DNA Ligase I Mediates Essential Functions in Mammalian Cells

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DNA replication, repair, and recombination are essential processes in mammalian cells. Hence, the application of gene targeting to the study of these DNA metabolic pathways requires the creation of nonnull mutations. We have developed a method for introducing partially defective mutants in murine embryonic stem cells that circumvents the problem of cellular lethality of targeted mutations at essential loci. Using this approach, we have determined that mammalian DNA ligase I is essential for cell viability. Thus, DNA ligases II and III are not redundant with DNA ligase I for the function(s) associated with cell proliferation. Partial complementation of the lethal DNA ligase I null mutation allowed the creation of deficient embryonic stem cell lines. We found that a wild-type DNA ligase I cDNA, as well as a variant DNA ligase I cDNA, was able to rescue the lethality of the homozygous null mutation, whereas an N-terminal deletion mutant consisting of the minimal DNA ligase I catalytic domain was not. This observation demonstrates that sequences outside the DNA ligase I catalytic domain are essential for DNA ligase I function *in vivo*.

DNA ligase activity is a presumptive requirement in essentially all DNA repair and recombination mechanisms, as well as in the completion of lagging-strand DNA synthesis. Three distinct mammalian DNA ligase activities have been identified: DNA ligases I, II, and III (41). DNA ligases I and III are expressed at high levels and together compose the majority of the DNA ligase activity found in proliferating cells (42). Whereas the physiological role of DNA ligase I has been extensively characterized from biochemical and genetic analysis, the functions of DNA ligases II and III remain unclear. Conditional *Saccharomyces cerevisiae* DNA ligase I (*CDC9*) mutants exhibit DNA replication defects, in addition to generalized DNA repair deficiency (23, 31). Mammalian DNA ligase I has been similarly implicated in DNA replication by its ability to mediate the joining of Okazaki fragments during lagging-strand DNA synthesis *in vitro* (15, 21, 45).

Further insight regarding the physiological role of mammalian DNA ligase I comes from the phenotypes of DNA ligase I-deficient cells. Two distinct human DNA ligase I deficiency syndromes have been described: Bloom syndrome and a genetically distinct syndrome represented by the cell line 46BR (13, 46). These syndromes are associated with DNA replication defects, consistent with the biochemical evidence for DNA ligase I function in DNA replication, and further indicate a role for DNA ligase I in the repair of DNA damage. Mutation of DNA ligase I in 46BR results in a 10- to 20-fold reduction in DNA ligase I activity, resulting in increased sensitivity to DNA-alkylating agents and DNA lesions induced by UV light (4, 20, 27, 34, 36). DNA ligase I deficiency has also been associated with Bloom syndrome (9, 10, 47, 48), although analysis of the structure and expression of the DNA ligase I gene in Bloom syndrome cells reveals no mutation, and highly purified preparations of Bloom syndrome DNA ligase I appear unaffected (32, 42). Like 46BR, Bloom syndrome cells exhibit defects in DNA replication (11). In addition, these cells exhibit an extraordinarily high rate of mutation and chromosome loss

and elevated sister chromatid exchange frequency and show broad sensitivity to DNA-damaging agents, suggesting a generalized deficiency in DNA repair (8, 14, 26). The observation that DNA ligase I deficiency affects both DNA replication and DNA repair suggests that certain functions of DNA ligases II and III are redundant with DNA ligase I.

In this study, we utilized a gene-targeting strategy that is generally applicable to the analysis of any essential mammalian gene. We show that an ectopically expressed DNA ligase I cDNA is required in order to derive homozygous null DNA ligase I mutants. This result indicates that the gene for DNA ligase I is an essential gene in mammalian cells and suggests that the function of DNA ligase I in DNA replication cannot be replaced by DNA ligase II or III. Our data further demonstrate that the N-terminal region of DNA ligase I, which lies outside the minimal catalytic domain, is indispensable for *in vivo* function in mammalian cells.

