The structure-specific endonuclease Ercc1–Xpf is required for targeted gene replacement in embryonic stem cells

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The Ercc1–Xpf heterodimer, a highly conserved structure-specific endonuclease, functions in multiple DNA repair pathways that are pivotal for maintaining genome stability, including nucleotide excision repair, interstrand crosslink repair and homologous recombination. Ercc1–Xpf incises double-stranded DNA at double-strand/single-strand junctions, making it an ideal enzyme for processing DNA structures that contain partially unwound strands. Here we demonstrate that although Ercc1 is dispensable for recombination between sister chromatids, it is essential for targeted gene replacement in mouse embryonic stem cells. Surprisingly, the role of Ercc1–Xpf in gene targeting is distinct from its previously identified role in removing nonhomologous termini from recombination intermediates because it was required irrespective of whether the ends of the DNA targeting constructs were heterologous or homologous to the genomic locus. Our observations have implications for the mechanism of gene targeting in mammalian cells and define a new role for Ercc1–Xpf in mammalian homologous recombination. We propose a model for the mechanism of targeted gene replacement that invokes a role for Ercc1–Xpf in making the recipient genomic locus receptive for gene replacement.

Keywords: D-loops/double-strand breaks/heterology/homologous recombination

Introduction

hERCC1 was originally identified as the human gene that corrects hypersensitivity to UV irradiation in one of 11 Chinese hamster ovary (CHO) cell complementation groups (Westerveld *et al.*, 1984), hence the name excision repair cross complementation group 1. The gene is highly conserved with homologs in mouse, *mErcc1* (Selfridge *et al.*, 1992), *Saccharomyces cerevisiae*, *RAD10* (van Duin *et al.*, 1986) and *Schizosaccharomyces pombe*, *swi10* (Rodel *et al.*, 1992). In cells, the Ercc1 protein exists in a tight complex with Xpf (Biggerstaff *et al.*, 1993; van Vuuren *et al.*, 1993). Heterodimerization is required to

stabilize both proteins and for complementation of DNA repair defects. Xpf is also highly conserved (Sijbers *et al.*, 1996), as is heterodimeric interaction. The Rad10–Rad1 protein complex was discovered to be an endonuclease (Tomkinson *et al.*, 1993) that cleaves double-stranded (ds) DNA only on the strand with a 3′ single-strand (ss) tail (Bardwell *et al.*, 1994). This substrate specificity has helped to define the role of Ercc1–Xpf in correcting UV sensitivity. The complex is responsible for making a cut in the damaged strand of duplex DNA 5′ to a lesion in nucleotide excision repair (NER) (Mu *et al.*, 1995; Sijbers *et al.*, 1996). In addition, the complex cleaves stem-loop and splayed arm structures within duplex DNA and only on the strand with the 3′ ss tail (de Laat *et al.*, 1998a).

Although ERCC1 plays an essential role in NER, it is unable to complement the UV sensitivity of any of the eight complementation groups of fibroblast cell lines derived from NER-deficient xeroderma pigmentosum patients (van Duin *et al.*, 1989). Nor has a viable mutation in *hERCC1* been described. Furthermore, *Ercc1*^{−/−} mice display a much more severe phenotype than other NER-deficient mouse models (McWhir *et al.*, 1993; Weeda *et al.*, 1997). These observations imply additional function(s) for Ercc1 outside NER. Hints as to this additional function(s) come from studies of mutant cell lines. Ercc1- and Xpf-deficient mammalian cells, unlike other NER-deficient cells, are sensitive to DNA crosslinking agents (Westerveld *et al.*, 1984; Hoy *et al.*, 1985). However, they are not ionizing radiation (IR)-sensitive. This is also true for *rad10*, *rad1* (Moore, 1978) and *swi10* mutants (Hang *et al.*, 1996). These observations indicate a role for Ercc1–Xpf in NER-independent interstrand crosslink repair but not in double-strand break (DSB) repair.

In addition to involvement in NER and interstrand crosslink repair, there is evidence for a role of Ercc1–Xpf in recombination. For example in *S.cerevisiae*, mutations in *rad1* or *rad10* suppress mitotic recombination between direct or inverted repeats (Klein, 1988; Schiestl and Prakash, 1988, 1990; Prado and Aguilera, 1995). Furthermore, recombination between repeats yields different products in *rad1* and *rad10* compared with wild-type (wt) strains (Klein, 1988; Aguilera and Klein, 1989; Zehfus *et al.*, 1990). Excisional products in which the sequence between the repeats is lost are less frequently recovered from the mutant strains. Similar results were obtained in Ercc1-deficient CHO cells (Sargent *et al.*, 2000). The *rad1* phenotype is not exaggerated by a mutation in *rad10* (Schiestl and Prakash, 1990), implying that the role these proteins play in recombination is dependent upon protein heterodimer formation and therefore most likely its endonuclease function.

Intrachromosomal recombination between directly repeated sequences occurs most efficiently via single-strand annealing (SSA) (Paques and Haber, 1999). SSA requires

Fig. 1. Targeting strategy for sequential inactivation of both *mErcc1* alleles in mouse ES cells. (A) The top of the scheme depicts the 3' end of the *mErcc1* genomic locus with relevant restriction sites. Numbered boxes indicate exons. Beneath, the two targeting constructs are depicted. Exon 7 of *mErcc1* was disrupted by insertion of either a *neo*^R or *hygro*^R marker containing a diagnostic *Bam*HI restriction site. ES cells were transfected first with the *mErcc1*^{neoR} construct. Genomic DNA was isolated from resistant clones, digested with *Bam*HI and analyzed by DNA hybridization using a 5' external probe (indicated above the constructs). The lengths of the expected genomic fragments are indicated with horizontal arrows below the constructs. *mErcc1*^{+/-} cells were subsequently transfected with the *mErcc1*^{hygroR} construct and grown under hygromycin B selection. Positive clones were analyzed by DNA blotting. (B) An example of a DNA blot of *Bam*HI-digested genomic DNA isolated from ES clones and probed with the 5' external probe is shown. The probe detects either an 8-kb fragment (wt allele), 4.8-kb fragment (*hygro*^R mutant) or 4.2-kb allele (*neo*^R mutant). Two independent double mutant clones were identified.

a DSB between or within one of the repeats followed by exonuclease resection from both ends revealing complementary 3' ss overhangs. The regions of homology within the two overhangs align and anneal, ultimately yielding a product in which one of the repeats and all intervening sequences are lost. The proposed role for Ercc1-Xpf in SSA is to remove nonhomologous termini from the 3' ss overhangs after the overhangs have realigned. Indeed, Rad1 and Rad10 do cleave nonhomologous ss termini from the ends of a DSB to facilitate repair via homologous recombination (HR) in yeast (Fishman-Lobell and Haber, 1992; Ivanov and Haber, 1995; Prado and Aguilera, 1995; Paques and Haber, 1997), as do the hamster homologs in CHO cells (Sargent *et al.*, 2000). Similarly, Ercc1 is required to remove nonhomologous tails from ends-in-targeting constructs in CHO cells (Adair *et al.*, 2000).

