of the reaction, nicked circular and linear M13mp18GTG-derived DNA were present in addition to cccDNA. T5 exonuclease treatment with exclusion

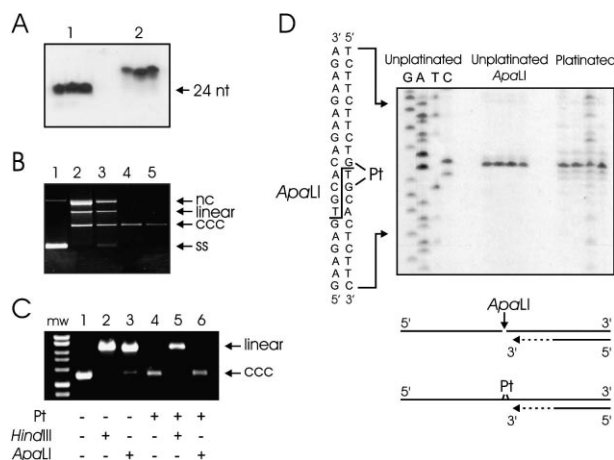


Fig. 1. Analysis of control (Con-GTG) and platinated (Pt-GTG) cccDNA substrates. A: Electrophoretic mobility of control (1) and platinated (2) oligonucleotides. The platinated 24-mer has a mobility of approximately +1 DNA nucleotide relative to the control. B: Purification of cccDNA. M13mp18GTG ssDNA prior to circularization (lane 1), then after circularization with control (lane 2) and platinated (lane 3) oligonucleotide primers are shown. Con-GTG (lane 4) and Pt-GTG (lane 5) cccDNA are demonstrated following treatment with T5 exonuclease. Mobilities of nicked circular (nc), linear, covalently closed circular (ccc) and single-stranded (ss) DNA are indicated. C: Restriction digestion analysis of purified cccDNA. Undigested (lane 1), *Hind*III- (lane 2) and *Apa*LI- (lane 3) treated Con-GTG are indicated, as well as undigested (lane 4), *Hind*III- (lane 5) and *Apa*LI- (lane 6) treated Pt-GTG. D: Primer extension analysis. Sequencing ladder of RF M13mp18GTG DNA and primer extension of *Apa*LI-treated Con-GTG and untreated Pt-GTG are indicated.

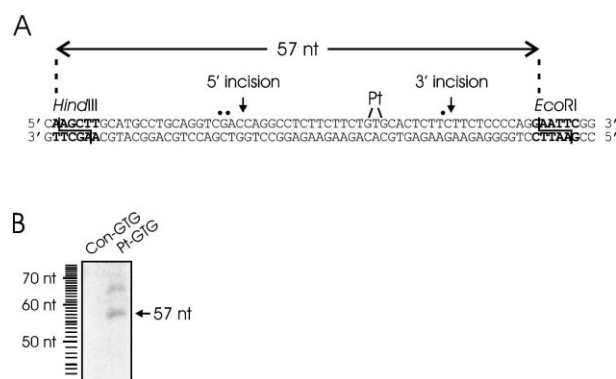


Fig. 2. Detection of repair patch synthesis. A: Sequences surrounding the cisplatin adduct together with 5' and 3' incision sites generated by fetal hepatocyte extracts during NER. Solid circles above the sequence indicate secondary incisions generated by HeLa whole cell extracts. *HindIII* and *EcoRI* restriction sites used to detect the repair synthesis step of NER are also shown. B: Autoradiograph of *HindIII/EcoRI* restriction fragments from Con-GTG and Pt-GTG DNA following repair reactions. The sizes in nucleotides (nt) of the fragments from a DNA sequencing ladder are shown alongside.

chromatography removed all but the desired Pt-GTG and Con-GTG cccDNA products (Fig. 1B).

ApaLI digestion and primer extension analysis confirmed the presence of the 1,3-intrastrand d(GpTpG)-cisplatin crosslink in purified Pt-GTG cccDNA. The platinated d(GpTpG) sequence is located within a unique *ApaLI* recognition sequence of the cccDNA, and renders the site resistant to restriction by *ApaLI* (Fig. 1C). Also, the cisplatin crosslink arrests primer extension at the targeted d(GpTpG) triplet on Pt-GTG and this corresponds to the site of termination when using the *ApaLI*-digested Con-GTG DNA as template (Fig. 1D). Taken together, these data confirm that site-specific platination has been achieved at the intended d(GpTpG) triplet of the purified covalently closed circular Pt-GTG NER substrate. Pt-GTG DNA with a unique 1,3-intrastrand d(GpTpG)-cisplatin crosslink has been shown to be a good substrate for vertebrate NER [13]. The single adduct allows detailed analysis of NER, and is thus ideally suited to defining steps of this pathway in previously uncharacterized cell extracts.

