DNA ligase I null mouse cells show normal DNA repair activity but altered DNA replication and reduced genome stability

Darren J. Bentley[1], Caroline Harrison[2], Ann-Marie Ketchen[2], Nicola J. Redhead[2], Kay Samuel[3], Martin Waterfall[4], John D. Ansell[4] and David W. Melton[2,*]

[1]Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, UK
[2]Sir Alastair Currie CRC Laboratories, Molecular Medicine Centre, University of Edinburgh, Edinburgh EH4 2XU, UK
[3]Cell Therapy Group, Scottish National Blood Transfusion Service, Edinburgh EH4 2XU, UK
[4]John Hughes Bennett Laboratory, Department of Oncology, University of Edinburgh, Edinburgh EH4 2XU, UK
*Author for correspondence (e-mail: David.Melton@ed.ac.uk)

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Summary

DNA ligase I is the key ligase for DNA replication in mammalian cells and has also been reported to be involved in a number of recombination and repair processes. Our previous finding that *Lig1* knockout mouse embryos developed normally to mid-term before succumbing to a specific haematopoietic defect was difficult to reconcile with a report that DNA ligase I is essential for the viability of cultured mammalian cells. To address this issue, we generated a second *Lig1* targeted allele and found that the phenotypes of our two *Lig1* mutant mouse lines are identical. Widely different levels of *Lig1* fusion transcripts were detected from the two targeted alleles, but we could not detect any DNA ligase I protein, and we believe both are effective *Lig1* null alleles. Using foetal liver cells to repopulate the haematopoietic system of lethally irradiated adult mice, we demonstrate that the haematopoietic defect in DNA-ligase-I-deficient embryos is a quantitative deficiency relating to reduced proliferation rather than a qualitative block in any haematopoietic lineage. DNA ligase I null fibroblasts from *Lig1* mutant embryos showed an accumulation of DNA replication intermediates and increased genome instability. In the absence of a demonstrable deficiency in DNA repair we postulate that, unusually, genome instability may result directly from the DNA replication defect. *Lig1* null mouse cells performed better in the survival and replication assays than a human *LIG1* point mutant, and we suggest that the complete absence of DNA ligase I may make it easier for another ligase to compensate for DNA ligase I deficiency.

Key words: DNA ligases, DNA repair, DNA replication, Genome instability

Introduction

The requirement for DNA ligase activity is common to all pathways in mammalian cells by which DNA is replicated or rearranged or genomic integrity is maintained. Three distinct DNA ligase genes have been identified in humans (*LIG1*, *LIG3* and *LIG4*). However, the division of roles between the encoded proteins has not yet been completely defined. Formerly it had been suggested that DNA ligase I is involved in a number of DNA replication, recombination and repair processes, but recent evidence has led to a re-evaluation of the role of this protein (for a review, see Timson et al., 2000).

Inherited human disorders that result in chromosome instability and cancer predisposition have been central to the understanding of many mammalian DNA metabolism and repair pathways. The paradigm for genome instability disorders is Bloom's syndrome (BS). Patients with BS have growth retardation, immune deficiency and are predisposed to a range of cancers, and cells from BS patients show a very high level of sister chromatid exchange, increased mutation and somatic recombination rate and increased sensitivity to a range of DNA damaging agents (German, 1993). Although BS is caused by mutations in the *BLM* helicase gene (Ellis et al., 1995), mutations in *LIG1* were identified in a patient with similar physical retardation, immune deficiency and hypersensitivity to sunlight to patients with BS (Webster et al., 1992). A Glu566 to Lys mutation at the enzyme active site inactivated one allele, whereas an Arg771 to Trp mutation on the second allele resulted in 20-fold reduction in enzyme activity (Barnes et al., 1992). A fibroblast cell line isolated from this patient (46BR) showed increased sensitivity to a wide variety of DNA damaging agents, delayed joining of DNA replication intermediates and increased somatic recombination, although not to the level seen in BS (Teo et al., 1983; Lehmann et al., 1988; Prigent et al., 1994; Henderson et al., 1985). As a consequence, it was speculated that DNA ligase I plays roles in multiple DNA replication, recombination and repair pathways.

There is a wealth of evidence confirming that DNA ligase I is the main ligase involved in joining DNA replication intermediates. DNA ligase I expression and activity correlate closely with the rate of cell proliferation; the protein localises to multiprotein replication complexes and interacts with proliferating cell nuclear antigen (PCNA) (e.g. Levin et al., 2000; Tom et al., 2001) and functions in lagging-strand DNA replication in vitro (Waga et al., 1994). Perhaps unsurprisingly, DNA ligase I has been reported to be an

essential gene for mammalian cells growing in vitro (Petrini et al., 1995). There is also strong evidence supporting the role of DNA ligase I in the long-patch form of base excision repair (BER) (for a review, see Lindahl et al., 1997), and it has also been implicated in nucleotide excision repair (NER) by in vitro reconstitution (Shivji et al., 1995; Aboussekhra et al., 1995). However, DNA ligase I is not the only DNA ligase that functions in BER. The ubiquitously expressed form of DNA ligase III (IIIa) complexes with the single-strand break repair protein, XRCC1, and is involved in the predominant (short-patch) form of BER (Cappelli et al., 1997). Similarly, although DNA ligase I is active in a cell-free V(D)J recombination system (Ramsden et al., 1997), it cannot carry out double-strand break repair in ligase IV-defective cells (Grawunder et al., 1998). Instead, cultured cells lacking DNA ligase IV are both defective in V(D)J recombination and hypersensitive to ionising radiation (Frank et al., 1998). Hence there is uncertainty about the roles of DNA ligase I in recombination and repair and the level of redundancy with other DNA ligases.

To study the function of DNA ligase I in mammalian cells, and as the first stage in the production of a mouse model for the 46BR patient, we knocked out the mouse *Lig1* gene (Bentley et al., 1996). As expected, homozygosity for the targeted *Lig1* allele resulted in embryonic lethality. However, mutant embryos developed much further than anticipated and were indistinguishable from their wild-type siblings up to E10.5. Only from E11.5 onwards, when the liver takes over from the yolk sac as the major site of blood production, was a difference observed. Mutant embryos failed to achieve normal foetal liver haematopoiesis, leading to a severe deficiency of mature enucleated erythrocytes in the peripheral circulation, anaemia and death by E16.5. Although the foetal livers lacked normal erythropoietic islands, both primitive multipotent (CFU-A) and erythroid (BFU-E) progenitors were present, but at a reduced level compared with controls, and they also gave rise to smaller colonies in vitro. This indicated that there was not a qualitative block on haematopoietic differentiation but rather a quantitative impediment leading to reduced proliferation. We therefore postulated that another ligase can compensate for the lack of DNA ligase I in other foetal tissues but that this compensation fails to meet the high replicative demands of foetal liver erythropoiesis.

The phenotype of our DNA ligase I knockout mice is clearly not compatible with the report that DNA ligase I is a cell-essential gene (Petrini et al., 1995), and it has been suggested (Mackenney et al., 1997) that we may not have completely inactivated the allele, despite targeting having removed the 3′ end of the gene and our inability to detect either *Lig1* transcripts or DNA ligase I protein in mutant embryos. To address this issue we have now generated a second *Lig1* targeted allele. In this report we compare the phenotypes of the two *Lig1* mutant mouse lines and characterise the products from the targeted alleles. We also investigated the particular requirement for DNA ligase I in haematopoiesis by studying the ability of haematopoietic precursors from mutant embryos to rescue lethally irradiated recipients. Finally, we have isolated DNA-ligase-I-deficient fibroblasts from mutant embryos and used them to study the role of DNA ligase I in DNA replication, repair and the preservation of genomic stability in vitro.