MATERIALS AND METHODS

Molecular biology. Plasmid construction, Southern blotting, and Northern (RNA) blotting were carried out according to standard procedures (2). The NBA probe is a 477-bp *Bam*HI-*Nco*I fragment derived from the murine DNA ligase I cDNA (nucleotides 837 to 1313; numbering from GenBank no. MMU04674). The MoBN probe is a 281-bp *Bgl*II-*Nae*I murine DNA ligase I cDNA fragment (nucleotides 1808 to 2088). These molecules were radiolabeled for use as hybridization probes (11), and hybridizations were carried out at 42°C in 5× SSCPE (5× SSCPE is 0.75 M NaCl, 0.075 M sodium citrate, 50 mM NaH₂PO₄, and 5 mM disodium EDTA)–50% formamide–10× Denhardt's solution–2% sodium dodecyl sulfate (SDS). Sonicated herring sperm DNA (50 µg/ml) was included in the hybridization solution, but we found that omitting it did not reproducibly affect the signal-to-noise ratio of the ensuing autoradiograms. Filters were washed three times at 68°C for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS.

For Western blotting (immunoblotting), 5×10^5 cells were lysed in SDS lysis buffer containing β-mercaptoethanol, boiled, and aspirated through a 22-gauge needle to shear the genomic DNA before fractionation on SDS–7.5% polyacrylamide gel electrophoresis (PAGE) gels. Immunoblot analysis was carried out by standard procedures (17), with F6F, a polyclonal rabbit antiserum raised against a polypeptide (KGISLRFPFRFIRVREDKQP) which corresponds to residues 866 to 884 at the carboxyl terminus of DNA ligase I (the N-terminal Lys has been added to facilitate coupling to beads during peptide synthesis). Antiserum to a similar peptide has been raised by other investigators (40).

Construction of the DNA ligase I targeting vector. The left arm of ΔRLPGK is a 2.7-kb *Eco*RI-*Sal*I DNA ligase I genomic fragment flanked by a herpes simplex virus thymidine kinase gene for negative selection and the *neo* gene for positive selection. Both markers are under the control of the murine phosphoglycerate kinase promoter and are transcribed in the antisense orientation. The

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neo gene contained in this construct is defective as evidenced by the lack of a *Bst*YI site at nucleotide 2096. This alteration changes Glu-182 to aspartic acid (numbering as in reference 50). We found that the selection of spontaneously arising homozygotes required the use of this mutant *neo* gene, as targeted murine embryonic stem (ES) cell clones containing a wild-type *neo* cassette were resistant to extremely high levels of G418 (≥ 16 mg/ml), precluding the selection of homozygotes. The right arm of Δ RLPGK is a 5.7-kb *Eco*RI-*Spe*I DNA ligase I genomic fragment. The construct is cloned into pSKII (Stratagene) and is linearized at the *Not*I site flanking the left arm. Approximately 3 kb of the DNA ligase I locus from the *Sal*I site to the *Spe*I site is deleted in this construct.

Construction of DNA ligase I expression vectors. Human DNA ligase I expression vectors were assembled in pBluescript (Stratagene) by using the mouse phosphoglycerate kinase promoter (6) in conjunction with the bovine growth hormone poly(A) site excised from pRC/CMV (Invitrogen). *lig231* was derived from lig32B2 (32) by cleavage with *Bam*HI to delete the 5' 903 bp. The cohesive *Bam*HI terminus was converted to a blunt end by treatment with Klenow fragment and deoxynucleoside triphosphates (dNTPs). pBluescript containing the 3' human DNA ligase I *Eco*RI fragment (nucleotides 1543 to 3083; numbering according to the work of Barnes et al. [3]) was cut with *Nco*I and *Bam*HI to liberate all but the 5' 13 bp of this DNA ligase I segment from the plasmid backbone, gaattccaccagccATG, which contains an optimal methionine codon (25). Following treatment with Klenow fragment and dNTPs, the blunt 2,180-bp *Bam*HI fragment derived from lig32B2 was then ligated to the blunt 13-bp segment, thus creating the *lig231* coding sequence initiating at the optimal methionine. For expression in *S. cerevisiae*, *lig231* was placed in pDB20 (5) under the control of the alcohol dehydrogenase promoter. The yeast *lig32B2* and wild-type DNA ligase I expression constructs have been described previously (32).

