INTRODUCTION

The nucleotide excision repair (NER) pathway has evolved principally to deal with u.v. light-induced DNA damage, but is also active against a variety of other endogenously generated bulky DNA lesions. Components of the NER system are defective in patients with the rare autosomal recessive disease, xeroderma pigmentosum (XP), which is characterised by photosensitivity and a thousandfold increased incidence of skin cancer. There are seven complementation groups in classical XP and most of the genes involved have now been isolated. NER genes have also been isolated independently by their ability to complement u.v. light-sensitive rodent cell lines. There is extensive overlap between these ERCC (excision repair cross-complementing) genes and XP genes (for review see Wood, 1996). The NER pathway has now been reconstructed in vitro using proteins purified from mammalian cells (Aboussekhra et al., 1995). It consists of an initial damage recognition step followed by the removal of the damaged DNA sequence. This is achieved by the action of the XPE protein, which is essential for NER in mammalian cells and is also believed to be involved in mitotic recombination. ERCC1-deficient mice, with their extreme runting and polyploid hepatocyte nuclei, have a phenotype that is more reminiscent of a cell cycle arrest/premature ageing disorder than the classic DNA repair deficiency disease, xeroderma pigmentosum. To understand the role of ERCC1 and the link between ERCC1-deficiency and cell cycle arrest, we have studied primary and immortalised embryonic fibroblast cultures from ERCC1-deficient mice and a Chinese hamster ovary ERCC1 mutant cell line. Mutant cells from both species showed the expected nucleotide excision repair deficiency, but the mouse mutant was only moderately sensitive to mitomycin C, indicating that ERCC1 is not essential for the recombination-mediated repair of interstrand cross links in the mouse. Mutant cells from both species had a high mutation frequency and the level of genomic instability was elevated in ERCC1-deficient mouse cells, both in vivo and in vitro. There was no evidence for an homologous recombination deficit in ERCC1 mutant cells from either species. However, the frequency of S-phase-dependent illegitimate chromatin exchange, induced by ultra violet light, was dramatically reduced in both mutants. In rodent cells the G1 arrest induced by ultra violet light is less extensive than in human cells, with the result that replication proceeds on an incompletely repaired template. Illegitimate recombination, resulting in a high frequency of chromatid exchange, is a response adopted by rodent cells to prevent the accumulation of DNA double strand breaks adjacent to unrepaired lesion sites on replicating DNA and allow replication to proceed. Our results indicate an additional role for ERCC1 in this process and we propose the following model to explain the growth arrest and early senescence seen in ERCC1-deficient mice. In the absence of ERCC1, spontaneously occurring DNA lesions accumulate and the failure of the illegitimate recombination process leads to the accumulation of double strand breaks following replication. This triggers the p53 response and the G2 cell cycle arrest, mediated by increased expression of the cyclin-dependent kinase inhibitor p21cip1/waf1. The increased levels of unrepaired lesions and double strand breaks lead to an increased mutation frequency and genome instability.

Key words: DNA repair, Genome instability, Recombination, Xeroderma pigmentosum

SUMMARY

The ERCC1 protein is essential for nucleotide excision repair in mammalian cells and is also believed to be involved in mitotic recombination. ERCC1-deficient mice, with their extreme runting and polyploid hepatocyte nuclei, have a phenotype that is more reminiscent of a cell cycle arrest/premature ageing disorder than the classic DNA repair deficiency disease, xeroderma pigmentosum. To understand the role of ERCC1 and the link between ERCC1-deficiency and cell cycle arrest, we have studied primary and immortalised embryonic fibroblast cultures from ERCC1-deficient mice and a Chinese hamster ovary ERCC1 mutant cell line. Mutant cells from both species showed the expected nucleotide excision repair deficiency, but the mouse mutant was only moderately sensitive to mitomycin C, indicating that ERCC1 is not essential for the recombination-mediated repair of interstrand cross links in the mouse. Mutant cells from both species had a high mutation frequency and the level of genomic instability was elevated in ERCC1-deficient mouse cells, both in vivo and in vitro. There was no evidence for an homologous recombination deficit in ERCC1 mutant cells from either species. However, the frequency of S-phase-dependent illegitimate chromatin exchange, induced by ultra violet light, was dramatically reduced in both mutants. In rodent cells the G1 arrest induced by ultra violet light is less extensive than in human cells, with the result that replication proceeds on an incompletely repaired template. Illegitimate recombination, resulting in a high frequency of chromatid exchange, is a response adopted by rodent cells to prevent the accumulation of DNA double strand breaks adjacent to unrepaired lesion sites on replicating DNA and allow replication to proceed. Our results indicate an additional role for ERCC1 in this process and we propose the following model to explain the growth arrest and early senescence seen in ERCC1-deficient mice. In the absence of ERCC1, spontaneously occurring DNA lesions accumulate and the failure of the illegitimate recombination process leads to the accumulation of double strand breaks following replication. This triggers the p53 response and the G2 cell cycle arrest, mediated by increased expression of the cyclin-dependent kinase inhibitor p21cip1/waf1. The increased levels of unrepaired lesions and double strand breaks lead to an increased mutation frequency and genome instability.

Key words: DNA repair, Genome instability, Recombination, Xeroderma pigmentosum
light-induced DNA damage, the cell cycle arrest in and show uncoupling of S phase and mitosis.

NER does not proceed in isolation, but is precisely co-ordinated with other essential cellular processes. In addition to genome-wide NER, there is also preferential repair of the transcribed DNA strand (Mellon et al., 1987), with the XP-B and XP-D proteins being essential components of the RNA polymerase II transcription factor TFIH (Schaeffer et al., 1993). u.v. light, ionising radiation and other DNA damaging agents lead to p53 stabilisation and a cell cycle arrest, during which the damage may be repaired (Hartwell and Kastan, 1994). This G1 arrest, which induces apoptosis in some cell types (Clarke et al., 1993) and can be prolonged in other cell types, even at low doses of ionising radiation (Di Leonardo et al., 1994), prevents cells from initiating DNA replication on a still-damaged template, which would lead to the fixation of mutations and the accumulation of DNA strand breaks.