There is also mounting evidence that Ercc1-Xpf (and their homologs) participate in the processing of recom-

bination intermediates in which the DNA substrates are not identical, but the sequence nonhomology is positioned between homologous domains rather than at an end. For example, gene conversion products resulting from recombination between direct repeats that differ by distally positioned deletions are not recovered in a *rad1* strain (Klein, 1988). Similarly, in *rad1* and *rad10* strains, gene targeting using a linear construct is suppressed 40-fold (Schiestl and Prakash, 1988, 1990; Saparbaev *et al.*, 1996). Furthermore, mutations in *rad1* or *rad10* suppress meiotic repair of loops created by patches of nonhomology between homologous chromosomes (Kirkpatrick and Petes, 1997; Kearney *et al.*, 2001) as do mutations in the *S.pombe* homologs (Fleck *et al.*, 1999). Finally, in Ercc1-deficient CHO cells intrachromosomal recombination between direct repeats separated by 600 bp of intervening sequence yields many more gene rearrangements compared with Ercc1-proficient cells (Sargent *et al.*, 1997), in particular deletions beginning at the borders between the repeat and the intervening sequence.

In the experiments described below, we discovered that Ercc1 is absolutely required for HR between gene replacement constructs and genomic loci in mouse embryonic stem (ES) cells. We tested linear constructs consisting of a selectable marker flanked by completely homologous arms and constructs including additional nonhomologous termini. This allowed us to determine whether the essential function of Ercc1 in gene replacement was removal of 3' nonhomologous termini, similar to SSA, or processing of looped-out heteroduplex intermediates. Our data are consistent with the latter and have implications for the mechanism of targeted gene replacement in mammalian cells.

Results

Generation of *mErcc1*^{-/-} and rescued ES cells

A clonal *mErcc1*^{+/-} [neomycin-resistant (*neo*^R)] ES cell line (Weeda *et al.*, 1997) was transfected with a linearized *mErcc1* targeting construct containing a hygromycin selectable marker (*hygro*^R) with the goal of disrupting the second *mErcc1* allele and thereby generating a nullizygous cell line (Figure 1A). More than 300 *hygro*^R clones from multiple transfection experiments were analyzed by DNA blot analysis (Figure 1B). The absolute targeting efficiency of the *mErcc1* locus was 16% as previously reported (Weeda *et al.*, 1997). However, there was disequilibrium between targeting the wt and mutant alleles of the *mErcc1*^{+/-} cells. In only two out of 48 targeted clones was the wt *mErcc1* allele replaced by the *hygro*^R construct. Thus, the frequency of generating *mErcc1*^{-/-} cells was 12-fold lower than expected, suggesting selection against Ercc1-deficient ES cells. Indeed, the *mErcc1*^{-/-} cells required daily passaging to maintain morphology and growth characteristics typical of wt ES cells.

To verify that the phenotype of the *mErcc1*^{-/-} cells was directly attributable to the loss of functional Ercc1, we generated rescued mutant lines. *mErcc1*^{-/-} ES cells were transfected with a plasmid harboring the *hERCC1* cDNA fused in-frame with a downstream sequence encoding yellow fluorescent protein (YFP). Transformants were screened for expression of ERCC1-YFP by fluorescence

Fig. 2. Immunoblot analysis of wt, *mErcc1*^{-/-} and rescued mutant ES cell lysates. **(A)** Immunoblot of WCE using α-hERCC1 polyclonal antibody. The antibody recognizes a 39 kDa band in the positive control (HeLa cells, lane 6). Lanes 2–4 contain lysates from three cell lines (#8, 9 and 10) expanded from individual MMC-resistant clones established by stable transfection of the *mErcc1*^{-/-} ES cells with a construct containing the *hERCC1* cDNA coupled in-frame with YFP. Expression of only the full-length fusion protein is seen as a single band with the predicted molecular mass of 66 kDa (39 kDa ERCC1 + 27 kDa YFP). Lane 5 contains a lysate from *mErcc1*^{-/-} ES cells. **(B)** Immunoblot of WCE using α-hXPF polyclonal antibody. A band corresponding to XPF (115 kDa) is visible in the HeLa cell and wt ES cell lysates (lanes 2 and 3, respectively) but not in the *mErcc1*^{-/-} mutant line (lane 4). Rescue of the mutant line with *hERCC1*-YFP restores the Xpf band to physiological levels (lane 5). The presence of a cross-reactive band in the ES cells (~75 kDa) serves as a loading control.

microscopy. In all of the clones screened, the fluorescent signal was low and restricted to the nucleus. These characteristics are inconsistent with expression of YFP alone. Immunoblot analysis of whole cell extracts (WCE) from the individual clones with α-hERCC1 antibody indicated expression of full-length ERCC1-YFP fusion protein but not ERCC1 by itself (Figure 2A). Furthermore, fusion protein levels were similar to levels of ERCC1 in HeLa cells, indicating physiological expression levels. The α-hERCC1 antibody does not recognize mouse Ercc1, therefore the absence of a protein signal in *mErcc1*^{-/-} cell extract does not formally prove that there is no endogenous Ercc1 present in the mutant cell lines. However, immunoblot analysis with α-hXPF antibody, which does recognize the endogenous mouse protein,