Cell culture and transfection. For gene targeting, 25 μ g of *Not*I-linearized Δ RLPGK was electroporated into 5×10^6 D3 ES cells in a volume of 800 μ l of phosphate-buffered saline or normal growth medium with a Bio-Rad Gene Pulser at 575 V/cm (230 V in a 0.4-mm cuvette) and 500 μ F as described previously (19). Following electroporation, cells were plated onto two gelatinized 10-cm plates and placed under selection at 230 μ g of G418 per ml–0.2 μ M FIAU [1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil] in Dulbecco modified Eagle medium supplemented with 17% heat-inactivated fetal calf serum and 1% conditioned medium derived from leukemia inhibitory factor (LIF)-producing CHO cells (provided by Gordon Wong, Genetics Institute). For transfection of DNA ligase I expression constructs, 50 to 100 μ g of *Xho*I-linearized plasmid was cotransfected with 1 μ g of closed circular PGKpuro (provided by Jianzhu Chen) as described above. Transfected cells were plated on one 10-cm gelatinized plate and placed under puromycin selection at 1 μ g/ml for 8 to 12 days. Puromycin-resistant colonies were pooled, expanded to 2×10^6 to 4×10^6 cells, and subjected to 2.5-mg/ml G418 selection for 12 to 14 days. Resistant clones were expanded and analyzed. For the analysis of 3-aminobenzamide (3AB) sensitivity, duplicate ES cell cultures were seeded at 200 cells per well on gelatinized six-well plates and grown in the presence or absence of 8 mM 3AB for 8 days. Colonies were visualized by staining with crystal violet.

DNA ligase assays. Transformation of *cde9* was carried out as described by Ito et al. (22). Assays for DNA ligase I activity were carried out as previously described (32). Crude extracts were prepared from *cde9* transformants by physical disruption (31), and reactions were carried out at 37°C in 60 mM Tris (pH 8)–10 mM MgCl₂–5 mM dithiothreitol–1 mM ATP–50 μ g of acetylated bovine serum albumin (BSA) per ml containing 5' ³²P-labeled oligo(dT₂₅₋₃₀) annealed to poly(dA) (Pharmacia) in a 1:1 molar ratio as described previously (1). Reactions were stopped by heating at 68°C for 30 minutes and then treated with alkaline phosphatase (3 U) (Boehringer-Mannheim) for 3 h at 55°C and trichloroacetic acid precipitated, and Cerenkov counts were determined. One unit of DNA ligase activity corresponds to the conversion to an alkaline phosphatase-resistant form of 1 nmol of ³²PO₄ μ g of protein⁻¹ min⁻¹. The assay was linear over 10 min with the assay conditions described.

Analysis of DNA replication intermediates. Analysis of DNA replication intermediates was carried out by the method of Burhans et al. (7). Cells were trypsinized, pelleted, and resuspended in 100 μ l of ice-cold Dulbecco modified Eagle medium without serum. One hundred microliters of ice-cold 2 $\times$ replication cocktail (60 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES]–KOH [pH 7.8]; 0.2 mM dGTP, dTTP, and dATP; 0.4 mM GTP-CTP-UTP; 8 mM ATP; 20 mM MgCl₂; 0.2 mg of BSA per ml; 2 mM dithiothreitol; 30% glycerol) containing 5 to 20 μ l of [α -³²P]dCTP (10 mCi/ml, 3,000 Ci/mmol) was added, and cells were permeabilized by adding Nonidet P-40 to 0.4% and shifted to 34°C for 150 s. To stop reactions, 800 μ l of OKPK (50 mM Tris [pH 7.8], 10 mM EDTA [pH 8], 0.4 M NaCl, 0.6% SDS, 0.2 mg of proteinase K per ml) was added and incubated for 1 to 2 h at 37°C. Residual peptides were then precipitated by adding 330 μ l of saturated NaCl per ml of lysate. DNA was precipitated from the supernatant by addition of 2 volumes of absolute ethyl alcohol and fractionated on 6% polyacrylamide–8 M urea gels. Quantitation of radioactivity was carried out with a Betascope 603 (Betagen).