ERCC1 was the first mammalian NER gene to be cloned (Westerveld et al., 1984). In a complex with XP-F (ERCC4), it makes the incision 5′ to the damage site. Although ERCC1 is essential for NER in vitro, an ERCC1 defect has not been identified in XP, or indeed in any known human disease. To study the consequences of ERCC1-deficiency in a whole animal system, we used gene targeting to inactivate the ERCC1 gene (Selfridge et al., 1992). Our ERCC1-deficient mice were severely runted and died before weaning with an unusual liver pathology (McWhir et al., 1993). At birth, some hepatocyte nuclei were already enlarged and polyploid; by three weeks of age, the frequency of cells with enlarged nuclei had increased and nuclei with both polyploid and aneuploid DNA content were apparent. These changes were associated with increased levels of p53 protein in some hepatocyte nuclei. ERCC1 knockout mice and animals with a C-terminal ERCC1 truncation have also been generated independently (Weeda et al., 1997). In addition to the same runting and liver pathology, these animals, which survived for up to six months, also showed additional changes to the skin, spleen and kidneys.

We, and Weeda and co-workers, have suggested that the phenotype seen in ERCC1-deficient mice is more reminiscent of the consequences of cell cycle arrest and premature ageing than NER deficiency per se. This view is reinforced by the striking similarity between the ERCC1-deficient phenotype and that reported for transgenic mice overexpressing the cyclin-dependent kinase inhibitor, p21cip1/waf1, in hepatocytes (Wu et al., 1996). p21cip1/waf1 is one of the main effectors of p53, being induced as part of the response to DNA damage, leading to cell cycle arrest at the G1/S checkpoint (el-Deiry et al., 1993). Furthermore, cells lacking (Waldman et al., 1996), or overexpressing, p21cip1/waf1 (Wu et al., 1996) arrest in G2 and show uncoupling of S phase and mitosis.

We have speculated that, in the obvious absence of any u.v. light-induced DNA damage, the cell cycle arrest in ERCC1-deficient mice might result from the inability of these mice to use the NER pathway to repair endogenously generated (oxidative) DNA damage (McWhir et al., 1993). In the interim, XP-A (de Vries et al., 1995; Nakane et al., 1995) and XP-C (Sands et al., 1995) knockout mice, have been generated with a phenotype that is not consistent with this hypothesis. They do not exhibit the growth arrest characteristic of ERCC1-deficiency, but do show the characteristic sensitivity to u.v. light found in XP patients.

The other possibility to explain the ERCC1-deficient phenotype that we have considered, and the one favoured by Weeda and co-workers, is that ERCC1, in common with many of the other NER proteins, may have an additional function and that it is the lack of this function, rather than the NER deficiency, that is responsible for the phenotype. One prime candidate for such an additional function for ERCC1 is in mitotic recombination. This is based on two observations: the homology between ERCC1 and the S. cerevisiae RAD10 protein, which is involved in both NER and mitotic recombination (Fishman-Lobell and Haber, 1992) and the hypersensitivity of ERCC1 (and ERCC4)-deficient cells, but not other ERCC mutants, to the DNA cross-linking agent, mitomycin C. (Interstrand cross links are believed to be repaired by a process involving mitotic recombination.)

To improve our understanding of the link between ERCC1-deficiency and cell cycle arrest, we have carried out studies on primary and immortalised embryonic fibroblast cultures from ERCC1-deficient mice and on a Chinese hamster ovary ERCC1 mutant cell line. ERCC1-deficient cell lines from both species showed the expected hypersensitivity to u.v. light, but the mouse mutant was considerably less sensitive to mitomycin C than its hamster equivalent. Both ERCC1-deficient cell lines had a mutation frequency 100-times higher than the species controls. ERCC1-deficient mouse embryos, primary fibroblast cultures and immortalised cell lines all had a higher level of genome instability than wild-type controls. Using both a plasmid recombination and a gene targeting assay, we were unable to detect an homologous recombination deficit in ERCC1-deficient cells from either species. However, the frequency of S-phase-dependent illegitimate chromosome exchange induced by u.v. light was dramatically reduced in both mutants. Primary fibroblasts from ERCC1-deficient mice showed the same growth rate, cell cycle distribution and p53 response following u.v. light as control cultures, with no indication of the polyploid cells found in hepatocytes in vivo.

MATERIALS AND METHODS

Cells

Primary fibroblasts were isolated from individual day 10.5 or 11.5 mouse embryos by a combination of maceration and trypsinisation. The resulting cell suspension was plated into 60 or 90 mm culture dishes, with cultures becoming confluent within 3 days. Spontaneously immortalised mouse fibroblast cell lines were isolated from senescing primary cultures. PF20 arose from a wild-type embryo; PF18 arose from an HPRT-deficient embryo; PF24 arose from an embryo that was both ERCC1-deficient and HPRT-deficient. ERCC1 genotyping of embryos and resulting cell lines was carried out as described (McWhir et al., 1993). The Chinese hamster ovary cell line, CHO-9, and its ERCC1 mutant derivative, 43-3B (Wood and Burki, 1982), were kindly provided by Rick Wood (ICRF, Clare Hall).

Cell culture and HPRT selection

Cells were grown in Glasgow-modified Eagle’s medium, supplemented with foetal calf serum (10% for primary cultures, 7% for other cell lines). Selection for HPRT expression was in HAT medium (Littlefield, 1964); selection against HPRT expression was in medium containing 5 μg/ml 6-thioguanine (6-TG).
Survival assays
Survival following u.v. (254 nm) irradiation or 24 hours exposure to mitomycin C was determined as described (McWhir et al., 1993). For the u.v. survival, cells were plated at 2×10^4/30 mm dish. For the mitomycin C survival, cells were plated in 24-well microtitre plates at 10^4/well.