Fig. 3. Effect of UV, MMC and IR on wt and *mErcc1*^{-/-} ES cells. **(A)** Clonogenic survival of wt (squares), *mErcc1*^{-/-} (triangles) and a rescued mutant line (circles) after UV irradiation. The percent of irradiated cells that were able to form a colony compared with untreated cells is plotted against the dose of UV. **(B)** Clonogenic survival of wt, *mErcc1*^{-/-} and a rescued mutant line after treatment with MMC for 1 h. **(C)** Clonogenic survival of ES cells after treatment with IR. Since the *mErcc1*^{-/-} line was not hypersensitive to irradiation, a rescued line was not tested. An *mRad54*^{-/-} ES line (diamonds) was used as a positive control for irradiation.

demonstrates markedly reduced levels of Xpf protein in the *mErcc1*^{-/-} lines (Figure 2B). This is consistent with previous findings of destabilization of Xpf in the absence of Ercc1 (Sijbers *et al.*, 1996; Houtsmuller *et al.*, 1999; Gaillard and Wood, 2001). Xpf protein levels in the rescued mutant line were comparable to wt ES cells.

Characterization of *mErcc1*^{-/-} ES cells

To characterize the *mErcc1*^{-/-} cells, we examined their sensitivity to three types of DNA damaging agents: UV, mitomycin C (MMC) and IR. UV causes lesions that are substrates for NER, while MMC induces interstrand crosslinks and IR generates DSBs that in ES cells are efficiently repaired by HR (Dronkert *et al.*, 2000). We observed that the *mErcc1*^{-/-} cells are 2.7-fold more sensitive to UV than wt cells (Figure 3A). In fact, the Ercc1 mutant was equally as sensitive to UV irradiation as an *Xpa*^{-/-} ES line (H.de Waard, unpublished data) consistent with a complete NER defect. The cell lines stably transfected with *hERCC1* cDNA behaved identically to the parental wt line, demonstrating a complete restoration of NER. The *mErcc1*^{-/-} cells were 10-fold more sensitive to low doses of MMC than the wt and rescued mutant cells (Figure 3B). These results were not crosslink agent-specific, as the *mErcc1*^{-/-} ES cells were also 16-fold more sensitive to the crosslinking agent cisplatin and 12-fold more sensitive to 8-methoxypsoralen + UVA than the wt cells (data not shown). The *mErcc1*^{-/-} cells were not

Fig. 4. Strategy for quantitating gene replacement in wt and *mErcc1*^{-/-} ES cells. (A) Top, the central portion of the *mRad54* genomic locus is shown. Exons are depicted as numbered boxes. The targeting construct was generated by inserting a *puro*^R expression cassette into intron 3. Once homologously recombined into the genome, the *StuI* fragment encompassing this region is shortened from 9.0 to 8.5 kb. The targeting construct was isolated from a vector as either an *EcoRI* fragment (completely homologous to the *mRad54* locus, with the exception of the selectable marker), or a *ClaI*-*NotI* fragment (which contains 32 bp of bacterial sequence on the 5' and 14 bp on the 3' end of the construct, indicated as dashed lines; not drawn to scale). Stable transformants were selected with puromycin. (B) Genomic DNA was isolated from individual resistant clones and analyzed by DNA hybridization after *StuI* digestion. Targeting was detected by the appearance of an 8.5-kb DNA fragment using a 3' external probe from exons 7 and 8 of *mRad54* cDNA.

sensitive to IR (Figure 3C). This was in contrast to the HR-defective *mRad54*^{-/-} ES. Finally, to screen further for a role for Ercc1 in HR, the frequency of sister chromatid exchange (SCE) was measured in the different ES cell lines. The *mErcc1*^{-/-} cells displayed the same number of SCEs as the parental wt line or the rescued mutant lines (data not shown), indicating that the absence of functional Ercc1 does not preclude HR. This was true whether spontaneous or MMC-induced SCEs were measured. Together, these data define the *mErcc1*^{-/-} ES cells as NER-deficient, crosslink repair-deficient but DSB repair- and HR-proficient.

Measuring gene replacement frequency in *mErcc1*^{-/-} ES cells

To explore the possibility of a role for Ercc1 in recombination between non-identical substrates, we examined the capacity of the mutant cells for gene targeting. In order to do this, the mutant, wt and rescued ES cells were transfected with a linear targeting construct consisting of a

puromycin resistance (*puro*^R) selectable marker flanked on both sides with ~4 kb of sequence homologous to the *mRad54* genomic locus (Figure 4). The construct was isolated either with or without 14–32 bp of heterologous sequences at each end.

In the wt cell line, the construct with the nonhomologous termini targeted the *mRad54* locus with 30% efficiency (Table I). In stark contrast, transfection of the same construct into *mErcc1*^{-/-} cells did not yield a single targeting event in >225 *puro*^R clones. This represents a >78-fold drop in targeting efficiency. In the *mErcc1*^{-/-} ES line rescued with a tagged *hERCC1* cDNA, the targeting efficiency returned to wt levels. Unexpectedly, the complete absence of targeting events in the *mErcc1*^{-/-} ES cells was also observed when the ends of the transfected linear construct were completely homologous to the genomic locus. Thus, these data define a new role for Ercc1 in gene targeting in mammalian cells that is distinct from the previously identified role in removing nonhomologous termini from recombinational intermediates.

To exclude the possibility that the abrogation of gene targeting in *mErcc1*^{-/-} ES cells was construct related, we also tested other linear 'ends-out' constructs that target the murine retinoblastoma (*mRb*) or Cockayne syndrome B (*mCsb*) locus (Table I). With these additional constructs, we again never observed a single event of gene replacement (0/212 *puro*^R clones and 0/160 *puro*^R clones, respectively) in the Ercc1-deficient cells, although the targeting efficiency was 18–20% in wt ES cells. Nor was the abrogation of gene targeting peculiar to the particular *mErcc1*^{-/-} cell line studied, as examination of a second *mErcc1*^{-/-} and two additional rescued mutant lines yielded results in accordance with Table I (0/165 *puro*^R clones in the mutant cell line; 7/38 and 6/30 *puro*^R clones in the rescued line). By way of comparison, we also determined the targeting frequency of these same constructs in an HR-deficient *mRad54*^{-/-} ES cell line. In the *mRad54*^{-/-} cells, targeted integration was reduced by 5- to 15-fold, but not abolished. The difference in the level of suppression of gene replacement between the *mErcc1*^{-/-} and *mRad54*^{-/-} ES cells was statistically significant (*p* < 0.001).