Materials and Methods

Mice

The production of mice containing the original ($Lig1^{-(\#53)}$) null allele has been described previously (Bentley et al., 1996). Mice containing a new ($Lig1^{-(\#12)}$) null allele were generated in exactly the same way except that the gene targeting vector used contained the *Hprt* minigene, DWM110 (Magin et al., 1992), instead of PGK-HPRT (RI).

Cells

The isolation and culture of spontaneously immortalised mouse embryonic fibroblast lines PF20 (wild type) and PF24 (*Ercc1*-deficient) has been described (Melton et al., 1998). The DNA-ligase-I-deficient immortalised mouse embryonic fibroblast lines, PFL10 and PFL13, were isolated and cultured in the same way. The source of the transformed human control cell line MRC5 (full name MRC5V1) and the *LIG1* point mutant 46BR (full name 46BR.1G1) have been described (Somia et al., 1993). BrdU incorporation and detection to measure DNA replication and flow cytometry of DNA content for cell cycle distribution were carried out as described (McWhir et al., 1993). Micronuclei were detected and scored in situ in cells growing on plastic culture dishes as described (Melton et al., 1998).

Assay for DNA replication intermediates

Fibroblasts, grown to 80% confluency in 30 mm dishes were rinsed twice in warm serum-free medium and then incubated at 37°C for 10 minutes in serum-free medium containing 4 μCi of [methyl-^{3}H]-thymidine (25 Ci/mole). After chase times ranging from 0-30 minutes cells were scraped into ice cold PBS, washed and resuspended in 20 μl 10mM Tris-HCl (pH 8.0), 20 mM NaCl, 0.1 M EDTA. 60 μl of molten 1.5% low-melting point agarose was then added to the suspension, which was mixed and left to set on ice for 5 minutes. The DNA in the plugs was denatured, and single-stranded DNA replication intermediates were separated on 1% agarose gels as described (Sambrook et al., 1989). After electrophoresis the gels were neutralised and stained with ethidium bromide to visualise the DNA under UV illumination. The gels were sliced into 1 cm fractions, ranging from <0.2 to >23 kb, using size markers as a guide. Gel fractions were transferred to scintillation vials, soaked in 0.1 M HCl for 1 hour, melted in a microwave and the [^{3}H] present was counted in a scintillation counter using an aqueous scintillant.

Sister chromatid exchange assay

The fluorescence plus Giemsa technique (Perry and Wolff, 1974) with modifications (Goto et al., 1978) was used to detect SCEs. Fibroblasts were grown in medium supplemented with 10 μM BrdU for 36-96 hours. The precise time, which varied between lines, was sufficient for two rounds of DNA replication. Metaphase chromosome preparations, prepared from colcemid-arrested cultures, were immersed in Hoechst 33258, rinsed and sealed in 100 mM Na phosphate buffer, pH 8.0. Spreads were exposed to a blacklight (18W Blacklight blue special fluorescent lamp, peak emission 365 nm, Philips) at a distance of 5 cm for 30 minutes at 55°C, followed by incubation in 2×SSC for 2 hours at 65°C, rinsing in phosphate buffer pH 6.8, staining in Giemsa for 30 minutes, mounting and scoring.

Northern and western blotting

Total RNA was extracted and subjected to northern analysis as described previously (Thompson et al., 1989). Protein lysates were made and subjected to western analysis as described by Bentley et al. (Bentley et al., 1996). Antibodies to DNA ligases I (TL5) (Tomkinson et al., 1990), III (TL25) and IV (TL18) were kindly supplied by T. Lindahl and D. Barnes (ICRF, Clare Hall Laboratories).

Quantitative RT-PCR

RNA (20 μg) from wild type and *Lig1* null mouse cells was used for cDNA synthesis. Samples were made up to a volume of 10 ml with sterile distilled water, denatured at 70°C for 2 minutes, then snap-cooled on ice. Reactions were carried out at 43°C for 1 hour in 50 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT supplemented with 20 units of RNasin, dNTPs to a final concentration of 1.25 mM, 0.1 μg of random hexamers, 50 ng oligo dT and 50 units of M-MuLV reverse transcriptase. A second round of reverse transcription was then carried out. The mixture was heated to 70°C for 2 minutes, snap-cooled on ice and an additional 20 units of RNasin and 50 units of reverse transcriptase added. After incubation at 43°C for a further 1 hour, the mixture was diluted with 200 μl of TE and stored at 4°C. PCR reactions were then carried out as described (Tomescu et al., 2001) using primers for *Lig1* exons derived from the mouse *Lig1* cDNA sequence (Jessop and Melton, 1995) (GenBank Acc. No. U19604): exon 4 F5284 (CTGTGTCAGACTCTGAACAGAGCTCTCC) – exon 7 F5285 (GTCTTGGCACCTCTAGCAGGAGGTTTGC), 429 bp; exon 16 F5286 (CTGACCTGGATCGAATCATCCCTGTGC) – exon 19 F5287 (CTTGCGCGTGGTGAGTACTTGGAATGG), 404 bp; exon 21 F8744 (GGGTGAGTTTGTCTTCACCACCTCTTTGG) – exon 22 F8745 (AAGGTCTTCACCATCAGGCCCTCACAGG), 110 bp; exon 22 N2469 (CCTGTGAGGGCCTGATGGTGAAGACCTTGG) – exon 23 M5176 (CCGGAATTCCGGCTTGCATATAGCCTGAAGCTCTTC), 243 bp.

The cycle conditions were: 26-28 cycles, 94°C for 1 minute, 69°C for 1 minute, 72°C for 1 minute. The quantitative nature of these PCR conditions was confirmed by using three different amounts of each cDNA, varying over a four-fold range, with scanning densitometry of the products obtained. The amounts of *Lig1* transcripts in the cell lines were standardised against a separate quantitative RT-PCR reaction for the *Ercc1* gene (mouse *Ercc1* cDNA sequence, GenBank Acc. No. X07414): exon 4 033M (CCCGTGTTGAAGTTTGTGCG) – exon 6 159M (TAGCCAGCTCCTTGAGAGCC), 228 bp.

Non-quantitative (35 cycle) PCR reactions using the *Lig1* exon 21 primer in combination with a primer from the 3′ end of the *Hprt* minigene (mouse *Hprt* cDNA sequence; GenBank Acc. No. J00423; exon 9 M1748, GCAGATGGCCACAGGACTAGAAC) were also used to characterise the fusion transcripts from the *Lig1* targeted alleles. After gel purification products were sequenced directly using an ABI PRISM dye terminator sequencing reaction-ready kit on an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corporation). Sequence analysis was with the Genetics Computer Group programmes, version 9.

Quantitative PCR

Male donor cells were detected by a PCR reaction for the *Zfy-1* gene (Kunieda et al., 1992): primers Zfy1 (GACTAGACATGTCTTAACATCTGTCC) and Zfy2 (CCTATTGCATGGACTGCAGCTTATG), product 0.15 kb, 20 cycles of 95°C for 1 minute, 65°C for 0.5 minutes, 72°C for 0.5 minutes. This reaction was run in parallel with a separate reaction for the *Ercc1* gene: primers 033M (sequence already given) and 035M (CGAAGGGCGAAGTTCTTCCC), product 0.6 kb, 20 cycles of 94°C for 1 minute, 70°C for 1 minute, 72°C for 1 minute. PCR products were separated by agarose gel electrophoresis, blotted and hybridised to [^{32}P]-labelled probes specific for the two products. Levels of bound radioisotope were determined by phosphorimagery, and the ratio of the two signals calculated. To estimate the percentage of male cells present in a female tissue, the calculated ratios were compared against a standard curve produced by spiking varying amounts of male cell DNA into female cell DNA and carrying out PCR quantification in parallel to the experimental samples.