RESULTS

Disruption of the DNA ligase I locus. We used gene targeting to disrupt the DNA ligase I locus in ES cells. The targeting

DNA Ligase I locus

FIG. 1. Targeting strategy for the DNA ligase I locus. Vertical boxes correspond to DNA ligase I exons, the narrow line represents genomic regions, and the thick line represents Δ RLPGK DNA. The inserted *neo* gene carries a *Hind*III site, and so homologous integration of Δ RLPGK changes the size of the *Hind*III fragment containing the active site (K-583) from 9 kb to a 6.5-kb fragment that lacks K-583. The mouse and human DNA ligase I genes are highly conserved; the active site of the mouse enzyme is inferred from the location of the human one, where the two sequences are greater than 94% identical. The 9-kb genomic segment is detected by the NBA and MoBN probes, whereas the 6.5-kb fragment is detected by the NBA probe but not the MoBN probe. Restriction site abbreviations: B, *Bam*HI; H, *Hind*III; D, *Dra*I; E, *Eco*RI; S, *Sal*I; Sp, *Spe*I; N, *Not*I.

vector, Δ RLPGK, was designed to create a null allele, as the disrupted DNA ligase I locus contains the *neo* gene in place of the sequence that encodes the active site of the DNA ligase I enzyme at Lys-583 (Fig. 1) (41). Previous studies indicate that mutation of the analogous residue in human DNA ligase I results in complete inactivation of the enzyme (24). Linearized Δ RLPGK was transfected into ES cells, and G418-FIAU-resistant clones were analyzed by Southern blotting to identify homologous integrants with the NBA probe (see Fig. 3A). This probe detects the 6.5-kb *Hind*III fragment created by homologous integration of Δ RLPGK and the 9.0-kb *Hind*III fragment corresponding to the untargeted wild-type allele. One of the clones, 36PGK, that contains a DNA ligase I gene insertion is shown. The 6.5-kb *Hind*III fragment corresponding to the targeted allele is not detected by the MoBN probe, which comprises the sequences that have been deleted by gene targeting (see Fig. 3B). Detection of both bands by NBA and of the 9.0-kb fragment by MoBN thus defines the heterozygous null genotype. The frequency of homologous Δ RLPGK integration was approximately 1 in 50. We subsequently attempted to isolate spontaneously arising homozygous DNA ligase I mutant cell clones by culturing 36PGK, one of the heterozygous null mutant cell lines, in elevated levels of G418 (2.5 mg/ml) according to the method of Mortensen et al. (30). A heterozygous mutant contains a single *neo* gene and is sensitive to elevated levels of G418, whereas the homozygous mutant contains two copies of *neo* and thereby survives the stronger selection. From 3×10^6 36PGK cells placed under high-G418 selection, no viable clones arose, suggesting that homozygous null mutants of DNA ligase I are nonviable.

DNA ligase I expression constructs. To prove that the gene for DNA ligase I is an essential gene, we tested whether the homozygous null mutation could be preemptively rescued by transfection of a DNA ligase I cDNA expression construct.

FIG. 2. Strategy for preemptive complementation of a homozygous null mutation. Step 1, heterozygous null mutants are created by conventional gene targeting. Step 2, a mutant cDNA expression construct is introduced. Step 3, selection for spontaneous homozygous null mutants in high-G418 conditions is used to test for lethality of the homozygous mutant. The extent of deficiency in the ensuing homozygous clone is dependent upon the intrinsic properties of the mutant cDNA and its level of expression.

Further, we designed variant DNA ligase I expression constructs to test whether partially active DNA ligase I cDNAs could be used to rescue homozygous null mutants and thereby establish viable DNA ligase I-deficient ES cell lines (Fig. 2). Prior to application in the ES cell assay, variant DNA ligase I expression constructs were tested for their ability to rescue the conditionally lethal DNA ligase mutation in *S. cerevisiae*, *cdc9-1*. The wild-type human cDNA complements this mutation (3, 32), and so the ability of the variant cDNAs to rescue the *cdc9-1* mutant was used as a criterion of suitability for subsequent use in the ES cell assay. cDNA expression constructs were prepared from a wild-type human DNA ligase I cDNA (LIGI) as well as *lig32B2*, an alternative splice variant of DNA ligase I isolated from a human tonsillar cDNA library which lacks the 30 amino acids encoded by exon 2 (32). Ligase variant *lig231* is a 72-kDa truncation mutant of DNA ligase I that lacks sequences encoding the 231 N-terminal amino acids. The predicted *lig231* protein is approximately the same size as the minimal catalytic domain of DNA ligase I (24, 40). *cdc9-1* transformants were grown at the nonpermissive temperature (37°C) to test the ability of the human cDNA variants to complement the yeast defect (Table 1). As previously observed for *lig32B2*, *lig231* activity was sufficient to permit growth of *cdc9-1* at the nonpermissive temperature (Table 1). Measurement of the human DNA ligase I activity present in extracts prepared from the *cdc9-1* transformants showed that *lig231* and *lig32B2* encode 18 and 16% of the activity expressed by the wild-type DNA ligase I cDNA, respectively (Table 1) (32).