Discussion

Generation of *mErcc1*^{-/-} and rescued ES cell lines

Both alleles of the *mErcc1* locus were disrupted in ES cells via sequential rounds of gene targeting (Figure 1). In the second round of targeting, disruption of the remaining wt *Ercc1* allele occurs 12-fold less frequently than re-targeting of the previously replaced allele, suggesting selection against Ercc1-deficient ES cells, as previously reported in primary mouse embryonic fibroblasts (MEFs) (Weeda *et al.*, 1997). A rescued mutant line (*mErcc1*^{-/-} cells stably transfected with *hERCC1*-YFP cDNA) was also generated for use as a positive control during phenotypic analysis of the *mErcc1*^{-/-} ES cells. The heterodimeric partner of Ercc1, Xpf, is not detected in *mErcc1*^{-/-} ES cells by immunoblotting, but is detected in the rescued line (Figure 2B). This result demonstrates that in ES cells, like in CHO cells (Sijbers *et al.*, 1996), the absence of Ercc1 destabilizes Xpf. This result also provides evidence for the absence of functional Ercc1 protein in the *mErcc1*^{-/-} cells. The *mErcc1* nullizygous

Table I. Frequency of gene replacement events in ES cells of the indicated genotypes

ES cell genotype	Targeted locus:	<i>mRad54</i>		<i>mRb</i>		<i>mCsb</i>	
	Construct ends:	non-homologous	homologous	non-homologous	non-homologous	non-homologous	non-homologous
wt		30.9% ^a (30/97)	25.7% (37/144)	20.0% ^{c,f} (19/95)	17.9% ^e (10/56)		
<i>mErcc1</i> ^{-/-}		<0.4% ^b (0/227)	<0.4% ^b (0/269)	<0.5% ^b (0/212)	<0.6% ^b (0/160)		
<i>mErcc1</i> ^{-/-} + <i>hERCC1</i>		14.7% ^{c,d} (21/143)	26.1% ^{c,d} (36/138)	n.d.	n.d.		
<i>mRad54</i> ^{-/-}		1.6% (4/249)	2.1% (6/284)	3.3% ^e (2/61)	4.3% ^e (3/69)		

n.d., not determined.

^aPercent of homologous integration events amongst the total number of integration events. Absolute numbers are shown in parentheses.

^bThe difference between the *mErcc1*^{-/-} ES cells and the wt cells is statistically significant for all targeting constructs ($p < 0.001$ by χ^2 analysis).

^cThe difference between the rescued *mErcc1*^{-/-} cell line and the wt cell line is not statistically different ($p > 0.01$). The difference between the rescued *mErcc1*^{-/-} cell line and the *mErcc1*^{-/-} ES cells is significant ($p < 0.001$).

^dThe difference between the results obtained with the two targeting constructs in the rescued ES line is not statistically significant ($p > 0.05$).

^ePreviously reported (Essers *et al.*, 1997).

^fThis data was collected in a *mRad54*^{+/-} ES cell line (Essers *et al.*, 1997).

cells are as sensitive to UV irradiation (Figure 3A) as a complete NER-deficient ES cell line (*Xpa*^{-/-} ES cells; H.de Waard, unpublished results). This level of sensitivity corresponds to that of immortalized MEFs derived from a knock-out mouse and to a CHO mutant line (Melton *et al.*, 1998). The mutant ES cells are also extremely sensitive to the crosslinking agent MMC (Figure 3B), much more so than are NER- or HR-deficient ES cells (Essers *et al.*, 1997). This is consistent with the hypothesis that DNA interstrand crosslinks are repaired via a mechanism that can involve both NER and HR (Sladek *et al.*, 1989) and that Ercc1 is involved in both contributory pathways (Dronkert and Kanaar, 2001). CHO Ercc1 mutants are as sensitive to MMC as the ES mutant cells (Westerveld *et al.*, 1984). However, primary (Weeda *et al.*, 1997) and immortalized *Ercc1*^{-/-} MEFs (Melton *et al.*, 1998) are less sensitive, possibly reflecting differential utilization of the various recombinational repair pathways by the different cell types.

Role of Ercc1 in homologous recombination

Since SCE and repair of DSBs can occur via HR (Paques and Haber, 1999; Sonoda *et al.*, 1999), assays to measure either are used to screen for novel genes involved in recombination. The *mErcc1*^{-/-} ES cells are not sensitive to DSBs induced by IR (Figure 3C), similar to *rad10* yeast (Moore, 1978) and *Ercc1*^{-/-} CHO cells (Darroudi and Natarajan, 1985). Thus, Ercc1 is not essential for DSB repair in any organism yet tested. Furthermore, the wt and *mErcc1*^{-/-} ES cells display identical frequencies of spontaneous and MMC-induced SCEs (data not shown). Thus, HR appears not to be suppressed in Ercc1-deficient mammalian cells. Our findings are consistent with the demonstrated HR-proficiency of Ercc1^{-/-} CHO cells (Nairn *et al.*, 1991; Sargent *et al.*, 1997) and immortalized MEFs (Melton *et al.*, 1998). Together, these data support the notion that if Ercc1 does play a role in HR, it is limited to a selected subset of recombinational events.

Indeed, Ercc1 is absolutely essential for a particular class of HR in ES cells: gene replacement. We screened a total of 868 puro^R clones derived from transfection of *mErcc1*^{-/-} ES cells with constructs carrying a puro^R selectable marker and targeting three different genomic loci, yet we never observed a single gene replacement

event (Table I). In total, these data reflect a >200-fold drop in targeting efficiency in the Ercc1-deficient cells compared with wt ES cells. Stable transfection of the Ercc1-deficient cells with a tagged *hERCC1* cDNA corrects the targeting efficiencies to wt levels. Surprisingly, failure to homologously recombine targeting constructs in the *mErcc1*^{-/-} cells is not limited to constructs carrying heterologous terminal sequences. This provides the first evidence that in mammalian cells, Ercc1 is required for HR between non-identical DNA substrates in a capacity other than removing nonhomologous termini.