Cell cycle distribution
Nuclei from primary mouse fibroblasts were prepared for flow cytometry and stained with propidium iodide as described (Vindelov et al., 1983). DNA content was determined by FACScan (5×10^4 nuclei analysed/sample). To identify cells in S-phase, cultures growing on glass cover slips, or in 30 mm dishes, were pulsed for 2 hours in medium containing 10 μM bromodeoxyuridine (BrdU). Cells were then fixed (methacarn for coverslips, cold ethanol for dishes), processed and incubated with an anti-BrdU monoclonal antibody (Harlan, Sera-Lab). Detection was with a biotinylated secondary anti-sheep antibody and a streptavidin/biotin peroxidase conjugate (Dako). Positively stained (i.e. S-phase) nuclei were scored by eye following photography of the cultures.

p53 determination
Total protein lysates were made from primary mouse embryonic fibroblast cultures as described (Lasko et al., 1990) and samples were run on 8% SDS-PAGE gels. Gels were western blotted using a semi- dry horizontal system (Bio-Rad). p53 was detected using a p53 (Ab- complex/horseradish peroxidase conjugate (Dako). Detection of micronuclei
Cells growing on glass coverslips were treated in mild hypotonic solution (0.81% NaCl + 0.056% KCl; Iskandar, 1979) for 10 minutes before being fixed in 90% methanol. Centromere-containing micronuclei were identified as described previously (Nuesse et al., 1989) by treating coverslips with CREST antiserum (Antibody Inc., Davis, CA, USA); FITC-conjugated goat anti-human IgG (Sigma) was used as the secondary antibody. Micronuclei and nuclei were counterstained with propidium iodide. The numbers of CREST+ and CREST– micronuclei present (expressed/10^3 cells) were determined by direct observation under a fluorescence microscope at ×1,000 magnification.

Plasmid homologous recombination assay
The construction and expression of the functional mouse Hprt minigene used for this assay, pBt/PGK-HPRT(RI), have been described (Magin et al., 1992). In the referenced publication this minigene was described as PGK/pDWM1. The 2.7 kb minigene is under the control of the mouse phosphoglycerate kinase (PGK-1) gene promoter. The minigene contains part of the Hprt 5¢ untranslated region, the entire coding region and 3¢ untranslated region, with the coding region being interrupted by introns 7 and 8. The minigene was excised from PGK/pDWM1 as an EcoRI fragment and cloned into the EcoRI site of pBluescript II SK(+) (Stratagene), to give pBt/PGK-HPRT(RI).

The two substrates for the plasmid homologous recombination assay were derived from pBT/PGK-HPRT(RI). A 5¢ deletional derivative, pBT/PGK- HPRT (XhoI), was constructed by excising the 3¢ 1.9 kb of the minigene as an XhoI (site within exon 3)-EcoRI (site at 3¢ end of minigene) fragment and recloning it into pBluescript II SK (+) cut with XhoI/EcoRI. A 3¢ deletional derivative, pBT/PGK-HPRT (Scal-EcoRVΔ), was constructed by restricting pBT/PGK- HPRT (RI) with Scal (site within exon 8) and EcoRV (site at 3¢ end of minigene), to liberate the 3¢ end of the minigene, and then religating the plasmid. The deletional derivatives are both non-functional, but share a 0.6 kb region of homology, extending from the XhoI site in exon 3 to the Scal site in exon 8. Homologous recombination between these regions of the two plasmids will generate a functional minigene. Plasmids were introduced into Hprt-deficient cultured cells by the calcium phosphate method (Graham and Van der Eb, 1973), with HAT selection for Hprt expression (Littlefield, 1964) imposed 24 hours later.

Gene targeting assay
The basis of the Hprt gene targeting assay has been described (Thompson et al., 1989). The Hprt-deficient mouse fibroblasts used here have the same defective Hprt allele, where a spontaneous deletion has removed the promoter region and exons 1 and 2. The Hprt correction vector, pDWM102, can be used to correct this deficiency by homologous recombination. The vector contains the 5¢ end of the Hprt gene, comprising the promoter and exons 1-3. The vector is cut with XhoI to liberate plasmid sequences prior to electroporation. The XhoI site is within a region of homology between the vector and the target locus. Insertion of the vector into the target locus will restore gene function, with correctant clones being directly selected in HAT medium. Random integrations of the vector will not yield HAT® survivors. Conditions for electroporation were as described (Thompson et al., 1989), with HAT selection added 24 hours later.

RESULTS
ERCC1-deficient immortalised mouse fibroblasts are hypersensitive to u.v. light but only moderately sensitive to mitomycin C
We have previously reported that primary fibroblasts, isolated from day 10.5 ERCC1-deficient mouse embryos, were hypersensitive to u.v. light with virtually no demonstrable nucleotide excision repair, but were only moderately sensitive to the DNA cross-linking agent, mitomycin C (McWhir et al., 1993). Similar observations have been made on primary embryonic fibroblasts from independently generated Ercc1 knockout mice and mice with a C-terminal Ercc1 truncation (Weeda et al., 1997). To permit extended duration assays on cultured ERCC1-deficient mouse cells, we isolated spontaneously immortalised lines from ERCC1-deficient (PF24) and wild-type (PF20) primary embryonic fibroblast cultures. The immortalised ERCC1-deficient mouse lines retained the expected hypersensitivity to u.v. light, with a very similar survival curve to the ERCC1-deficient Chinese hamster ovary cell line, 43-3B (see Fig. 1). Control mouse (PF20) and hamster (CHO-9) cell lines gave the same response to mitomycin C, with a D50 value (concentration of mitomycin C for 50% survival) of 310 ng/ml for PF20 and 325 ng/ml for CHO-9 (see Fig. 2). The hamster ERCC1 mutant cell line (43-
3B) was 40-fold more sensitive to mitomycin C (D$_{50}$ 7.5 ng/ml) than the control hamster cell line, while the mouse ERCC1-deficient cell line (PF24) was only 8-fold more sensitive (D$_{50}$ 41 ng/ml) than its species control.

Thus, immortalised cell lines with the predicted nucleotide excision repair deficiency can be isolated from ERCC1-deficient mouse embryos for further analysis. The different response of ERCC1-deficient mouse and hamster cells to mitomycin C suggests that an alternative (i.e. non-ERCC1-dependent) pathway for the repair of DNA interstrand cross-links may operate in mouse, but not hamster cells.