The gene for DNA ligase I is an essential gene. 36PGK was transfected with each of the DNA ligase I expression constructs: a wild-type human DNA ligase I cDNA, PGKLIGI, PGKlig32B2, PGKlig231, and an inert plasmid as a negative

control. Expression of the cDNAs in mammalian cells was under the control of the phosphoglycerate kinase promoter, which is highly active in ES cells (37). cDNA transfectant pools were established by selection in puromycin, expanded to approximately 3×10^6 cells, and subjected to elevated levels of G418. Following 12 to 14 days in culture, the PGKLIGI and PGKlig32B2 transfectants gave rise to colonies in 2.5 mg of G418 per ml. These colonies were expanded and analyzed by Southern blotting to assess the status of their DNA ligase I loci. The wild-type PGKLIGI transfectants gave rise to eight resistant colonies which were shown to be homozygous for the targeted DNA ligase I allele by Southern blotting (Table 1 and Fig. 3A). In addition, five PGKlig32B2 transfectant clones were found to lack the 9.0-kb *HindIII* fragment that corresponds to the wild-type allele, indicating that these clones were also homozygous for the Δ RPLGK-disrupted, null DNA ligase I allele (Table 1 and Fig. 3A). The homozygous mutant genotype of the PGKLIGI-expressing clones along with those of three *lig32B2* clones, 36-32B2.6, 36-32B2.9, and 36-32B2.21, was confirmed with the MoBN probe (Fig. 3B), where only hybridization to the transfected cDNA was observed. Thus, ectopic expression of the wild-type DNA ligase I cDNA was sufficient to effect preemptive complementation of the homozygous null mutation.

Given the reduced activity of *lig32B2* in the yeast system, we tested the level of expression required to enable this partially active variant to complement the homozygous DNA ligase I mutation. The expression level of *lig32B2* in the homozygous mutants was assessed by Northern and Western blotting. Northern blotting was carried out with the MoBN probe so that only mRNA derived from the *lig32B2*-transfected cDNA would be detected (Fig. 3C). The levels of DNA ligase I mRNA in 36-32B2.6, 36-32B2.9, and 36-32B2.21 were significantly reduced in relation to the mRNA levels seen in the wild-type control (Fig. 3C). Although the three homozygous mutants tested showed reduced levels of DNA ligase I protein, the differences observed were less significant than those seen at the mRNA level (Fig. 3D).

The N terminus of DNA ligase I is required for in vivo function. Pooled *lig231* transfectants failed to yield homozygous mutants upon selection in elevated levels of G418 (Table 1), although *lig32B2* and *lig231* encode similar levels of DNA ligase I activity in the heterologous yeast system. *lig231*-transfected cells were cloned and screened to identify clones with high levels of *lig231* expression to test whether overexpression of this mutant could compensate for its reduced ability to rescue viable homozygous mutants. Three clones, 36-231.2, 36-231.8, and 36-231.11, expressed high levels of the 2.2-kb *lig231* mRNA as well as the 72-kDa *lig231* protein (Fig. 3C and D). These highly expressing clones also failed to yield homozygous colonies upon selection under high-G418 conditions (Table 1). Although *lig231* complements the *cdc9* defect, the deleted N-terminal domain in this variant appears to include sequence that is critical for the function of DNA ligase I in mammalian cells. This result demonstrates that the preemptive complementation strategy can be used to carry out structure-function analysis of the protein encoded by the targeted locus.