The data obtained with the *mErcc1*^{-/-} ES cells can be contrasted with the results obtained with *mRad54*^{-/-} ES cells. Rad54-deficient cells are sensitive to IR (Figure 3 and Essers *et al.*, 1997) and have a reduced capacity for spontaneous and DNA damage-induced SCE (Dronkert *et al.*, 2000), yet gene replacement is not abrogated in *mRad54*^{-/-} ES cells (Table I and Essers *et al.*, 1997). This serves to highlight the differential roles of the proteins in HR; whereas Rad54 facilitates the general mechanism of HR, Ercc1 has a limited, yet essential function in a subset of recombinational events.

The finding that Ercc1 plays a role in gene replacement in ES cells is consistent with observations made in *S.cerevisiae*, the first organism for which targeted gene replacement was developed (Orr-Weaver *et al.*, 1981; Rothstein, 1983). Replacement is suppressed 40-fold in *rad1* or *rad10* deletion strains compared with wt strains (Schiestl and Prakash, 1988, 1990; Saparbaev *et al.*, 1996). In contrast, however, the role of Ercc1 in mammalian cells appears to be essential to gene replacement, whereas in yeast there is either another protein that can substitute for Rad1–Rad10 or there is an additional Rad10-independent mechanism of gene replacement.

A model for Ercc1-dependent gene replacement

Based on our findings in ES cells, we propose a model for the mechanism of gene replacement that incorporates an essential role for the endonuclease function of Ercc1–Xpf (Figure 5). Currently, at least three models exist for the mechanism of gene replacement using ends-out targeting constructs: double cross-over, SSA and break-induced replication (Paques and Haber, 1999). We implement the double cross-over mechanism as the basis of our model

(Szostak *et al.*, 1983). Although our data are in no way inconsistent with the other mechanisms, we found them more difficult to reconcile with the incorporation of large stretches of heterologous DNA into a mammalian genome.

Our proposed model is applicable to the replacement of a continuous stretch of genomic DNA (recipient) of indeterminate length with a large stretch of nonhomologous sequence (donor DNA; typically a selectable marker). The novel feature of our model is that Ercc1 is required for initiating what becomes, in effect, a DSB in the recipient DNA. We propose that cleavage by the Ercc1-Xpf endonuclease is required for resolution of the heteroduplex intermediate formed between the donor and recipient DNA when branch migration becomes stalled at the point of sequence nonhomology. Accurate localization of the endonuclease cleavage is directed by the junctions between heterology and homology.

Our model proceeds as follows (Figure 5). First, both processed 3' ends of the linear donor DNA identify homologous sequences in the recipient DNA with the aid of the general HR machinery, including Rad51, Rad52, Rad54 and the ss DNA binding protein RPA (Paques and Haber, 1999). Strand invasion at each end creates two D-loops (Figure 5B). Observations that are consistent with the hypothesis that gene replacement is initiated via invasion by both ends of the donor DNA include: (i) the frequency and the fidelity of gene replacement increase dramatically if the donor DNA is linear and both ends are homologous to the recipient DNA (Berinstein *et al.*, 1992; Thomas *et al.*, 1992; Negritto *et al.*, 1997); and (ii) the length of nonhomologous donor sequence does not affect targeting efficiency (Suroskey and Tye, 1985; Mansour *et al.*, 1990; Deng *et al.*, 1993; Li and Baker, 2000).

Secondly, the extent of the heteroduplex DNA is maximized (Li and Baker, 2000). This occurs by branch migration in an inward direction via pairing of homologous sequences between the donor and recipient DNA (Figure 5Ci) and perhaps also outwardly by leading strand DNA synthesis from the 3' invading ends using the donor as a template (Figure 5Cii). D-loop expansion continues until heterology between the donor and recipient DNA

Fig. 5. Model for Ercc1-Xpf-dependent gene replacement. The two strands of a linear targeting construct or donor DNA are depicted in shades of blue. The central portion of the donor DNA (shown in red) indicates a stretch of sequence without homology to the recipient DNA. The domain of nonhomology is typically a selectable marker gene. The two strands of genomic or recipient DNA are pictured in shades of purple. New DNA synthesis is indicated by arrows with a gradient-fill pattern. (A) Exonucleolytic processing of the donor to reveal 3' ss ends. (B) Strand invasion by both 3' ss ends of the donor DNA into homologous sequences of the recipient DNA to create two D-loops. (C) Expansion of the D-loops proximally by branch migration (i), and possibly also distally by DNA replication using the invading donor strand as a primer and the recipient strand as a template (ii). (D) Branch migration continues until the region of nonhomology in the donor DNA blocks further heteroduplex formation. Substrates for the structure-specific Ercc1-Xpf endonuclease are indicated by arrows. The slanted green arrow indicates the predicted site of primary Ercc1-Xpf cleavage. The dashed green arrow indicates an alternative cleavage site that can also result in gene replacement. The thin green arrow indicates a third potential substrate for Ercc1-Xpf that is nonproductive because it cannot result in gene replacement. (E) Ercc1-Xpf incises the stalled intermediate at characteristic ds/ss junctions just 5' to a 3' ss domain (at bold arrows shown in D). (F) Conversion of the nicks in the recipient DNA to a DSB. The break is stabilized by the heteroduplex DNA holding the two genomic ends in proximity. (G) Gene replacement is achieved by nonconservative insertion of the nonhomologous sequence into the DSB either with or without polymerization from the new 3' ends in the recipient DNA by Ercc1-Xpf incision. Resolution requires the activities of a flap endonuclease and DNA ligase.

sequences is reached, at which point branch migration is stalled and the intermediate stabilized (Negritto *et al.*, 1997; Belotserkovskii *et al.*, 1999; Constantinou *et al.*, 2001).