Flow cytometry

Foetal liver cells were prepared and transplanted into γ-irradiated recipients and cell suspensions from haematopoietic tissues were prepared as previously described (Bentley et al., 1996), counted electronically (Beckman Coulter) and diluted to 10^7 per ml in PBS. 10^6 cells were stained with the desired fluorochrome-linked antibody, washed twice to remove unbound antibody and data for 10,000 cells were acquired using a FACScan (Becton Dickinson) with CellQuest software. The monoclonal antibodies used were obtained from various suppliers. Caltag Ltd.: Anti-mouse IgM F(ab′)2 PE-conjugated affinity purified goat IgG, anti-mouse CD4 PE-conjugated rat monoclonal IgG2a (clone CT-CD4), anti-mouse CD8a FITC-conjugated rat monoclonal IgG2a (clone CT-CD8a), anti-mouse B220 PE-conjugated rat monoclonal IgG2a (clone RA3-6B2), anti-mouse GR1 FITC-conjugated rat monoclonal IgG2a (clone RB6-8C5). Pharmingen: Anti-mouse CD45 PE-conjugated rat monoclonal IgG2b (clone 30F 11.1), anti-mouse TCRαβ FITC-conjugated rat monoclonal IgG2b (clone H57-797), anti-mouse Ter-119 FITC-conjugated rat monoclonal IgG2b (clone Ter-119), anti-mouse H-2Kd FITC-conjugated rat monoclonal IgG2a (clone SF1-1.1), anti-mouse H-2Kb PE-conjugated rat monoclonal IgG. Sigma-Aldrich Company Ltd.: Anti-mouse CD5 FITC-conjugated rat monoclonal IgG (clone 53-7.3).

Results

Lig1 gene targeting produces mice with no detectable DNA ligase I

The structure of our original and new *Lig1* targeted alleles is shown schematically in Fig. 1. In both cases the 3′ end of the *Lig1* gene (exons 23-27), encoding 174 residues of the 916

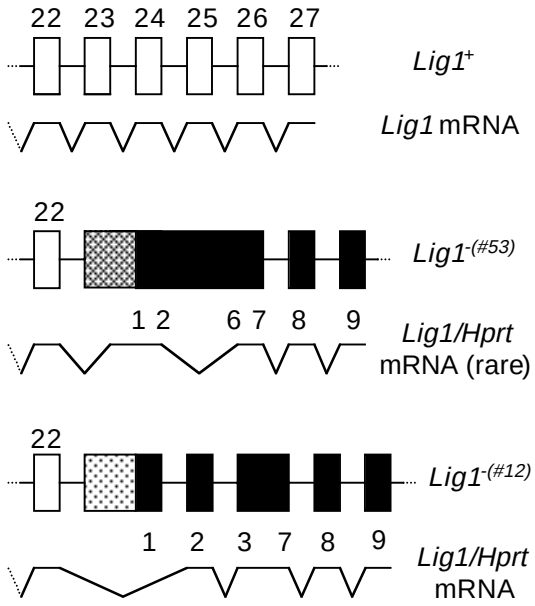

Fig. 1. Structure and expression of *Lig1* targeted alleles. The structure of the 3′ end of the wild-type and *Lig1* targeted alleles is shown schematically. *Lig1* exons are depicted as numbered open boxes. In the $Lig1^{-(\#53)}$ allele, exons 23-27 have been replaced by the PGK-HPRT(RI) *Hprt* minigene. *Hprt* exons are shown as numbered closed boxes with the *Pgk* promoter cross-hatched. In the $Lig1^{-(\#12)}$ allele, exons 23-27 have been replaced by the DWM110 *Hprt* minigene, with the *Hprt* promoter stippled. The normal RNA splicing pattern for *Lig1* mRNA is indicated and the sequences present in the *Lig1*/*Hprt* fusion transcripts from the *Lig1* targeted alleles are shown. Note that the fusion transcript from the $Lig1^{-(\#53)}$ allele is rare.

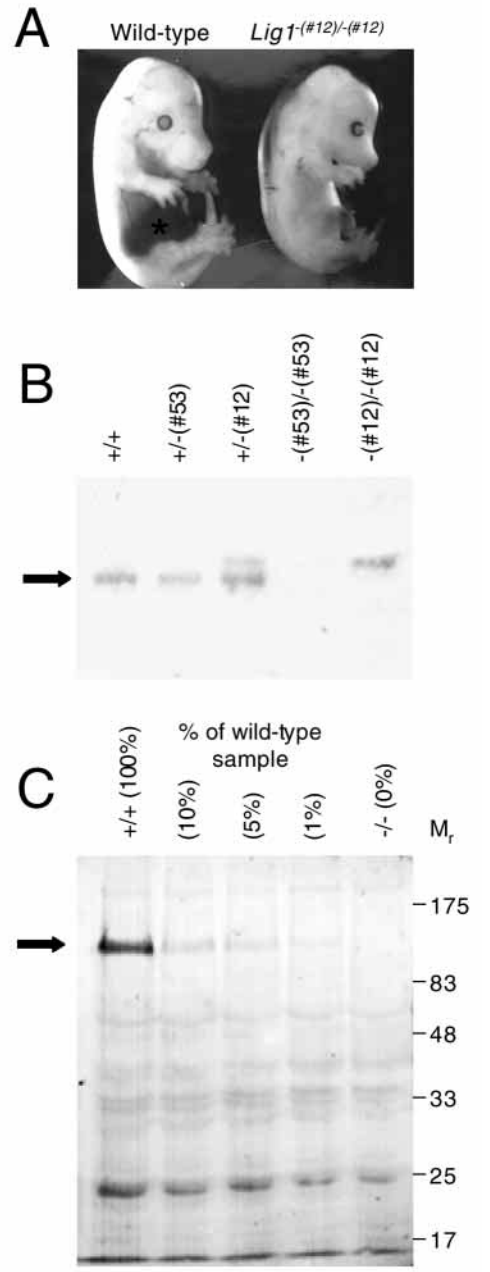

Fig. 2. Phenotype of *Lig1* gene targeted mice and expression from the targeted alleles. (A) Morphology of wild-type (left) and *Lig1*$^{-(\#12)/-(\#12)}$ (right) embryos at E16.5. Note that the mutant embryos are smaller, anaemic and lack the erythropoiesis clearly evident in the wild-type foetal liver (*). (B) Northern analysis of *Lig1* transcripts. RNA (30 μg) extracted from primary embryonic fibroblasts (wild-type, heterozygous and homozygous for the *Lig1*$^{-(\#12)}$ and *Lig1*$^{-(\#53)}$ targeted alleles) was probed with a 2.1 kb fragment of mouse *Lig1* cDNA (Bentley et al., 1996). The position of the 3.2 kb wild-type *Lig1* mRNA is indicated. Note the larger (4.1 kb) transcript from the *Lig1*$^{-(\#12)}$ allele, whereas a transcript is undetectable from the original *Lig1*$^{-(\#53)}$ allele by northern analysis. Equivalent RNA loadings were confirmed by ethidium bromide staining of the gel prior to transfer. (C) Western analysis of DNA ligase I in wild-type and homozygous *Lig1*$^{-(\#53)}$ mutant embryos. Protein extracted from E13.5 embryos was probed with the TL5 rabbit polyclonal antibody raised against purified bovine DNA ligase I. This antibody predominantly recognises epitopes in the N-terminus of the protein. 100 μg of protein was loaded in each lane, but in addition to pure wild-type and mutant extracts, mutant extracts were also spiked with the percentages indicated of the wild-type sample. The position of the 102 kDa wild-type DNA ligase I protein is indicated by the arrow. The mobility of M_r markers (kDa) on the same gel is shown.