DNA replication and repair phenotypes of DNA ligase I-deficient ES cells. We examined whether the LIGI or *lig32B2* activity ectopically expressed in the ES cell mutants was sufficient to mediate efficient joining of lagging-strand DNA during DNA replication. Given the role of DNA ligase I in lagging-strand DNA synthesis (15, 21, 45), reduced DNA ligase I activity might impair Okazaki fragment joining and lead to an accumulation of cells in the S or G₂ phase of the cell cycle. Using propidium iodide staining and flow cytometry, we found

TABLE 1. Properties of DNA ligase I cDNA expression constructs

cDNA	<i>cdc9</i> growth ^a	% wild-type activity ^b	No. of cells (high G418) ^c	No. of homozygotes isolated ^d
LIGI	+	100	18×10^6	8
Vector	-	0	20×10^6	0
<i>lig32B2</i>	+	16	3×10^6	5
<i>lig231</i> ^e	+	18	50×10^6	0

^a Yeast strain provided by J. Haber; *cdc9-1 ura3*.

^b Determined by heterologous expression in *S. cerevisiae*.

^c High-G418 selection was carried out on semiconfluent cultures of 2×10^6 to 4×10^6 cells per well. Values shown are based on the number of wells tested for a given transfectant, assuming an average of 3×10^6 cells per culture.

^d As determined by Southern blotting.

^e Number of cells tested includes transfectant pool and all tests on 12 ensuing clones.

FIG. 3. Analysis of DNA ligase I structure and expression in targeted ES cell clones and derivatives. (A) Five micrograms of genomic DNA prepared from the ES cell clones was digested with *Hind*III, fractionated on 0.8% agarose gels, and analyzed by Southern blotting with the NBA probe. D3 and 36PGK are wild-type and heterozygous controls, respectively. Sizes are indicated in kilobases. (B) The same filter was stripped and rehybridized to the MoBN probe. Note that hybridization to the transfected cDNA was observed but is cropped out of the photographs in panels A and B. (C) For Northern blots, 25 µg of total RNA from ES cell clones was fractionated on 1% agarose-formaldehyde gels and hybridized to the MoBN probe (upper) or the glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH) (lower). The lower band of the doublet seen in the *lig231* lanes corresponds to the *lig231* transcript which is approximately 700 bp shorter than the full-length one. (D) Cells (5×10^5) were harvested from subconfluent wells and lysed in sample buffer under reducing conditions. Proteins were fractionated by SDS-7.5% PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis with a 1:250 dilution of rabbit anti-DNA ligase I peptide antiserum, F6F. Secondary detection of antibody complexes was effected with horseradish peroxidase-coupled A+G protein and complexes were visualized by chemiluminescence (Dupont).

TABLE 2. Phenotypes of DNA ligase I-deficient ES cells

Cell line	Genotype and DNA ligase I locus	% Survival (8 mM 3AB) ^a	% Newly synthesized DNA ^b
D3	+/+	59	41
V2 ^c	+/-	21	47
LIGI-7.15	-/-/FL ^d	35.9	50
LIGI-12.3	-/-/FL	41.7	48
LIGI-12.4	-/-/FL	47.6	46
36-32B2.6	-/-/32B2	9	55
36-32B2.9	-/-/32B2	19.5	ND ^e
36-32B2.21	-/-/32B2	8	63

^a Values shown correspond to the ratio of colony numbers with or without 3AB and are based on two to four independent cultures.

^b Values shown are the percentages of total incorporated dCTP found in 75- to 150-bp DNA fragments.

^c V2 is a clone derived from 36PGK by transfection of the nonrecombinant vector as a negative control in the test for DNA ligase I lethality.

^d FL, full-length DNA ligase I (LIGI).