**ERCC1-deficient mouse and Chinese hamster cells have a high spontaneous mutation frequency**

The frequency with which mutants at the X-chromosome-linked hypoxanthine phosphoribosyltransferase (HPRT) locus arise in cell cultures provides an indication of the mutation rate. HPRT mutants are conveniently identified by their ability to grow in the presence of the purine analogue, 6-thioguanine (6-TG). The spontaneous frequency of 6-TG$^R$ was higher than 90-fold higher in the CHO ERCC1 mutant (43-3B) than in its parental wild-type line (CHO-9) (see Table 1). An elevated mutation rate for ERCC1 mutant cells (Wood and Burki, 1982) and for Chinese hamster cells where the ERCC1 gene has been inactivated by gene targeting (Rolog et al., 1997) have been previously reported.

The immortalised ERCC1-deficient mouse fibroblast line (PF24) was isolated from an HPRT$^{-}$-deficient embryo. This was because ERCC1 gene targeting was originally carried out in the HPRT$^{-}$-deficient embryonic stem (ES) cell line, HM-1 (Selfridge et al., 1992), with the result that the HM-1-derived HPRT$^{-}$ null allele is segregating in our ERCC1 knockout mouse stock. While the HPRT deficiency was ideal for a comparison of homologous recombination frequency between wild-type and ERCC1-deficient mouse cell lines (see later section), it prohibited the same simple comparison of HPRT mutation frequency used on the CHO cell lines. However, the HM-1-derived HPRT null allele can be corrected by gene targeting to restore function to the endogenous HPRT gene (see later section). HPRT correction was carried out in ERCC1-deficient (PF24) cells and in another immortalised mouse embryonic fibroblast line (PF18), which was ERCC1 wild type and HPRT-deficient. The spontaneous frequency of 6-TG$^R$ was determined in independent HPRT correctants of PF18 and PF24 (see Table 1). The mean 6-TG$^R$ frequency for the PF24 correctants was 180-fold higher than for their PF18 equivalents. Thus, both ERCC1-deficient mouse and hamster cell lines have a high spontaneous mutation frequency.

**ERCC1-deficient mouse fibroblasts show increased genomic instability**

The high spontaneous HPRT mutation frequency found in ERCC1-deficient cells could arise from local events, such as point mutations or deletions, and also from genome-wide events, such as chromosome breakage and loss. To assess the latter events, the frequency of micronuclei was determined in both primary and immortalised, wild-type and ERCC1-deficient, mouse fibroblast cultures. Micronuclei were divided into two categories: those containing whole chromosomes (or, more rarely, centromeric fragments), which stained with CREST antibodies (directed against kinetochores), and arose more rarely, centromeric fragments), which stained with CREST antibodies (directed against kinetochores), and arose more rarely, centromeric fragments), which stained with CREST antibodies (directed against kinetochores), and arose more rarely, centromeric fragments), which stained with CREST antibodies (directed against kinetochores), and arose.
immortalised wild-type and ERCC1-deficient cultures, with the total number of micronuclei being 3-fold higher in mutant than control cultures. Both chromosome loss and chromosome breakage were significantly elevated in the mutant cell line, with the elevation in chromosome breakage (5-fold) being particularly pronounced.

The effect of u.v. light on the frequency of micronuclei in pre-crisis primary mouse fibroblast cultures was also determined (see Table 3). From 48–72 hours after u.v. light, the frequency of micronuclei was significantly elevated for both irradiated wild-type and ERCC1-deficient cultures, compared to non-irradiated controls. The increase in total micronuclei following u.v. light was significantly higher for ERCC1-deficient than wild-type cultures, with both chromosome loss and chromosome breakage being significantly elevated.

Thus, developing ERCC1-deficient mouse embryos have a significantly higher spontaneous incidence of genome instability than control embryos. This difference becomes progressively more pronounced on extended primary culture and in immortalised cell lines. u.v. light also causes increased genome instability in ERCC1-deficient cultures compared to controls.

### The homologous recombination frequency is not reduced in immortalised ERCC1-deficient cells

The homology between ERCC1 and the *S. cerevisiae* RAD10 protein, which is involved in mitotic recombination, and the mitomycin C sensitivity of ERCC1-deficient cell lines, has led to the suggestion that the phenotype of ERCC1-deficient mice could arise from defective mitotic recombination, rather than defective nucleotide excision repair (Weeda et al., 1997). To address this question, we have used two assays to compare the frequency of homologous recombination in ERCC1-deficient and control mouse and hamster cells.

The first assay measured the ability for homologous recombination between two plasmids, introduced into the same cell, using an HPRT selection assay. The HPRT-deficient mouse cell lines PF18 (*ERCC1* wild-type) and PF24 (*ERCC1*-deficient)

### Table 1. The spontaneous frequency of HPRT mutations is elevated in immortalised ERCC1-deficient cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell line</th>
<th>HPRT+ colonies/10^5 cells</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster wild type</td>
<td>CHO-9</td>
<td>0.3±0.3 (n=10)</td>
<td></td>
</tr>
<tr>
<td>Hamster <em>ERCC1</em> mutant</td>
<td>43-3B</td>
<td>23.3±2.6 (n=10)(93±10)</td>
<td></td>
</tr>
<tr>
<td>Mouse wild type</td>
<td>PF18/DWM102#1</td>
<td>4.9±1.3 (n=10)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>PF18/DWM102#2</td>
<td>1.7±0.5 (n=10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF18/DWM102#3</td>
<td>3.2±2.1 (n=10)</td>
<td></td>
</tr>
<tr>
<td>Mouse <em>ERCC1</em> −/−</td>
<td>PF24/DWM102#1</td>
<td>1592±152 (n=2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF24/DWM102#2</td>
<td>61.7±4.5 (n=3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF24/DWM102#4</td>
<td>19.7±15.1 (n=3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF24/DWM102#5</td>
<td>95.2±48 (n=2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PF24/DWM102#6</td>
<td>85.7±7.9 (n=5)</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

*Cultures were maintained in HAT medium to prevent the accumulation of pre-existing HPRT mutants, before being transferred to non-selective medium for 8 days to allow HPRT mutants to accumulate. Cells were then plated (10^5 cells/90 mm dish) in 6-thioguanine (5 μg/ml) to determine the number of HPRT mutants.