amino acid protein, has been deleted and replaced with an *Hprt* minigene: PGK-HPRT(RI) in the case of the original targeted allele (*Lig1*$^{-(\#53)}$) and the more highly expressed DWM110 in the case of the new allele (*Lig1*$^{-(\#12)}$). Embryos homozygous for the new *Lig1*$^{-(\#12)}$ allele had a phenotype identical to one that we have previously reported for embryos homozygous for the original *Lig1*$^{-(\#53)}$ allele: embryos were indistinguishable from wild-type controls up until E10.5 but then became progressively more anaemic, failing to make the transition in erythropoiesis from yolk sac to foetal liver and dying at E15.5/16.5 (Fig. 2A).

Northern analysis on RNA extracted from primary embryonic fibroblasts cultured from *Lig1*$^{-(\#53)/-(\#53)}$ embryos confirmed the lack of the normal 3.2 kb *Lig1* mRNA (Fig. 2B). However, a larger 4.1 kb transcript, produced from the new targeted allele, was evident in both *Lig1*$^{-(\#12)/-(\#12)}$ and *Lig1*$^{+/-(\#12)}$ cultures. RT-PCR using primers derived from *Lig1* and *Hprt* exons, followed by DNA sequencing, was used to show that the transcript from the *Lig1*$^{-(\#12)}$ allele was a *Lig1*/*Hprt* hybrid, splicing from *Lig1* exon 22 onto *Hprt* exon 2 and then following the normal pattern of splicing to the end of the *Hprt* minigene (Fig. 1). This hybrid transcript could potentially encode a fusion protein, comprising 742 residues up to the end of exon 22 of the 916 residue DNA ligase I, in frame with 209 residues from the start of *Hprt* exon 2 through to its normal translational stop. The predicted size of a DNA ligase I fusion protein from the *Lig1*$^{-(\#12)}$ allele would be 106 kDa.

Quantitative RT-PCR detected a *Lig1* transcript from the original *Lig1*$^{-(\#53)}$ allele where northern analysis had failed. Signals for three different *Lig1* primer pairs (exons 4-7, 16-19 and 21-22), when standardised against a reaction for exons 4-6 of the *Ercc1* gene, were <1% of the wild-type level. No signal was obtained with a *Lig1* exon 22-23 primer pair. Sequencing of the PCR product again indicated that this very rare transcript was a *Lig1*/*Hprt* hybrid. Splicing from *Lig1* exon 22 occurred into the promoter of the *Hprt* minigene. A second cryptic splice occurred between the middle of *Hprt* exons 2 and 6 before following the normal *Hprt* splicing pattern to the end of the minigene. This hybrid transcript could potentially encode a fusion protein, again comprising 742 residues of DNA ligase I up to the end of exon 22, with just four additional residues from the *Hprt* promoter region before an in-frame stop signal. The predicted size of a DNA ligase I fusion protein from this allele would be 83 kDa.

Western blotting had previously failed to detect DNA ligase I in *Lig1*$^{-(\#53)/-(\#53)}$ embryos (Bentley et al., 1996). This result was confirmed and extended in Fig. 2C where no normal or altered size DNA ligase I protein could be detected in extracts from *Lig1*$^{-(\#53)/-(\#53)}$ embryos under conditions in which wild-type DNA ligase I could be detected at 1% of the normal concentration. DNA ligase I of normal or altered size was also not detected in *Lig1*$^{-(\#53)/-(\#53)}$ fibroblasts and *Lig1*$^{-(\#12)/-(\#12)}$ embryos and primary fibroblasts (data not shown).

Thus, embryos homozygous for the two *Lig1* targeted alleles have identical phenotypes despite the very large difference in levels of transcripts encoded by the two alleles. No DNA ligase I protein could be detected from either targeted allele, and we conclude that both are effectively *Lig1* null alleles. All subsequent work was carried out with animals containing the original $Lig1^{-(\#53)}$ targeted allele, henceforth described as the $Lig1^{-}$ or *Lig1* null allele.

Lig1 null haematopoietic cells can rescue lethally irradiated recipients

We have previously shown that haematopoietic progenitors, which are present in a cell suspension from single *Lig1* null foetal livers, were unable to repopulate the haematopoietic system and rescue lethally irradiated recipients (Bentley et al., 1996). The presence of haematopoietic progenitors, albeit in reduced numbers, in *Lig1* null foetal liver indicated that there was not a fundamental, qualitative haematopoietic defect associated with DNA ligase I deficiency, but rather that the replicative impairment imposed a quantitative defect. We investigated this further by assessing the ability of foetal liver cell suspensions, pooled from multiple *Lig1* null embryos, to rescue lethally irradiated recipients.

E13.5 embryos were collected from matings between *Lig1* heterozygotes, and anaemic *Lig1* null embryos were identified. Livers were removed and stored on ice while a rapid PCR reaction was carried out on the remainder of each embryo to determine both the *Lig1* genotype and sex of the embryo (using primers for the *Zfy-1* gene). Foetal liver suspensions from male embryos were used for transplantation with female recipients so that the fate of the donor cells could be followed by PCR. Nine recipients were each injected with a foetal liver suspension from a single wild-type embryo. Five recipients were each injected with a foetal liver suspension pooled from three null embryos. Six irradiated control animals received no donor cells. All control animals died within two weeks. Two out of five recipients transplanted with pooled null cells survived for at least ten weeks along with two out of nine recipients of wild-type cells. Peripheral blood samples were taken from surviving animals at 4 and 6 weeks, and all animals were killed between 10 and 12 weeks for detailed analysis of haematopoietic tissues.

Animals rescued by *Lig1* null cells are anaemic with macrocytosis and reticulocytosis

Although all surviving animals were overtly healthy, with stable bodyweights, when blood samples were taken 4 weeks after irradiation it was found that animals rescued with *Lig1* null cells were anaemic. Haematocrits for recipients of wild-type cells were in the normal range (42-50%), but the mean value for recipients of null cells was only 26%. The reduced haematocrit was accompanied by macrocytosis (enlarged erythrocytes) and reticulocytosis (increased numbers of immature erythrocytes). As the haematocrits in animals rescued by *Lig1* null cells dropped as the experiment progressed, the proportion of reticulocytes increased. These data indicated that a high proportion of red cells was being released prematurely into the circulation and that the situation was deteriorating with time in animals rescued by *Lig1* null cells.

Table 1. Contribution of wild-type and *Lig1* null donor cells to haematopoietic tissues of rescued animals

	Wild type		*Lig1* null	
Recipient tissue*	Total cell number $\times 10^{-7}$	% Donor contribution	Total cell number $\times 10^{-7}$	% Donor contribution
Peripheral blood (4 weeks)	2.9	54	1.3	36
Peripheral blood (6 weeks)	3.7	58	2.1	22
Peripheral blood (10/12 wks)	2.5	51	1.9	41
Bone marrow	3.1	54	0.7	86
Mesenteric lymph nodes	2.8	46	2.3	13
Peripheral lymph nodes	1.8	35	1.0	7
Peritoneal cells	0.7	29	0.4	ND‡
Spleen	22.0	63	41.0	65
Thymus	7.8	49	2.4	10

*Single cell suspensions were prepared from the peripheral blood (sampled at the times indicated) and from haematopoietic tissues of irradiated mice rescued with wild-type or *Lig1* null foetal liver cells 10-12 weeks after transfer. Cells were counted, and the mean values for the total number of cells in each tissue were calculated for the animals rescued with cells of each genotype. DNA was also prepared from each sample and quantitative PCR was used to estimate the mean percentage contribution of donor cells to each tissue.