3.2. DNA repair patch synthesis by fetal liver extracts

The cisplatin lesion is located between *HindIII* and *EcoRI* restriction sites of Pt-GTG (Fig. 2A). These restriction digestion sites flank the previously reported 5' and 3' NER incisions of HeLa extracts [13] and are thus ideally suited to locating repair patch synthesis. To demonstrate repair synthesis in the area of the cisplatin adduct, Pt-GTG and Con-GTG DNA were initially incubated with the fetal liver extract in the presence of [α - 32 P]dCTP. Thereafter, digestion of the purified cccDNA with *HindIII* and *EcoRI* revealed that most of the repair synthesis occurred in a fragment of 57 nucleotides in length (Fig. 2B). This size corresponds to that of the *HindIII/EcoRI* restriction fragment that encompasses the repair patch. The labeled fragment of 65 nucleotides was only observed with the damaged substrate and may result from restriction star activity by the combination of *EcoRI* and *HindIII*. No labeling of the 57 nucleotide fragment from Con-GTG was detectable (Fig. 2B) and verifies that specific repair synthesis occurs in the platinated cccDNA substrate.

3.3. Mapping of NER 3' and 5' incisions by fetal liver extracts

To map 3' NER incision sites generated by the fetal liver extract, a primer extension assay using the Pt-GTG(+38) oligonucleotide was performed. This oligonucleotide is complementary to a sequence 38 nucleotides downstream of the unique platinated site of Pt-GTG. Together with sequencing of the M13mp18GTG RF DNA, primer extension reveals that a single 3' incision is located nine phosphodiester bonds from the site of platination (Fig. 3A). This is similar to the main 3' incision that has been documented when using HeLa extracts [13]. Primer extension took place efficiently when using Con-GTG DNA as the template. Intensively labeled high molecular weight DNA fragments were generated, but only the area of interest for detection of incision sites is shown in Fig. 3A. The secondary 3' incision effected by HeLa extracts, which is eight nucleotides from the cisplatin adduct, was undetectable. The proportion of substrate with the 3' incision represented approximately 20% of the total Pt-GTG that was included in the reaction mixture. In the remaining 80% of substrate DNA, primer extension was terminated at the platination site (Fig. 3A). The efficiency of 3' incision effected by the fetal liver extracts is lower than that under similar conditions reported using HeLa extracts [13]. The reasons for this are not entirely clear but are likely to reflect slight differences in reaction conditions, variations in the preparation of cell extracts or a lower overall NER competence in fetal hepatocytes. Nuclear extracts of fetal hepatocytes were used here, whereas whole cell extracts were prepared for the study of HeLa NER. In our hands, the nuclear preparation from hepatocytes carried out NER more efficiently than whole cell extracts (data not shown). Diminished hepatocyte viability induced by termination of pregnancy is also likely to contribute to the lower NER efficiency that we have observed.

3.4. Location of the 5' incision sites and detection of excised platinated oligonucleotides produced by dual incision

Coupled incision and mapping of the 5' incision site were demonstrated by analysis of excised fragments containing the

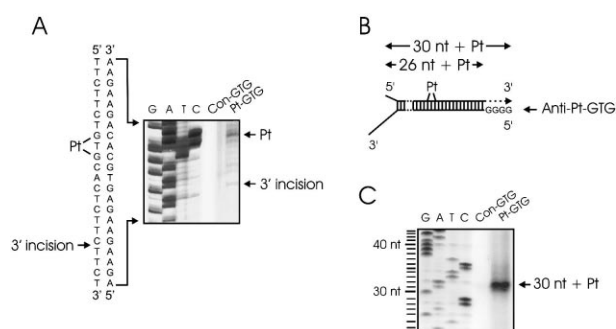


Fig. 3. Detection of 5' and 3' NER incision sites. A: Mapping the 3' incision site. Primer extension on Con-GTG and Pt-GTG template recovered from the repair reaction reveals the position of the 3' incision relative to the cisplatin adduct. B: Strategy for the detection of NER excision fragments. Excision fragments are hybridized to a 34 nt synthetic oligonucleotide (anti-Pt-GTG) containing sequences complementary to the excised fragment. The 3' terminal non-complementary nucleotides prevent labeling of anti-Pt-GTG. A 5' terminal poly-G provides template for labeling of the excised fragment using [α - 32 P]dCTP and modified T7 DNA polymerase (Sequenase). C: Autoradiograph showing sequencing ladder as molecular weight marker, and platinated oligonucleotide excised from Pt-GTG.