†For each cell line, the number of 6-thioguanineR colonies/10^5 cells is given, ± s.d.; n is the number of individual determinations. The mean frequency of 6-TG® colonies is also given for the PF18 and PF24 correctant series.

‡The increase in the frequency of HPRT mutants in *ERCC1*-deficient cells, relative to the wild-type species control, is given in square brackets.

controls (see Table 2), the difference being confined to CREST+ micronuclei, arising from chromosome loss. The spontaneous frequency of micronuclei was also determined after extensive in vitro cell division, but prior to the characteristic crisis that primary cultures undergo. The frequency of micronuclei was higher in this pre-crisis material than in the day one cultures (see Table 2), with the frequency again being significantly higher in *ERCC1*-deficient cultures than in controls. In this case, both chromosome loss (CREST+ micronuclei) and chromosome breakage (CREST− micronuclei) were significantly elevated in the mutant cultures. A still higher frequency of micronuclei was found in the immortalised *ERCC1*-deficient primary fibroblasts, with the total number of micronuclei being 3-fold higher in mutant than control cultures. Both chromosome loss and chromosome breakage were significantly elevated in the mutant cell line, with the elevation in chromosome breakage (5-fold) being particularly pronounced.

### Table 2. The spontaneous frequency of micronucleus formation is higher in ERCC1-deficient mouse primary fibroblasts and immortalised fibroblasts

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cell culture</th>
<th>Cells scored</th>
<th>Total micronuclei no. (MN/10^5 cells)</th>
<th>CREST+ MN no. (MN/10^5 cells)</th>
<th>CREST− MN no. (MN/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>Day 1</td>
<td>11,132</td>
<td>197 (17.7)</td>
<td>122 (11.0)</td>
<td>75 (6.7)</td>
</tr>
<tr>
<td><em>ERCC1</em> −/−</td>
<td>Day 1</td>
<td>14,698</td>
<td>372 (25.3)</td>
<td>250 (17.0)</td>
<td>122 (8.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.0001‡</td>
<td>P&lt;0.0001</td>
<td>n.s.‡</td>
</tr>
<tr>
<td>Wild type</td>
<td>Pre-crisis</td>
<td>6,161</td>
<td>183 (29.7)</td>
<td>114 (18.5)</td>
<td>69 (11.2)</td>
</tr>
<tr>
<td><em>ERCC1</em> −/−</td>
<td>Pre-crisis</td>
<td>9,922</td>
<td>451 (45.5)</td>
<td>262 (26.4)</td>
<td>189 (19.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Wild type</td>
<td>Immortalised</td>
<td>4,214</td>
<td>157 (37.3)</td>
<td>122 (29.0)</td>
<td>35 (8.3)</td>
</tr>
<tr>
<td><em>ERCC1</em> −/−</td>
<td>Immortalised</td>
<td>3,598</td>
<td>426 (118.4)</td>
<td>281 (78.1)</td>
<td>145 (40.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
<td>P&lt;0.0001</td>
</tr>
</tbody>
</table>

*The total number of micronuclei, and the number of CREST+ micronuclei (indicating chromosome loss) and CREST− micronuclei (indicating chromosome breakage), were determined for primary fibroblasts isolated from 10.5 day wild-type and *ERCC1*-deficient embryos. The determination was made 1 day after plating (before in vitro cell division) and after in vitro cell division had commenced, on pre-crisis cultures at passage 3. The day 1 data come from 4 wild-type and 7 *ERCC1*-deficient embryos, the pre-crisis data are from single wild-type and *ERCC1*-deficient primary cultures. The determination was also made on immortalised wild-type (PF20) and *ERCC1*-deficient (PF24) fibroblasts.

†‡χ²-test on frequency of micronucleus formation, *ERCC1* −/− versus wild-type control.

‡‡n.s., not significant.
Table 3. The u.v.-induced frequency of micronucleus formation is higher in ERCC1-deficient primary fibroblasts*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>U.V. dose (Jm⁻²)</th>
<th>Cells scored</th>
<th>Total micronuclei no. (MN/10⁶ cells)</th>
<th>CREST+ MN no. (MN/10⁶ cells)</th>
<th>CREST– MN no. (MN/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>6,161</td>
<td>183 (29.7)</td>
<td>114 (18.5)</td>
<td>69 (11.2)</td>
</tr>
<tr>
<td>Wild type</td>
<td>2.5</td>
<td>3,408</td>
<td>206 (60.4)</td>
<td>98 (28.8)</td>
<td>108 (31.7)</td>
</tr>
<tr>
<td>ERCC1 --/--</td>
<td>0</td>
<td>9,922</td>
<td>451 (45.5)</td>
<td>262 (26.4)</td>
<td>189 (19.0)</td>
</tr>
<tr>
<td>ERCC1 --/--</td>
<td>2.5</td>
<td>5,150</td>
<td>535 (103.8)</td>
<td>282 (54.8)</td>
<td>253 (49.1)</td>
</tr>
<tr>
<td>P&lt;0.0001†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The frequency of micronuclei was determined for control primary cultures (pre-crisis at passage 3) of wild-type (PF20) and ERCC1-deficient (PF24) mouse fibroblasts and for cultures 48-72 hours after 2.5 Jm⁻² of u.v. irradiation.
†X²-test on u.v.-induced frequencies (control subtracted), ERCC1 --/-- (60.4–29.7 = 30.7) versus wild type (103.8–45.5 = 58.3).

and non-reverting HPRT-deficient derivatives of the hamster cell lines CHO-9 (ERCC1 wild-type) and 43-3B (ERCC1-deficient) were used. HPRT-deficient cells incorporating the functional HPRT minigene, pBT/PGK-HPRT(R), can be selected in HAT medium. Two non-functional but non-overlapping deletional derivatives of the minigene were used. No HAT colonies were obtained when either minigene was introduced singly into any of the HPRT-deficient cell lines. However, if co-introduced, homologous recombination between the plasmids would reconstruct a functional minigene and HAT colonies would arise. The plasmids were introduced in supercoiled form with the presumption that any ERCC-1 requirement for homologous recombination might involve its endonuclease function. The frequency of homologous recombination observed between plasmids in the four cell lines gave no indication of an homologous recombination deficit in ERCC1-deficient mouse or hamster cells (data not shown).