‡ND, not detected.

Lig1 null cells contribute to all haematopoietic tissues

Animals rescued by *Lig1* null cells had enlarged spleens (splenomegaly) compared with animals rescued by wild-type cells. This was reflected in an increase in the number of spleen cells recovered (Table 1). However, reduced numbers of cells were recovered from all other haematopoietic tissues removed from animals rescued by null as opposed to wild-type cells. This hypoplasia was most severe in bone marrow, which has nearly five-fold fewer cells.

Quantitative PCR for *Zfy-1* on DNA prepared from haematopoietic tissues of rescued animals was used to estimate the contribution of transplanted cells to each tissue (Table 1). High contributions (30-60%) of donor (male) cells were detected in all recipient (female) tissues from animals rescued with wild-type cells. These values are never 100% because there is always some recovery of the lethally irradiated recipient's haematopoietic system (Micklem et al., 1987). Donor-derived cells were also detected in all haematopoietic tissues from animals rescued by *Lig1* null cells but, apart from bone marrow and spleen, the contribution of null cells to repopulated tissues was lower than wild-type cells.

To determine whether this defect affected all haematopoietic lineages, we analysed haematopoietic tissues from rescued mice by flow cytometry using antibodies to: B220 (expressed on cells committed to the B lymphoid lineage); IgM and IgD (present on mature B lymphocytes which have undergone V(D)J recombination); CD4 and CD8 (present on thymocytes); TCRαβ (present on thymocytes with rearrangement of the T cell receptor gene); CD5 (present on T cells and a subset of B cells); CD45 (present on non-erythroid haematopoietic cells); GR1 (present on granulocytes); and Ter-119 (present on erythroid cells). The staining profiles from mice rescued with *Lig1* null cells were similar to, and in many cases indistinguishable from, wild-type controls. An accurate determination of the number of cells expressing a particular marker(s) in a particular haematopoietic tissue was impossible

because of the low numbers of labelled cells counted but, in general, cells of all lineages were present in reduced numbers in animals rescued with *Lig1* null cells compared with animals rescued by wild-type cells. The one notable exception was the spleen where the number of Ter-119-expressing (erythroid) cells was higher in the enlarged spleens from animals rescued with *Lig1* null cells. These data indicate that *Lig1* null haematopoietic stem cells can contribute to all lineages, but in lower numbers than control cells.

Lig1 null primary mouse fibroblasts are viable but have an elevated level of chromosome instability

To investigate the properties of DNA ligase I null cells in vitro, primary embryonic fibroblast cultures were established from E12.5 embryos obtained from matings between animals heterozygous for the original *Lig1*$^{-}$ targeted allele (Bentley et al., 1996). Cultures isolated from *Lig1*$^{-/-}$ embryos grew more slowly than control cultures from *Lig1*$^{+/+}$ embryos (generation time ~48 hours for mutant cultures compared to ~36 hours for controls; data not shown), but flow cytometry on propidium-iodide stained nuclei from cultures (passage 2-passage 5) showed the same cell cycle distribution for both genotypes (data not shown). Following a 2 hour pulse with BrdU, to identify cells in S-phase, 42±5% of cells from two independent control cultures (passage 2) showed nuclei stained with an anti-BrdU antibody. The corresponding value for three independent mutant cultures was only slightly lower (37±2%), confirming the similar cell cycle characteristics.

Defects in pathways involved in DNA maintenance are generally associated with genomic instability (Hoeijmakers, 2001). To investigate the possibility that DNA ligase I deficiency may result in chromosome breakage and loss, we measured the frequency of micronucleus formation in primary cultures of *Lig1* null and control embryonic fibroblasts. The mean frequency of micronuclei in six independent wild-type cultures (ranging from passage 2-5) was 0.49±0.21%. The corresponding frequency from six independent *Lig1* null cultures was four-fold higher, 2.00±0.79%. This difference was highly significant (p=0.001 by Student's *t* test).

Both wild-type and DNA ligase I null primary cultures senesced rapidly. To facilitate further analysis of DNA ligase I null cells, two spontaneously immortalised lines, PFL10 and PFL13, were isolated. Although the immortalised DNA ligase I null lines grew well in culture they were not as robust as wild-type lines. They were more difficult to establish from frozen stocks, did not plate well at low densities and their generation time was longer than control cultures (PFL13 ~36 hours, PFL10 ~48 hours, compared with ~24 hours for the wild-type PF20 control). DNA ligase I has been implicated in a number of different repair reactions and, a priori, a DNA repair deficiency would be the most likely explanation for the genome instability in *Lig1* null primary fibroblasts. Consequently we used the immortalised cell lines to assess the sensitivity of DNA ligase I null cells to a range of DNA damaging agents.

DNA ligase I is not essential for nucleotide excision repair

The human DNA ligase I point mutant cell line, 46BR, has been reported to show increased sensitivity to a range of DNA damaging agents, including UV light (Teo et al., 1983), and in vitro reconstitution of NER utilises purified DNA ligase I (Aboussekhra et al., 1995). However, in the UV survival experiment shown in Fig. 3A, the survival curves for all ligase mutant and control cell lines were very similar. The human 46BR point mutant was only marginally more sensitive than its species control (MRC5). Similarly, the two mouse *Lig1* null cell lines were only slightly more sensitive than the mouse (PF20) control. When these curves are compared with that of a mouse NER mutant (PF24, *Ercc1*-deficient) (Melton et al., 1998), it is clear that DNA ligase I is not essential for NER: the D_{50} for PF24 was 1.5 Jm^{-2}, whereas it was 7 Jm^{-2} for PFL10 and PFL13.