Thirdly, we propose that nicking by the structure-specific endonuclease Ercc1-Xpf is required to initiate resolution of the stalled intermediate. The substrate specificity of the endonuclease dictates the sites of incision which will occur in ds DNA just 5' to a 3' ss domain (de Laat *et al.*, 1998a). Within the proposed intermediate, cleavage of both strands of the recipient DNA favors the outcome of gene replacement (possible cut sites indicated with green arrows in Figure 5D). In NER, it has been established that RPA binds the undamaged strand and by virtue of its polarity, directs ERCC1-XPF cleavage exclusively to the opposite (damaged) strand (de Laat *et al.*, 1998b). In recombination, it is likely that RPA, bound to the invading ends of the donor DNA, similarly directs Ercc1-Xpf to cleave the strand with the opposite polarity (cleavage sites indicated by bold green arrows in Figure 5D). However, based on the currently available data, we cannot exclude the possibility that cleavage instead, or in addition, occurs immediately 5' to both of the D-loops (dashed green arrows in Figure 5D). Nevertheless, the net result is cleavage of both strands of the recipient genomic locus, making it receptive for productive gene replacement. It should also be noted that based on the substrate specificity of the Ercc1-Xpf endonuclease alone, the looped-out nonhomologous sequence of the donor DNA could also be a substrate (cleavage sites indicated with thin green arrows in Figure 5D). If, however, cleavage of the donor DNA occurs, the homologous arms are removed, eliminating the possibility of homologous gene replacement. In this case, the recipient DNA is not affected, therefore this outcome cannot be detected. The heteroduplex intermediate we propose as a substrate for Ercc1-Xpf (and therefore the concomitant role for the enzyme in HR) is fundamentally different from the role that Rad10-Rad1 plays in recombination between DNA substrates that differ by small mismatches (Nicholson *et al.*, 2000) because small patches of heterology are unlikely to stall branch migration.

Once the incisions are made by Ercc1-Xpf, there are a number of means by which the nicked intermediate might be resolved in order to achieve gene replacement. First, depending on the distance between the nicks, the DNA may be unwound either spontaneously or enzymatically by a helicase. Secondly, a 5'- to -3' exonuclease may remove the intervening sequence by chewing back from the nicks. Thirdly, another round of endonucleolytic cleavage immediately 5' to the D-loops may remove the intervening sequence. This site is also a potential substrate for Ercc1-Xpf, as indicated above. Regardless of the mechanism, the net result must be removal of the recipient DNA between the nicks, thereby creating a frank DSB in the chromosome (Figure 5F). After removal of the sequence between the Ercc1-Xpf-generated nicks, incorporation of the nonhomologous donor sequences into the genomic locus can be completed by enzymes involved in DNA replication (Figure 5G). A portion of the donor sequences may be replicated by leading strand synthesis initiated from the new 3' termini of the recipient DNA (Bardwell *et al.*, 1994). The activities of a flap endonuclease and

DNA ligase are required to restore continuity to both DNA strands of the replaced genomic locus.

In addition to the fact that creating incisions in the recipient DNA greatly facilitates conceptual understanding of how exogenous sequences can be incorporated into the genome, there is experimental evidence for this event. In mammalian cells, including ES cells, the introduction of a DSB in a chromosome stimulates targeting by exogenous DNA by several orders of magnitude (Rouet *et al.*, 1994; Smih *et al.*, 1995; Richardson *et al.*, 1999). In meiotic recombination in yeast, *rad1* or *rad10* strains fail to repair nonhomologous genomic insertions, as reflected by an increase in post-meiotic segregation compared with wt strains (Kirkpatrick and Petes, 1997; Kearney *et al.*, 2001). However, one class of segregation events is suppressed in the mutant strains: 2:6 conversions (the conversion of a wt allele to a mutant allele containing the inserted loop). This is consistent with the Rad1-Rad10 endonuclease mediating loop repair via cleavage of the recipient strand to allow integration of the nonhomologous sequence, similar to the mechanism proposed above for gene replacement in ES cells. Finally, precedence for a D-loop endonuclease that stimulates gene replacement exists in phages. Rap (for recombination adept with plasmid) is a phage protein that is a functional homolog of the *Escherichia coli* RusA Holliday junction resolvase. Rap preferentially cleaves branched structures and 3'-tailed D-loops identically to the proposed mechanism for Ercc1 (Sharples *et al.*, 1998).

Although the role we propose for Ercc1-Xpf in gene replacement is to initiate the formation of a DSB in the genome, there are several aspects of our model that contribute to the preservation of genomic integrity. First, the trigger for Ercc1-Xpf cleavage is an unresolved heteroduplex structure, which if left unresolved could itself trigger undesirable consequences via DNA and/or RNA polymerase blockage. Secondly, since the proposed cleavage is internal to two stretches of heteroduplex DNA, the break is stabilized and well-poised for rapid rejoining of the correct ends. This is consistent with the observation that there is a minimum amount of homology required to favor gene replacement in ES cells (Hasty *et al.*, 1991; Deng and Capecchi, 1992; Thomas *et al.*, 1992), which may be indicative of the amount of homology required to stabilize the proposed intermediate. Thirdly, the structure specificity of the endonuclease dictates that cuts are only made within ds sequences immediately adjacent to ds/ss borders (Bardwell *et al.*, 1994; de Laat *et al.*, 1998a). This minimizes the loss of sequence information from the chromosome. Similarly, sequence information is not lost from the targeting construct, which is supported by the observation that gene replacement is highly accurate even in the absence of gain-of-function selection (Zheng *et al.*, 1991; Li and Baker, 2000).

In conclusion, we have demonstrated that Ercc1 is essential for gene replacement in ES cells. This is true even if the replacement construct is completely homologous to the genomic target, with the exception of a centrally positioned selectable marker. Thus, the role for Ercc1 that we discovered is distinct from its previously established role in removing 3' overhangs of nonhomologous sequence from DSBs, SSA intermediates and gene insertion constructs. Considering the substrate specificity of the Ercc1-Xpf endonuclease, the most likely function

of Ercc1 in gene replacement is nicking of heteroduplex intermediates within D-loops. Therefore, in addition to defining a new role for Ercc1 in HR and providing evidence for a new substrate of the Ercc1-Xpf endonuclease, these data help to elucidate the mechanism of gene replacement in mammalian cells.