^e ND, not determined.

that the cell cycle distribution of 36PGK and the parent ES cell line, D3, was indistinguishable from that of *lig32B2* clones 36-32B2.6, 36-32B2.9, and 36-32B2.21 (data not shown). The ability of the products of the added LIGI and *lig32B2* genes to mediate Okazaki fragment joining was assessed directly. Exponentially growing cells were permeabilized and pulse-labeled with [³²P]dCTP; these were followed by isolation and electrophoresis of total genomic DNA under denaturing conditions to examine the abundance of low-molecular-weight, newly synthesized DNA (7) (see also Materials and Methods). Added LIGI expression yielded a fraction of Okazaki fragment joining that was indistinguishable from the D3 and V2 controls (Table 2). The impact of *lig32B2* upon Okazaki fragment joining was not severe, although we reproducibly observed an increased percentage of the newly synthesized DNA of 75 to 150 bp in the mutant cells relative to wild-type controls (Table 2). Thus, *lig32B2* expression is sufficient to mediate DNA replication and permit cell proliferation in the complete absence of endogenous DNA ligase I activity.

The DNA ligase I mutation in 46BR renders this cell line sensitive to killing by 3AB (28). The basis of 3AB toxicity has not been well defined, but it appears that the drug generally inhibits the repair of DNA strand breaks and is thereby differentially toxic to repair-deficient cells in which the steady-state level of DNA ends is constitutively elevated (35). We tested whether the LIGI- and *lig32B2*-expressing homozygous mutant cells were sensitive to killing by 3AB. LIGI-expressing clones yielded the same level of 3AB cell survival as D3 parental cells without ligase mutations. The homozygous *lig32B2* clone 36-32B2.9 and heterozygous mutant V2, which is a puromycin-resistant derivative of 36PGK, exhibited a threefold increase in 3AB sensitivity. 36-32B2.6 and 36-32B2.21 were sevenfold more sensitive to 3AB than the wild-type ES parent (Table 2). This observation may reflect that 3AB sensitivity is a more sensitive indicator of DNA ligase I function than the efficiency of lagging-strand DNA synthesis. Alternatively, given the minimal impact of *lig32B2* expression on DNA synthesis, the increased 3AB sensitivity of *lig32B2* expressors may indicate that DNA ligase I functions that are not associated with DNA replication, such as DNA repair, are differentially affected by this DNA ligase I variant.

DISCUSSION

Selective inactivation of genes by homologous recombination in murine ES cells is a powerful methodology. However, the analysis of essential loci is problematic since lethality of the null mutant precludes the application of conventional gene targeting strategies. As the genes which mediate mammalian DNA repair and replication functions become available, this limitation may be particularly significant since these functions are likely to be essential, and thus null mutants produced by targeted disruption would interfere with cell viability. Currently, no procedures are available to assess the cellular lethality of targeted mutations. Gene targeting strategies have been used to impart subtle, nonnull mutations by a stepwise inactivation and partial restoration of targeted loci (18, 43). However, these approaches are cumbersome for the creation of several different mutants because the creation of each new mutant requires an independent targeting procedure. An alternative strategy recently developed involves coupling the targeting event to an inducible system for tissue- or stage-specific inactivation of the targeted locus (16) but similarly requires a new targeting procedure for each mutant analyzed.

The strategy described in this report exploits the fact that homozygous mutant clones arise spontaneously from targeted heterozygous mutants (Fig. 2) (31). Ectopic expression of a cDNA in a heterozygous null mutant is established prior to testing whether the introduced cDNA is required to rescue the viability of spontaneously arising homozygous null mutants. An advantage of this approach is that partially active cDNAs can be introduced prior to the selection for homozygous mutants and thus a number of distinct mutant cell lines can be established from the original heterozygous knockout. The extent of deficiency in these cell lines is dependent upon the intrinsic properties of the ectopic cDNA and the level at which it is expressed.

In the present study, we have applied this strategy to demonstrate that DNA ligase I is an essential gene and further show that both a full-length DNA ligase I cDNA and a partially active DNA ligase I cDNA are able to preemptively complement the homozygous DNA ligase I null mutation. The DNA ligase I variant cDNAs used in this study (*lig32B2* and *lig231*) retain sufficient activity to mediate DNA replication in *S. cerevisiae* (32); both mutant proteins are stably expressed in ES cells (Fig. 3D), and yet only *lig32B2* was able to rescue viable homozygous DNA ligase I mutants. Hence, the N-terminal domain of DNA ligase I is essential in mammalian cells.