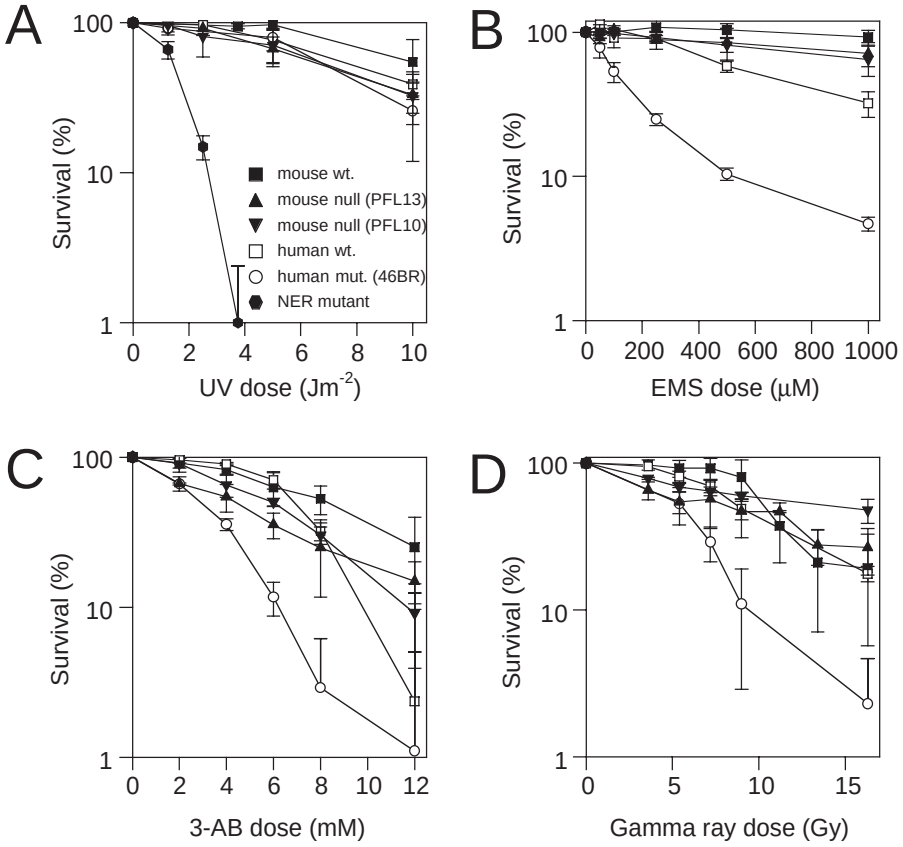

Fig. 3. Survival of DNA-ligase-I-deficient cells exposed to the following DNA damaging agents (A) UV; (B) EMS; (C) 3-aminobenamide (3-AB); and (D) γ-irradiation. Mean survival±s.e.m. is shown relative to non-treated controls. ■, mouse wild-type (PF20); ▲, mouse *Lig1* null (PFL13); ▼, mouse *Lig1* null (PFL10); □, human wild-type (MRC5); ○, human *LIG1* point mutant (46BR); closed hexagon, mouse *Ercc1* null (PF24).

DNA ligase I is not essential for repair of alkylation damage

DNA ligase I is involved in the long-patch form of BER, and 46BR cells have been found to show increased sensitivity to alkylating agents (Teo et al., 1983). Survival curves for EMS are shown in Fig. 3B. The 46BR point mutant was five-fold more sensitive to EMS than MRC5 (D_{50} 120 μM compared to 650 μM for MRC5). All the mouse lines were more resistant to EMS than the human control, with the two null lines (PFL10 and PFL13) indistinguishable from the PF20 control. Thus, as predicted from the existence of alternate pathways, DNA ligase I is not essential for repair of damage induced by alkylating agents.

Mouse DNA ligase I null cells are not hypersensitive to 3-aminobenzamide

Poly(ADP-ribose) polymerase (PARP) binds to DNA strand breaks and facilitates DNA ligation (Satoh and Lindahl, 1992). 3-aminobenzamide is a specific inhibitor of PARP that causes an accumulation of strand breaks. The reported increased sensitivity of 46BR cells to 3-aminobenzamide (Lehmann et al., 1988) was confirmed in Fig. 3C. 46BR cells were two-fold more sensitive than MRC5 (D_{50} 3 mM compared to 7 mM for MRC5). Although the mouse lines again showed greater resistance to 3-aminobenzamide, the *Lig1* null lines (PFL10 D_{50} 6 mM, PFL13 4.5 mM) were 1.5-fold more sensitive than the control (PF20 8.3 mM).

DNA ligase I is not essential for repair of ionising radiation damage

Although DNA ligase IV is the key ligase for double-strand break repair, 46BR cells have been reported to show increased sensitivity to ionising radiation (Teo et al., 1983). The increased sensitivity of 46BR compared with MRC5 was confirmed in Fig. 3D (46BR D_{50} 5.7 Gy, MRC5 9 Gy). The two mouse *Lig1* null lines were not more sensitive than the PF20 control. The survival curve for PFL13 (D_{50} 8.5 Gy) was very similar to PF20 (D_{50} 9 Gy). The curve for PFL10 was shallower and reproducibly lacked a conventional shoulder, resulting in an apparently higher D_{50} (15 Gy) than the wild-type control.

Replication intermediates accumulate in *Lig1* null cells

In the absence of a clear DNA repair defect in *Lig1* null cells, we next investigated DNA replication as a possible explanation for the genome instability. 40-300 base DNA replication intermediates (Okazaki fragments) are extremely transient in wild-type cells, but can be readily detected in 46BR cells by giving short pulses of [^{3}H]-thymidine, then chasing with unlabelled thymidine and measuring radioactivity in single-stranded DNA fractions of different sizes (Henderson et al., 1985). The results of such an experiment for fractions ranging from <0.2 to >23 kb are summarised in Fig. 4. Following the pulse, <2% of the total radioactivity incorporated into DNA in both wild-type human (MRC5) and mouse (PF20) cells was present in DNA molecules <2.0 kb and that which was present was rapidly chased into larger fractions. In contrast 23% of the radioactivity in the human DNA ligase I point mutant (46BR) was in the <2.0kb fraction after the pulse, and this did not completely disappear during the 30 minute chase. The situation in the mouse *Lig1* null line (PFL13) was intermediate between 46BR and the wild-type controls. 10% of the radioactivity was in the <2.0 kb fraction after the pulse, but this disappeared completely after a 15 minute chase. Thus, the ligation of replication intermediates is delayed in *Lig1* null mouse fibroblasts, but the delay is not so pronounced as in the human *LIG1* point mutant.

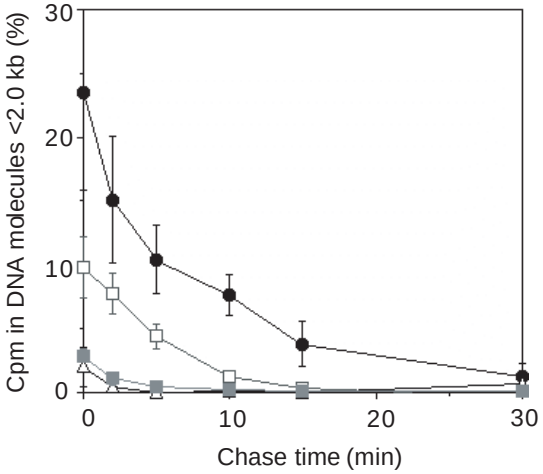

Fig. 4. Accumulation of DNA replication intermediates in DNA-ligase-I-deficient cells. Cultures were subjected to a short pulse with [^{3}H]-thymidine followed by a chase of up to 30 minutes. Cells were then harvested, lysed in agarose plugs and subjected to alkaline gel electrophoresis. Fractions from <0.2 kb to >23 kb were collected and counted. The figure shows the percentage of total radioactivity present in single-stranded DNA fragments <2.0kb for each cell line. Values shown are means±s.e.m. ■, mouse wild-type (PF20); △, human wild-type (MRC5); □, mouse *Lig1* null (PFL13); ●, human *LIG1* point mutant (46BR).

Sister chromatid exchange rates are normal in *Lig1* null mouse fibroblasts

One of the characteristics of Bloom's syndrome is a very high level of reciprocal interchanges between homologous chromatids (sister chromatid exchange, SCE). 46BR cells also show an increased frequency of SCEs and are hypersensitive to the induction of SCE by DNA damaging agents (Henderson et al., 1985). Because *Lig1* null fibroblasts showed increased chromosome instability, as determined by micronucleus formation, we also measured the spontaneous incidence of SCE in a range of primary cultures and immortalised cell lines (Table 2). All mouse and human wild-type samples displayed SCE rates consistent with previous studies (~10 SCEs per metaphase) (e.g. McDaniel and Schultz, 1992). As expected, the mean number of SCEs per metaphase was significantly elevated (2.4-fold) in 46BR cells compared with the human (MRC5) control (Mann-Whitney U test, $p < 0.05$). However, there was no significant difference in SCE frequency between primary (*Lig1*$^{-/-}$) or immortalised (PFL13) DNA *Lig1* null cells compared with wild-type controls (*Lig1*$^{+/+}$ and PF20).