The second assay for homologous recombination measured the frequency of HPRT gene targeting in the HPRT-deficient mouse cell lines PF18 (wild-type) and PF24 (ERCC1-deficient). Both contained the HM-1-derived HPRT null allele, which is deleted for the 5' end of the gene (Thompson et al., 1989). We have previously used an HPRT correction vector, pDWM102, to correct this allele in ES cells (Thompson et al., 1989). The same procedure was applied to PF18 and PF24. It could not be used on the HPRT-deficient derivatives of CHO-9 and 43-3B because they do not contain the equivalent HPRT mutation. The correction vector was linearised prior to gene transfer. This was essential, despite wishing to investigate the possible requirement for the endonuclease function of ERCC1 in homologous recombination, in order to obtain a measurable gene targeting frequency.

In Table 4, the frequency of HPRT gene targeting is expressed as the frequency of HAT colonies obtained with the correction vector, relative to the frequency of HAT colonies obtained in parallel experiments with a functional minigene. This introduces an important correction for any differences in gene transfer frequency between the cell types. The same gene targeting frequency (1.6 × 10⁻⁵) was obtained for both control and ERCC1-deficient cell types.

Thus, by both of these assays, there was no evidence for any homologous recombination deficit in ERCC1-deficient cells.

Table 4. The frequency of homologous recombination is not reduced in immortalised ERCC1-deficient mouse cells*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Homologous recombination (gene targeting) frequency‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse wild type</td>
<td>1.6×10⁻⁵±1.2×10⁻⁵ (n=4)</td>
</tr>
<tr>
<td>Mouse ERCC1 --/--</td>
<td>1.6×10⁻⁵±1.2×10⁻⁵ (n=4)</td>
</tr>
</tbody>
</table>

*HPRT-deficient cells, an HPRT minigene and an HPRT gene correction (gene targeting) vector were used in this study, with selection in HAT medium for HPRT expression to identify homologous recombination events. The wild-type (PF18) and ERCC1-deficient (PF24) immortalised mouse fibroblast lines, which were isolated from HPRT-deficient embryos, were used.
‡The gene targeting frequency was determined by introducing the linearised HPRT correction vector pDWM102 into HPRT-deficient mouse cells and counting the number of HAT colonies arising as a result of homologous recombination between the correcting vector and the chromosomal HPRT gene. This was expressed relative to the number of HAT colonies obtained with the minigene pBT/PGK-HPRT(RI) in each cell line. For each determination, 1.5×10⁶ cells were electroporated with 170 µg of DNA (linearised pDWM102 or supercoiled pBT/PGK-HPRT[RI]) and plated into 90 mm dishes (1×10⁶ cells for gene targeting, 3×10⁴ for gene transfer).

ERCC1-deficient primary mouse fibroblasts have a normal cell cycle distribution, growth rate and p53 response

The runted growth and polyploid liver cell phenotype in our ERCC1-deficient mice was more reminiscent of defective cell cycle progression and premature ageing than a simple consequence of DNA repair deficiency. This impression was reinforced by the very similar phenotype reported for transgenic mice overexpressing the cyclin-dependent kinase inhibitor, p21<sup>CIP1/WAF1</sup>, in the liver (Wu et al., 1996). A study on primary embryonic fibroblasts was carried out to see if the profound in vivo abnormality associated with ERCC1 deficiency was amenable to an in vitro analysis.

Primary cultures isolated from day 11.5 wild-type and ERCC1-deficient mouse embryos had the same growth rate, with a generation time of ~36 hours (data not shown). Following a 2 hour pulse with BrdU, to identify cells in S-phase, 42±5% of cells from two independent wild-type cultures showed nuclei stained with an anti-BrdU antibody. The corresponding value for two independent ERCC1-deficient cultures of the same passage (passage 2) was 44±7%, confirming the similar cell cycle characteristics. Following an extended 2 day pulse with BrdU, 90% of cells from the same wild-type cultures showed stained nuclei. The corresponding value for the equivalent ERCC1-deficient cultures was 87%, indicating that, in both cultures, most of the cells are actively cycling.

FACS analysis of wild-type and ERCC1-deficient cultures at passage 2 showed the same cell cycle distribution (see Fig. 3). Cultures of both genotypes senesced rapidly and, by passage 3, a discrete population of (presumably apoptotic) cells, with a sub-G<sub>1</sub> DNA content, was present to an equal extent.
Significantly, there was no indication in the ERCC1-deficient cultures of cells with a polyploid DNA content that would correspond to the in vivo situation in ERCC1-deficient liver.

Elevated levels of p53 protein were detected in isolated individual nuclei from ERCC1-deficient mice by immunohistochemistry (McWhir et al., 1993). To investigate the p53 response in vitro, the level of p53 protein present in control cultures of wild-type and ERCC1-deficient primary mouse embryonic fibroblasts was determined by western blotting. Contrary to the in vivo situation, p53 was readily detectable in wild-type extracts and was not elevated in ERCC1-deficient extracts (see Fig. 4). Both cultures showed the classic stabilisation of p53 response following u.v. irradiation. The kinetics of the p53 response were equivalent in the two cell types (data not shown).

Thus, the G2 cell cycle arrest seen in ERCC1-deficient hepatocytes in vivo is not reproduced in untreated cultured primary fibroblasts and there is no evidence for an altered p53 response.