This region of DNA ligase I may be a site of posttranslational regulation. DNA ligase I activity in vitro is increased by casein kinase II phosphorylation, whereas the activity of a truncation mutant similar to *lig231* lacking the N-terminal 250 amino acids is not affected by casein kinase II phosphorylation (24, 33). Recent characterization of bacterially produced DNA ligase I indicates that phosphorylation is not a requirement for DNA ligase I activity (38), so the loss of these phosphorylation sites in *lig231* is unlikely to account for its inability to rescue the homozygous null DNA ligase I mutation. Other aspects of posttranslational regulation may rely on the N-terminal domain of DNA ligase I. It has been proposed that a mutant DNA ligase I-associated protein may be responsible for the DNA ligase I deficiency associated with Bloom syndrome (32, 49). Consistent with this hypothesis, DNA ligase I-containing protein complexes have been identified (29, 49). Our data might reflect that the N-terminal region of DNA ligase I is required for these protein interactions in mammalian cells and that DNA ligase I function in vivo is dependent on its participation in these complexes. The strategy that we have designed

to analyze DNA ligase I is particularly advantageous in this regard, as the identification of functionally relevant domains does not rely upon heterologous expression systems in which relevant protein interactions may not occur.

It is surprising that reduced DNA ligase I function does not severely affect the growth properties of mutant ES cells or 46BR. Because DNA ligase I functions in DNA replication, it seemed likely that rapidly proliferating cells would be disproportionately affected by deficiency in this enzyme. In the case of logarithmically growing ES cells, more than 65% of the cells are in S phase, yet we did not observe a significant difference in the cell cycle profiles of control and mutant cells (data not shown). Similarly, in our hands untransformed 46BR and simian virus 40-transformed 46BR derivatives were quite robust. These observations suggest that the level of DNA ligase I activity in normal cells substantially exceeds the level required to mediate efficient lagging-strand DNA synthesis. It is also conceivable that long-lived breaks in nascent DNA strands resulting from reduced DNA ligase I activity may be repaired by DNA ligase II or III. In particular, DNA ligase III activity is high in proliferating cells (42), and cell cycle progression might not be overtly impaired in DNA ligase I-deficient cells if the repair of lagging strands is efficiently mediated by DNA ligase III. Since the role of DNA ligase I in lagging-strand DNA synthesis has been inferred largely from in vitro studies (15, 21, 29, 45), the ability of DNA ligase II or III to participate in the completion of nascent lagging strands would not have been detected. Our data indicate that DNA ligase I activity cannot be replaced by DNA ligase II or III but do not address whether DNA ligase I function is augmented by these activities in vivo. Consistent with this conclusion, Waga et al. have recently shown that DNA ligase III is unable to substitute for DNA ligase I in in vitro simian virus 40 DNA replication (44).

Alternatively, our findings may suggest that the three mammalian DNA ligase activities are unable to compensate for each other and that the extent of DNA ligase I deficiency tolerable to mammalian cells is low. DNA ligase I deficiency severe enough to significantly impair cell cycle progression might result in lethal genomic instability. Hence, application of the preemptive complementation strategy to rescue of the homozygous DNA ligase I mutant would require cDNAs encoding sufficient DNA ligase I activity to permit efficient lagging-strand DNA synthesis. Similarly, spontaneous DNA ligase I mutants, such as those in 46BR, would be inviable unless DNA replication was relatively unaffected.

Neither *lig32B2*-expressing ES cell clones nor heterozygous DNA ligase I null mutant cells exhibited sensitivity to ionizing or UV radiation (data not shown). Further, the DNA repair defects exhibited by 46BR are relatively subtle (20, 27, 34), indicating that DNA ligase I activity may be redundant with the other DNA ligases in DNA repair processes. Consistent with this idea is the observation that reduced DNA ligase III activity in the CHO mutant, EM9, is associated with sensitivity to ionizing radiation and reduced ability to repair DNA strand breaks (28, 39).

DNA ligase I deficiency syndromes are associated with growth defects, immune deficiency, and cancer predisposition (12, 46). The features of these human syndromes indicate that DNA ligase I deficiency constitutes a powerful model system to study the effects of generalized DNA repair deficiency on immune function and tumorigenesis. The use of ES cells to derive DNA ligase I-deficient mutants will allow us to further examine the impact of DNA ligase I deficiency in an animal model and to gain further insight into the physiological role of this enzyme.

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