Table 2. The frequency of sister chromatid exchanges in human and mouse fibroblast cells

Cell line	SCEs per metaphase*
MRC5	8.2±1.1
46BR	19.7±2.3
Lig1$^{+/+}$	9.8±1.0
Lig1$^{-/-}$	11.1±0.9
PF20	9.2±1.1
PFL13	8.7±1.3

*SCEs were scored on metaphase chromosome preparations from cultures of immortalised human wild-type (MRC5) and *LIG1* mutant (46BR), mouse wild-type (PF20) and *Lig1* null (PFL13) cell lines and primary mouse embryonic fibroblast wild-type and *Lig1* null cultures. Values are expressed as the mean number of SCEs per metaphase±s.e.m.

Levels of DNA ligases III and IV are not increased to compensate for DNA ligase I deficiency

DNA ligase I is highly expressed in proliferating cells, but corresponding levels of other DNA ligases are generally much lower. To investigate the possibility that increased expression of DNA ligases III or IV might be compensating for DNA ligase I deficiency, the levels of *Lig3* and *Lig4* transcripts and proteins were determined in mid-gestation embryos (*Lig1*$^{+/+,+/-,-/-}$) and immortalised cell lines (PF20, PFL10, PFL13). No differences in *Lig3* and *Lig4* transcripts were observed (data not shown). Ligase III and ligase IV proteins were readily detected by the antisera used, but there was no indication that the level of either was altered by DNA ligase I deficiency (Fig. 5).

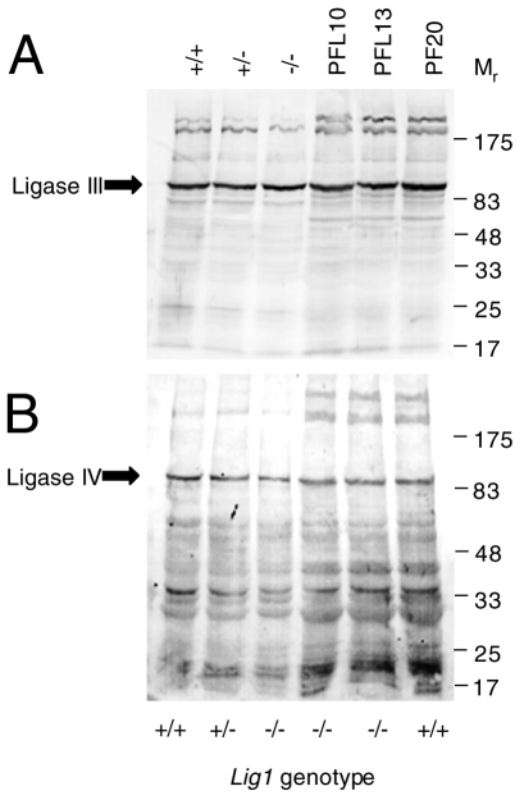

Fig. 5. Levels of DNA ligases III and IV are unaffected by DNA ligase I deficiency. Western analysis of protein (100 μg) extracted from mid-gestation embryos and immortalised embryonic fibroblasts. (A) Panel probed with TL25 rabbit polyclonal antibody raised against recombinant full-length human DNA ligase III. The position of the 103 kDa DNA ligase III is indicated by the arrow. (B) Panel probed with TL18 rabbit polyclonal antibody raised against a C-terminal peptide of human DNA ligase IV. The position of the 96 kDa DNA ligase IV is indicated by the arrow. The mobility of M_r markers (kDa) on the same gels is shown.

Discussion

Do the *Lig1* targeted alleles have some residual function?

Given the key role of DNA ligase I in replication (Timson et al., 2000) and the report that DNA ligase I was essential for the viability of cultured mammalian cells (Petrini et al., 1995), the issue of possible residual enzyme activity was critical for our understanding of the phenotype of our *Lig1* knockout mice and for the role of DNA ligase I in vivo. In both of our *Lig1* knockout alleles, targeting has removed the 3′ end of the gene, comprising exons 23-27 and encoding 174 residues of the 916 amino acid protein. A *Lig1* cDNA probe containing exons 8-27 had previously failed to detect a trancript from the original (*Lig1*$^{-(\#53)}$) targeted allele by northern blotting, and two different antibodies to epitopes in the N- and C-terminal regions of DNA ligase I could not detect any DNA ligase I protein in extracts from mutant embryos (Bentley et al., 1996). Although we detected a rare fusion transcript from the original *Lig1*$^{-(\#53)}$ allele and a more abundant fusion transcript from the new *Lig1*$^{-(\#12)}$ allele, there was no evidence for any DNA ligase I being produced in either case.

The low level of mRNA from the *Lig1*$^{-(\#53)}$ allele, detectable only by RT-PCR, could be because of a low level of transcription or the instability of the hybrid transcript. We have previously shown that transcription from the PGK-HPRT(RI) minigene is particularly unstable at the *Lig1* locus, with the mouse *Pgk* promoter becoming heavily methylated (Melton et al., 1997). Transcription from the mouse *Hprt* promoter in the DWM110 minigene, inserted at the identical position in the *Lig1*$^{-(\#12)}$ allele, was not similarly affected. Perhaps the silencing of the PGK-HPRT(RI) minigene extends to the *Lig1* promoter itself to result in the low level of transcripts from the *Lig1*$^{-(\#53)}$ allele.

Despite the presence of abundant transcripts from the new *Lig1*$^{-(\#12)}$ targeted allele, the phenotype of mutant embryos was identical to the one we have previously reported for the original *Lig1*$^{-(\#53)}$ allele. This strengthens the argument that our *Lig1* targeted alleles produce no functional DNA ligase I because, if there were residual ligase activity from the *Lig1*$^{-(\#53)}$ allele, greater activity and a milder phenotype would be expected from the *Lig1*$^{-(\#12)}$ allele with its much higher level of *Lig1* transcripts. There is additional evidence that, even if a DNA ligase I fusion protein were produced, it would have no catalytic activity. Removal of more than 16 amino acids from the C-terminus of human DNA ligase I rendered the protein enzymatically inactive (Kodama et al., 1991). Any fusion protein produced from our targeted alleles would lack the C-terminal 174 residues and should therefore be catalytically inactive. With the high levels of DNA ligase I activity normally found in proliferating cells, we consider it unlikely that an

undetectable amount of a theoretical protein that should have no activity could meet the catalytic requirements for ligase I activity in early mouse embryos. Consequently, we feel justified in describing our *Lig1* targeted alleles as effective nulls for DNA ligase I.

DNA ligase I is not essential for mammalian cell viability

How can our results be reconciled with the report that DNA ligase I is essential for mammalian cell viability (Petrini et al., 1995)? This conclusion arose from the failure to isolate mouse embryonic stem (ES) cells homozygous for a *Lig1* targeted allele following in vitro selection for gene conversion events in ES cells heterozygous for the targeted allele. We consider that this failure could result instead from the inherent difficulty of isolating ES cells homozygous for a disabling mutation under stringent selection conditions where single *Lig1* null cells may be viable but unable to proliferate sufficiently at low cell densities to form a colony and be detected. The fragility of our immortalised *Lig1* null fibroblasts, particularly at low plating densities, would support this suggestion. The failure of Petrini et al. to isolate ES cells homozygous for the *Lig1* targeted allele could also be because of the targeting strategy employed, which replaced exons 17-19 with a selectable marker. Although any product from this targeted allele would be catalytically inactive with the probes and antisera used, Petrini et al. did not exclude the possibility that an altered protein was produced from their targeted allele. We suggest that the presence of an inactive DNA-ligase-I-derived protein may actually hamper cell survival, possibly by preventing another ligase from acting instead of DNA ligase I (see below).