The frequency of S-phase-dependent chromatid exchange caused by u.v. light is reduced in immortalised ERCC1-deficient cells

Having failed to detect a homologous recombination deficit in ERCC-1 mutant cells, we investigated whether ERCC-1 mutations affected another recombination pathway responsible for the production of S-phase-dependent chromatid exchanges after u.v.-induced DNA damage. In hamster cells chromatid exchanges are a common consequence of u.v. irradiation (Bender et al., 1973; Darroudi and Natarajan, 1985), or after treatment with camptothecin, the S-phase-specific cytotoxic inhibitor of DNA topoisomerase I (Degrassi et al., 1989; Ryan et al., 1994). Through the action of DNA replication both u.v. irradiation and camptothecin induce the formation of DNA double strand breaks predominantly in the replicating fraction (Wang and Smith, 1986; Musk et al., 1990; S. Squires, J. A. Coates and R. T. Johnson, unpublished data; Avemann et al., 1988; Ryan et al., 1991, 1994), and these breaks are the likely precursors of the S-phase-dependent strand exchanges necessary for the development of chromatid exchanges.

The frequency of these S-phase-dependent chromatid exchanges, and also the frequency of chromatid breaks, were determined in immortalised wild-type and ERCC1 mutant mouse and hamster cell lines (see Table 5). In agreement with the frequency of micronuclei, following equitoxic doses of u.v. light, the number of chromatid breaks/cell was higher in both ERCC1 mutant cell lines than their wild-type species controls. However, the number of chromatid exchanges/cell was considerably (more than 6-fold) reduced in both mutant lines, to the extent that the ratio of chromatid exchanges/breaks was over 10-fold greater for both wild-type cell lines compared to the ERCC1 mutants.

This indicates that the ERCC1 protein may be playing an important role on replicating DNA after damage, promoting the illegitimate recombination reaction between S-phase chromosomes, and thus limiting the frequency of long-lived DNA breaks which give rise to chromatid breaks.
DISCUSSION

We have undertaken studies on cultured ERCC1-deficient cells to investigate whether it is a deficiency in nucleotide excision repair, in homologous recombination, or in some additional function of ERCC1, that is responsible for the unusual growth retardation/cell cycle arrest/premature ageing phenotype seen in ERCC1-deficient mice. We also sought to develop a cell culture system to study the mechanism of this cell cycle arrest.

Both primary (McWhir et al., 1993; Weeda et al., 1997) and immortalised (this report) fibroblasts derived from ERCC1-deficient mouse embryos were hypersensitive to u.v. light, with virtually no detectable NER. Although ERCC1 mutant Chinese hamster ovary cells were hypersensitive to the DNA cross linking agent, mitomycin C, ERCC1-deficient mouse cell lines were only moderately sensitive, indicating the existence of a second, non-ERCC1-dependent, homologous recombination pathway for the repair of DNA interstrand cross links.

Much of the ERCC1-deficient phenotype, both in vivo and in vitro, can potentially be attributed to the lack of these two repair functions. Spontaneous levels of endogenously generated oxidative damage in mammalian cells are constantly producing bulky DNA lesions. In addition, metabolic by-products, such as malondialdehyde, cause interstrand cross-links (Chaudhary et al., 1994). Both types of damage are normally dealt with in repair-proficient cells by NER and a repair system involving homologous recombination. In ERCC1-deficient cells, replication on an unrepaired template would result in the fixation of mutations and the accumulation of double strand DNA breaks. The elevated HRPT mutation frequency that we have observed in ERCC1-deficient cells and the increased genome instability, both in vivo and in primary and immortalised ERCC1-deficient cultures are compatible with this explanation. Furthermore, the DNA damage would trigger the p53 response, leading to the elevated p53 levels and cell cycle arrest, that we observe in vivo.

Contrary to the situation with ERCC1-deficient-mice, XP-A (de Vries et al., 1995; Nakane et al., 1995) and XP-C (Sands et al., 1995) knockout mice have proved to be a good model for xeroderma pigmentosum, showing the characteristic skin sensitivity to u.v. light and a high frequency of induced skin tumours, but with no evidence of growth retardation and premature ageing. Significantly, the XP-A and XP-C proteins are involved in NER, but not in mitotic recombination. Thus, as suggested by Weeda et al. (1997), the phenotype of ERCC1-deficient mice seems more likely to be due to a mitotic recombination, rather than an NER deficit.

However, using two different assays, we have found no evidence for any homologous recombination deficit in ERCC1-deficient mouse and hamster cells and none has been reported by Weeda and co-workers. This does not mean that ERCC1 is not involved in some homologous recombination pathway, merely that it is not essential for homologous recombination. Our data on mitomycin C sensitivity are compatible with this explanation. Indeed, the S. cerevisiae ERCC1 homologue, RAD10, is also involved in, but not essential for mitotic recombination (Schiestl and Prakash, 1990). The relative contribution of the NER and mitotic recombination deficits to the ERCC1-deficient phenotype will be resolved when the types of lesions which are believed to accumulate in ERCC1-deficient cells are identified directly.

We did find evidence for a profound alteration in S-phase-dependent illegitimate recombination in ERCC1-deficient cells, which we take to indicate an additional function for the ERCC1 protein. Despite the arrest of the cell cycle in hamster cells by u.v. (Orren et al., 1995), the induced delay is much less extensive than with human cells (Wang and Ellem, 1994; S. Squires, J. F. Gimenez-Abian, R. T. Johnson, unpublished data). One consequence of this difference is that replication of damaged S-phase cells recovers and proceeds more strongly in hamster than in human cells on an incompletely repaired template (Waters, 1979; Spivak and Hanawalt, 1992). However, it is likely that the nascent DNA double strand breaks that are generated at the sites of transient replication arrest in rodent cells are rapidly resolved by illegitimate recombination and that this results in the production of S-phase-dependent chromatid exchanges. Support for this data comes from results with the DNA topoisomerase I inhibitor, camptothecin. Here, the DNA double strand breaks that are induced by the drug in hamster replication forks are rapidly repaired, the cells move out of S-phase, but now they contain abundant S-phase-dependent chromatid exchanges (Ryan et al., 1994). The short-term gain of cycle progress is rapidly succeeded by cell death as the cells are unable to divide accurately. As expected, metaphases collected following u.v. irradiation of ERCC1-deficient cells showed increased levels of chromatid breaks. Of greater interest was the profound reduction in the level of chromatid exchange in the ERCC1-deficient cells, such that the ratio of exchanges to breaks was 10-fold greater for wild-type mouse and hamster cells than their ERCC1-deficient equivalents. We interpret this to indicate that, in addition to its role in NER and homologous recombination, ERCC1 also plays a key role in promoting chromatid exchanges in rodent cells, by its involvement in an illegitimate recombination process, which operates at u.v.-induced lesion sites, to channel the disappearance of DNA double strand breaks and thus allow.