1,3-intrastrand (GpTpG)-cisplatin adduct. After completing incubation of the NER reaction, an oligonucleotide (anti-Pt-GTG) was hybridized to complementary sequences of the excised cisplatin-containing oligonucleotide. The sequence of anti-Pt-GTG included a 5' overhang that serves as template for extension labeling of the 3' end of the excised Pt-containing fragment (Fig. 3B). A mismatch at the 3' end of anti-Pt-GTG prevents priming at this end and the reaction conditions thus allow only for the labeling of the cisplatin-containing oligonucleotide. The single labeled fragment that was detected using this methodology migrated with an equivalent molecular weight of a 31 nucleotide fragment (Fig. 3C). The excised fragment comprises 30 nucleotides since the platinum adduct increases its molecular weight by the equivalent of one nucleotide [16]. In addition to the cisplatin adduct, and using information on the location of the 3' incision (Fig. 3A), the detected fragment includes the four nucleotides that are complementary to the 5' overhang of anti-Pt-GTG. Thus, the unique NER excision product comprises 26 nucleotides together with the cisplatin adduct. The 5' incision of the Pt-GTG substrate is calculated to occur at a single site that is 16 phosphodiester bonds from the cisplatin adduct. Secondary incisions that occur 5' to the cisplatin adduct are undetectable. The locations of the 5' and 3' incisions that are generated by fetal hepatocyte preparations are depicted diagrammatically in Fig. 2A and are similar to the main incision sites that have been reported for HeLa cell extracts.

4. Discussion

Using platinated DNA similar to the substrate analyzed in this study, Moggs et al. demonstrated that HeLa whole cell extracts effect major and minor incisions both 5' and 3' to the cisplatin adduct during NER [13]. The main 5' incision is 16 phosphodiester bonds from the lesion and other 5' incisions occur less frequently at the 18th and 19th phosphodiester bonds from the cisplatin adduct. The major 3' incision was at the ninth phosphodiester bond, and a minor 3' incision occurred eight phosphodiester bonds from the lesion. The factors that govern exact positioning of the 5' and 3' incisions relative to the DNA lesion during NER are not completely understood [1]. Different lesions, such as UV-induced pyrimidine dimers, cisplatin and cholesterol adducts, are released within single-stranded oligonucleotides of similar size but the incision positions vary depending on the lesion. The helical distortions and altered base stacking arrangements that are caused by a particular lesion are thought to influence the protein–DNA and consequently protein–protein interactions to cause variable 3' and 5' incisions. Using cccDNA with a defined 1,3-intrastrand cisplatin adduct as template, primary human hepatocyte extracts consistently generate 5' and 3' incisions that correspond to the major incisions of HeLa extracts (Fig. 2A). It is not clear at this stage why the primary extracts effect a more precise endonucleolytic cleavage. However, our observations suggest that an accessory factor, not present in cell culture-selected HeLa cells, regulates precise incision.

The significance of tissue-specific NER activity and the importance of NER during mammalian development are not well established. Analysis of NER activity in rat tissues demonstrated incision and repair synthesis activities in several organs, and rat liver repair synthesis was more active than

in other tissues [17]. NER is also active in neuronal tissue, and defective NER in neurons may be responsible for the progressive neurodegeneration that is characteristic of some xeroderma pigmentosum patients [18]. NER has also been studied in embryonal stem (ES) cells [19]. Surprisingly perhaps, the repair of UV-induced lesions on transcribed and non-transcribed genes was relatively inefficient in these cells. ES cells commit easily to apoptosis following moderate UV light exposure and the role of NER in these cells may be limited. ES cells have an absolute requirement for replicative and transcriptional fidelity and it is thought that apoptosis with NER shutdown is the favored response to DNA damage. Our demonstration of efficient NER in hepatocyte extracts from fetuses of 14 weeks gestation suggests that the importance of NER is promoted after embryonic development is complete.

Hepatocytes play a central role in neutralizing the effects of a range of toxins. As a result of its anatomical location and portal blood supply from the intestine, the liver is exposed to potentially carcinogenic ingested mutagens. Also, common hepatocarcinogens, such as HBV, cause persistent necroinflammatory liver disease (cirrhosis) that is associated with increased formation of potentially carcinogenic mutations [8,20]. Moreover, in the case of HBV, the X protein (HBx) is thought to compromise NER and is responsible for the formation of oncogenic mutations in HBV-infected hepatocytes [9–12]. Successful reconstitution of NER using primary liver extracts presents several possibilities for the study of human hepatocyte DNA repair, as well as defects in the NER pathway that underlie hepatocarcinogenesis. The preparations described here have the advantage that they are not subjected to prior selection in culture and are functionally similar to hepatocytes in vivo. We have also shown that the human liver extracts described here are competent for in vitro transcription (data not shown). Using primary extracts of human fetal hepatocytes, our investigations are currently aimed at defining the role of HBx in NER as well as in the related transcription-coupled DNA repair pathway.

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