Lig1 null cells can repopulate the haematopoietic system of lethally irradiated recipients

Lig1 null embryos die as a consequence of failure to make the transition in erythropoiesis from yolk sac to foetal liver, despite the presence of haematopoietic progenitors in foetal liver. This led us to propose a model involving a quantitative, rather than a qualitative, defect in haematopoiesis in *Lig1* null embryos, which only manifested itself under the extremely high requirement for erythropoiesis in foetal liver. One prediction of this model that we have gone on to demonstrate is that *Lig1* null foetal liver cells, when transplanted in sufficient quantity, should be able to repopulate the haematopoietic system and rescue lethally irradiated recipients. Pooled *Lig1* null liver suspensions were as effective as individual wild-type liver suspensions in effecting rescue. The progressive anaemia and elevated levels of reticulocytes in animals rescued with $Lig1^{-/-}$ cells indicate that *Lig1* null haematopoietic cells are unable to meet demand for erythrocyte production. Similarly, cell numbers in all haematopoietic tissues from animals rescued by *Lig1* null cells were reduced compared with animals rescued by wild-type cells. However, all lineages were present in all haematopoietic tissues examined from animals rescued by *Lig1* null cells. The enlarged spleens in animals rescued by *Lig1* null cells were consistent with the known kinetics of haematopoietic repopulation coupled with the normal physiological response to chronic anaemia. These observations are in keeping with the phenotype of the developing *Lig1* null embryos themselves and are consistent with a quantitative deficiency relating to reduced proliferation rather than a qualitative block in any haematopoietic lineage. In particular, the normal ratio of B220 to IgM and IgD expression observed supports previous in vitro observations in 46BR cells (Petrini et al., 1994) that DNA ligase I is not necessary for V(D)J recombination.

DNA ligase I is not essential for DNA repair

The human *LIG1* point mutant 46BR has been reported to show increased UV sensitivity (Teo et al., 1983), although we could not demonstrate this in our study. DNA ligase I is a component of the NER reaction reconstituted in vitro (Aboussekhra et al., 1995), but our UV survival experiments on *Lig1* null mouse fibroblasts showed clearly that DNA ligase I was not essential for NER. Similarly, although DNA ligase I is involved in the long-patch form of BER (Lindahl et al., 1997) and 46BR cells show increased sensitivity to alkylating agents (Teo et al., 1983), the EMS survival curves for our *Lig1* null mouse fibroblasts were indistinguishable from the wild-type mouse control. Thus, as perhaps could be predicted from the involvement of DNA ligase III in the predominant short-patch form of BER, DNA ligase I is also not essential for repair of alkylation damage. DNA ligase IV is essential for double-strand break repair (Timson et al., 2000), but 46BR cells have been reported to show increased sensitivity to ionising radiation (Teo et al., 1983), and DNA ligase I is active in the V(D)J recombination mechanism in vitro (Ramsden et al., 1997). Our data provide no evidence for a role for DNA ligase I in double-strand break repair. In fact, the γ-irradiation survival curve for PFL10, our slowest growing *Lig1* null line, reproducibly indicated a slight increase in survival compared with the control, although this is more likely to be as a consequence of the slower growth rate and the resulting requirement to plate these cells at a higher density rather than a real increase in survival. Hence, in contrast to results from 46BR cells, we were unable to detect a measurable NER, BER or double-strand break repair defect in *Lig1* null cells. *Lig1* null cells did show a mildly increased sensitivity to 3-aminobenzamide compared with wild-type, although not to the level of 46BR cells. 3-aminobenzamide is an inhibitor of PARP. Although its precise role remains to be defined (for a review, see Herceg and Wang, 2001), PARP binds to DNA strand breaks and is known to stimulate DNA ligase activity (Satoh and Lindahl, 1992). Differences in 3-aminobenzamide sensitivity between 46BR and *Lig1* null cells may reflect an underlying difference in the number of DNA strand breaks occurring within the two cell types, correlating with observed differences in DNA replication.

Replication intermediates accumulate in *Lig1* null cells

46BR cells are defective in the joining of Okazaki fragments (Prigent et al., 1994), and this was clearly evident in our pulse-chase experiment as an accumulation after the pulse of short single-stranded DNA fragments that were slow to chase into high molecular weight DNA. The *Lig1* null cells also showed an accumulation of replication intermediates, but the accumulation was not as marked as in 46BR cells and the intermediates were chased away more rapidly. This accumulation of replication intermediates in *Lig1* null cultured

cells is compatible with the slower growth rate that we have observed and could provide the basis for the failure of *Lig1* null embryos when the replicative demands of erythropoiesis are highest.

Increased genome instability in *Lig1* null cells

One aim of our work with DNA ligase I is to produce a mouse model for the 46BR patient to study the relationship between DNA ligase I deficiency and cancer susceptibility. Since genome instability plays an important role in cancer development, we measured instability and found a four-fold higher level in primary cultures of *Lig1* null embryonic fibroblasts compared with wild-type controls. The micronucleus assay used detects whole chromosomes or chromosome fragments that have failed to segregate correctly at mitosis. The increased frequency of micronuclei was not mirrored in a similar increase in sister chromatid exchanges in *Lig1* null cells.

Genome instability usually results from mutation in cell cycle control genes, such as p53 (Levine, 1997), or from DNA repair deficiency (Hoeijmakers, 2001). The importance of NHEJ components, including DNA ligase IV, in the maintenance of genome stability has been demonstrated (Ferguson et al., 2000). The genome instability in *Lig1* null cells could result directly from the altered DNA replication, particularly since we have no evidence for any DNA repair defect that could also contribute to the instability. As such, Lig1 null cells would represent an unusual situation where there is genome instability in the absence of sensitivity to DNA damaging agents. A similar explanation could apply to at least a component of the instability observed in PARP-deficient cells (for a review, see Herceg and Wang, 2001).

Compensation for DNA ligase I deficiency

Another DNA ligase must be compensating for the lack of DNA ligase I in our *Lig1* null cells and embryos. We found no evidence for a compensatory increase in expression of the two obvious candidates, DNA ligases III and IV, but this does not exclude their involvement since both were readily detected and so increased expression may not be a prerequisite.

Lig1 null mouse cells were less sensitive to UV, EMS and γ-irradiation than human 46BR cells when compared with their species controls. The accumulation of replication intermediates was also more pronounced in 46BR than *Lig1* null cells, and only 46BR cells showed an elevated frequency of SCEs. This could be caused by ligase I playing different roles in the two species. Alternatively, in many respects, it may be better to have a complete absence of ligase I, rather than produce a partially functional enzyme as is the case with the 46BR cell line. This is not surprising given that interaction with PCNA has been demonstrated to be important for DNA ligase I function in joining Okazaki fragments and in long-patch BER (Levin et al., 2000). Perhaps only in the absence of DNA ligase I is another DNA ligase able to effectively complement for the missing ligase activity. Our results emphasise that the 46BR patient is a special case of DNA ligase I deficiency and that the 46BR phenotype is not necessarily representative of the effects of the complete absence of DNA ligase I. Nevertheless it must be remembered that, as with *Lig4* (Frank et al., 1998; Barnes et al., 1998), the *Lig1* null mutation is embryonic lethal, and so the importance of DNA ligase I should not be underestimated.

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