Materials and methods

Generation of *mErcc1*^{-/-} ES cells

mErcc1 targeting constructs and the production of *Ercc1*^{+/-} murine ES cells were as previously described (Weeda *et al.*, 1997). Briefly, a 12-kb *HindIII/SalI* genomic DNA fragment from a 129/CCE genomic library including the last four exons (exons 7–10) of *mErcc1* was subcloned into pBR322. A unique *ClaI* restriction site was created in exon 7 allowing disruption of the exon by introducing a neo^R expression cassette into the site. The neo^R construct was transfected into wt ES cells, G418-resistant colonies isolated and targeted clones identified by DNA blotting (Weeda *et al.*, 1997). To target the second *Ercc1* allele in the *mErcc1*^{+/-} ES line, the targeting construct was modified by replacing the neo^R marker with a hygro^R cassette. A linker containing a diagnostic *BamHI* site was included 3' to the selectable marker to facilitate identification of targeted clones. The construct was electroporated into the *mErcc1*^{+/-} ES cells and selected for with hygromycin. An *mErcc1*-specific probe that hybridizes to sequences outside (5') of the targeting constructs was used to distinguish between wt, neo^R and hygro^R alleles in DNA blot analysis of *BamHI*-digested genomic DNA isolated from hygro^R clones. Positive clones were further characterized using probes against the resistance markers and sequences external to the 3' homologous arm of the constructs.

Rescue of *mErcc1*^{-/-} ES cells with *hERCC1* cDNA

The cDNA encoding *hERCC1* was cloned into N-pEYFP (Promega) as an N-terminal in-frame fusion with eYFP. The fusion cDNA was subsequently cloned into a phosphoglycerate kinase promoter–poly(A) expression cassette (Essers *et al.*, 1997). The vector was electroporated into the *mErcc1*^{-/-} ES cells either alone or with a second plasmid carrying a puromycin expression cassette. Since the parental knock-out line is sensitive to crosslinking agents (see Results) selection of stably transformed cells was achieved by adding 5.0×10^{-8} M MMC to the media or in the case of cotransfection with puromycin (1 µg/ml). Resistant clones were screened for the presence of fluorescence by microscopy. Expression of the full-length fusion protein was confirmed by immunoblotting of 10 µg of WCE using a polyclonal α -*hERCC1* antibody (van Vuuren *et al.*, 1995).

DNA damage sensitivities and sister chromatid exchange

Sensitivity to various DNA damages was measured as colony-forming ability of the different ES cell lines after exposure to various agents as described (Essers *et al.*, 1997). All data points were measured in triplicate. The cloning efficiency of untreated cells was typically 20–30%. SCEs were measured as described previously (Dronkert *et al.*, 2000). More than 50 metaphase spreads were analyzed for each data point.

Quantitation of gene targeting events

Cloning of a 9-kb *EcoRI mRad54* genomic fragment (encompassing exons 3–8) into a bacterial vector was as described previously (Essers *et al.*, 1997). In an attempt to achieve high targeting efficiency but not necessarily disruption of Rad54 protein expression, a puro^R expression cassette was inserted into a unique *NcoI* site in intron 3 of the genomic fragment. When homologously recombined into the locus, the construct converts the 9.0-kb genomic *StuI* fragment to an 8.5-kb fragment, permitting diagnosis of targeting. The targeting construct was isolated from the vector as a linear *EcoRI* fragment with flanking arms completely homologous to the chromosomal *mRad54* target. Alternatively, the construct was isolated from the vector using a *ClaI*–*NotI* double digest, leaving 32 bp of vector sequence on the 5' end and 14 bp on the 3' end of the linear fragment, effectively creating short termini without homology to the *mRad54* locus. The linear fragments were electroporated into one wt, two *Ercc1*^{-/-} and three rescued mutant cell lines, as described above. Since all of the rescued mutant lines were phenotypically indistinguishable regardless of how they were generated (data not shown), only lines selected by MMC were used in this assay so that selection of targeted clones using the last selectable marker, puromycin, was possible.

Resistant clones were picked and expanded, then lysed and genomic DNA was isolated. DNA preparations were digested with *StuI* and analyzed by DNA blotting using a 3' external probe recognizing exons 7 and 8. Targeting frequencies are reported as the total number of HR events divided by the number of homologous plus nonhomologous integration events. The χ^2 test was used to evaluate the significance of the differences between the observed HR frequencies.

Acknowledgements

We thank H.de Waard, H.M.Kearney and T.D.Petes for communication of unpublished results and helpful discussion. This research was supported by the Netherlands Organization for Scientific Research (NWO), the Dutch Cancer Society (KWF), the Human Frontier Science Program Organization (grant RGP0298/2001-M) and the Association for International Cancer Research. G.W. was a fellow of The Netherlands Royal Academy of Arts and Sciences. L.J.N. was supported by the National Science Foundation (Grant INT-9900963; 6-1999 to 1-2000) and the American Cancer Society (Grant PF-99-142; 1-2000 to present).