### Table 5. The frequency of S-phase-dependent chromatid exchange caused by u.v. light is reduced in immortalised ERCC1-deficient cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Metaphases scored</th>
<th>Total chromosome exchanges</th>
<th>Exchanges /cell</th>
<th>Total chromatid breaks</th>
<th>Breaks /cell</th>
<th>Exchanges /breaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse wild type</td>
<td>51</td>
<td>176</td>
<td>3.45</td>
<td>25</td>
<td>0.49</td>
<td>7.04</td>
</tr>
<tr>
<td>Mouse ERCC1+/−</td>
<td>64</td>
<td>31</td>
<td>0.48</td>
<td>46</td>
<td>0.72</td>
<td>0.67</td>
</tr>
<tr>
<td>Hamster wild type</td>
<td>58</td>
<td>94</td>
<td>1.6</td>
<td>15</td>
<td>0.26</td>
<td>6.15</td>
</tr>
<tr>
<td>Hamster ERCC1 mutant</td>
<td>56</td>
<td>15</td>
<td>0.27</td>
<td>42</td>
<td>0.75</td>
<td>0.36</td>
</tr>
</tbody>
</table>

*Exponentially growing cultures of immortalised cell lines were irradiated with equitoxic doses of u.v. and the subsequent metaphases were collected and scored. For the wild-type hamster (CHO-9) and mouse (PF20) cell lines, the u.v. dose was 6.5 Jm⁻²; for the ERCC1-deficient hamster (43-3B) and mouse (PF24) cell lines, the u.v. dose was 1.5 Jm⁻².
replication and the cell cycle to proceed. The profound G2 arrest observed in ERCC1-deficient mice, could then be thought to arise as a response to the accumulation of double strand breaks during S-phase at the sites of unrepaired DNA lesions. Further rounds (or partial rounds) of DNA replication, in the absence of cell division, would result in the characteristic enlarged polyploid and aneuploid hepatocyte nuclei seen in our mice. Additional experiments to investigate the role of ERCC1 during DNA replication are planned.

The cell cycle arrest phenotype observed in ERCC1-deficient hepatocytes in vivo is strikingly similar to that in transgenic mice overexpressing the cyclin-dependent kinase inhibitor, p21cip1/waf1, in the liver (Wu et al., 1996). We have obtained preliminary evidence (data not shown) that p21cip1/waf1 mRNA levels are indeed elevated in ERCC1-deficient liver compared to wild-type controls. We and Weeda et al. (1997) have used primary embryonic fibroblast cultures to study the link between ERCC1-deficiency and the cell cycle arrest/senescence phenotype in vitro. Our ERCC1-deficient cultures, isolated from day 11.5 embryos, had a similar growth rate to control cultures, with BrdU pulses indicating that both cultures had the same frequency of cells in S-phase. The cell cycle distribution was identical in both cultures, with both senescing rapidly on subsequent passages. Significantly, FACS analysis on ERCC1-deficient cultures provided no evidence for the presence of the polyploid cells that are so characteristic of ERCC1-deficient hepatocytes in vivo. Contrary to the situation in vivo, p53 protein was readily detectable by western blotting in untreated cultures of both genotypes and both showed an equivalent p53 stabilisation response to u.v. irradiation. The high levels of p53 protein found in wild-type primary embryonic fibroblast cultures, compared to the in vivo situation in untreated normal tissues, where p53 protein is only just detectable, indicates the limitations of this in vitro system to study the role of ERCC1 deficiency in cell cycle arrest.

Weeda et al. (1997) have reported very different results from their studies of primary fibroblast cultures, established from day 12-13 mouse embryos with the C-terminal ERCC1 truncation. ERCC1-deficient cultures had a 3-fold longer generation time than wild-type cultures, with only half as many cells cycling. ERCC1-deficient cultures were also reported to senesce more rapidly and accumulate polyploid cells (although no comparative FACS data of wild-type and mutant cultures were shown). p21cip1/waf1 protein levels were also reported to be elevated in mutant cultures. It is difficult to reconcile the different results obtained. Cultures were isolated from different strains of ERCC1-targeted mice, but the in vivo phenotype of these animals is very similar. Culture conditions and different stage embryos used for the isolation could also have an effect. Additional analysis is needed to reconcile these differences. However, we would point out the following in support of our results that ERCC1-deficient cell cultures are not intrinsically inviable. ERCC1-deficient mouse embryos, from which primary fibroblast cultures are isolated, do develop to term (albeit with reduced viability) to produce live (but runted) pups after the normal gestation period. Immortalised ERCC1-deficient mouse fibroblasts and ERCC1 mutant CHO cells grow well in culture, only marginally slower than their wild-type controls.

In conclusion, we favour the following model to explain the growth arrest and early senescence seen in ERCC1-deficient mice. ERCC1 is essential for NER and is involved in, but is not essential for homologous recombination. In addition, ERCC1’s role in recombination is used by rodents to enhance a more promiscuous exchange behaviour, as part of a rodent-specific mechanism, which is adapted to the low overall level of NER in these cells, to prevent the accumulation of double strand breaks adjacent to unrepaired lesions on replicating DNA. In the absence of ERCC1, the failure of the illegitimate recombination process leads to the accumulation of double strand breaks following replication. This triggers the p53 response and the G2 cell cycle arrest, mediated by increased expression of the cyclin-dependent kinase inhibitor p21cip1/waf1. The increased levels of unrepaired lesions and double strand breaks would explain the increased mutation frequency and increased genome instability that we have observed.

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