References

- Adair,G.M., Rolig,R.L., Moore-Faver,D., Zabelshansky,M., Wilson,J.H. and Nairn,R.S. (2000) Role of ERCC1 in removal of long non-homologous tails during targeted homologous recombination. *EMBO J.*, **19**, 5552–5561.
- Aguilera,A. and Klein,H.L. (1989) Yeast intrachromosomal recombination: long gene conversion tracts are preferentially associated with reciprocal exchange and require the *RAD1* and *RAD3* gene products. *Genetics*, **123**, 683–694.
- Bardwell,A.J., Bardwell,L., Tomkinson,A.E. and Friedberg,E.C. (1994) Specific cleavage of model recombination and repair intermediates by the yeast Rad1–Rad10 DNA endonuclease. *Science*, **265**, 2082–2084.
- Belotserkovskii,B.P., Reddy,G. and Zarlino,D.A. (1999) DNA hybrids stabilized by heterologies. *Biochemistry*, **38**, 10785–10792.
- Berinstein,N., Pennell,N., Ottaway,C. and Shulman,M.J. (1992) Gene replacement with one-sided homologous recombination. *Mol. Cell. Biol.*, **12**, 360–367.
- Biggerstaff,M., Szymkowski,D.E. and Wood,R.D. (1993) Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects *in vitro*. *EMBO J.*, **12**, 3685–3692.
- Constantinou,A., Davies,A.A. and West,S.C. (2001) Branch migration and Holliday junction resolution catalyzed from mammalian cells. *Cell*, **104**, 259–268.
- Darroudi,F. and Natarajan,A.T. (1985) Cytological characterization of repair-deficient CHO cell line 43–3B. I. Induction of chromosomal aberrations and sister-chromatid exchanges by UV and its modulation with 3-aminobenzamide. *Mutat. Res.*, **149**, 239–247.
- de Laat,W.L., Appeldoorn,E., Jaspers,N.G. and Hoeijmakers,J.H.J. (1998a) DNA structural elements required for ERCC1–XPF endonuclease activity. *J. Biol. Chem.*, **273**, 7835–7842.
- de Laat,W.L., Appeldoorn,E., Sugawara,K., Weterings,E., Jaspers,N.G. and Hoeijmakers,J.H.J. (1998b) DNA-binding polarity of human replication protein A positions nucleases in nucleotide excision repair. *Genes Dev.*, **12**, 2598–2609.
- Deng,C. and Capecchi,M.R. (1992) Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.*, **12**, 3365–3371.
- Deng,C., Thomas,K.R. and Capecchi,M.R. (1993) Location of crossovers during gene targeting with insertion and replacement vectors. *Mol. Cell. Biol.*, **13**, 2134–2140.
- Dronkert,M.L.G. and Kanaar,R. (2001) Repair of DNA interstrand cross-links. *Mutat. Res.*, **486**, 217–247.
- Dronkert,M.L.G., Beverloo,H.B., Johnson,R.D., Hoeijmakers,J.H.J., Jasin,M. and Kanaar,R. (2000) Mouse RAD54 affects DNA double-strand break repair and sister chromatid exchange. *Mol. Cell. Biol.*, **20**, 3147–3156.
- Essers,J., Hendriks,R.W., Swagemakers,S.M., Troelstra,C., de Wit,J., Bootsma,D., Hoeijmakers,J.H.J. and Kanaar,R. (1997) Disruption of mouse RAD54 reduces ionizing radiation resistance and homologous recombination. *Cell*, **89**, 195–204.
- Fishman-Lobell,J. and Haber,J.E. (1992) Removal of nonhomologous DNA ends in double-strand break recombination: the role of the yeast ultraviolet repair gene *RAD1*. *Science*, **258**, 480–484.
- Fleck,O., Lehmann,E., Schar,P. and Kohli,J. (1999) Involvement of

- nucleotide-excision repair in msh2 pms1-independent mismatch repair. *Nature Genet.*, **21**, 314–317.
- Gaillard,P.H. and Wood,R.D. (2001) Activity of individual ERCC1 and XPF subunits in DNA nucleotide excision repair. *Nucleic Acids Res.*, **29**, 872–879.
- Hang,H., Hager,D.N., Goriparthi,L., Hopkins,K.M., Shih,H. and Lieberman,H.B. (1996) *Schizosaccharomyces pombe* rad23 is allelic with swi10, a mating-type switching/radioresistance gene that shares sequence homology with human and mouse ERCC1. *Gene*, **170**, 113–117.
- Hasty,P., Rivera-Perez,J. and Bradley,A. (1991) The length of homology required for gene targeting in embryonic stem cells. *Mol. Cell. Biol.*, **11**, 5586–5591.
- Houtsmuller,A.B., Rademakers,S., Nigg,A.L., Hoogstraten,D., Hoeijmakers,J.H.J. and Vermeulen,W. (1999) Action of DNA repair endonuclease ERCC1/XPF in living cells. *Science*, **284**, 958–961.
- Hoy,C.A., Thompson,L.H., Mopney,C.L. and Salazar,E.P. (1985) Defective DNA cross-link removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. *Cancer Res.*, **45**, 1737–1743.
- Ivanov,E.L. and Haber,J.E. (1995) *RAD1* and *RAD10*, but not other excision repair genes, are required for double-strand break-induced recombination in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **15**, 2245–2251.
- Kearney,H.M., Kirkpatrick,D.T., Gerton,J.L. and Petes,T.D. (2001) Meiotic recombination involving heterozygous large insertions in *S. cerevisiae*: formation and repair of large, unpaired DNA loops. *Genetics*, **158**, 1457–1476.
- Kirkpatrick,D.T. and Petes,T.D. (1997) Repair of DNA loops involves DNA-mismatch and nucleotide-excision repair proteins. *Nature*, **387**, 929–931.
- Klein,H.L. (1988) Different types of recombination events are controlled by the *RAD1* and *RAD52* genes of *Saccharomyces cerevisiae*. *Genetics*, **120**, 367–377.
- Li,J. and Baker,M.D. (2000) Mechanisms involved in targeted gene replacement in mammalian cells. *Genetics*, **156**, 809–821.
- Mansour,S.L., Thomas,K.R., Deng,C. and Capecchi,M.R. (1990) Introduction of a *lacZ* reporter gene into the mouse *int-2* locus by homologous recombination. *Proc. Natl Acad. Sci. USA*, **87**, 7688–7692.
- McWhir,J., Selfridge,J., Harrison,D.J., Squires,S. and Melton,D.W. (1993) Mice with DNA repair gene (*ERCC-1*) deficiency have elevated levels of p53, liver nuclear abnormalities and die before weaning. *Nature Genet.*, **5**, 217–223.
- Melton,D.W., Ketchen,A.M., Nunez,F., Bonatti-Abbondandolo,S., Abbondandolo,A., Squires,S. and Johnson,R.T. (1998) Cells from ERCC1-deficient mice show increased genome instability and a reduced frequency of S-phase-dependent illegitimate chromosome exchange but a normal frequency of homologous recombination. *J. Cell Sci.*, **111**, 395–404.
- Moore,C.W. (1978) Responses of radiation-sensitive mutants of *Saccharomyces cerevisiae* to the lethal effects of bleomycin. *Mutat. Res.*, **51**, 165–180.
- Mu,D., Park,C.-H., Matsunaga,T., Hsu,D.S., Reardon,J.T. and Sancar,A. (1995) Reconstitution of human DNA repair excision nuclease in a highly defined system. *J. Biol. Chem.*, **270**, 2415–2418.
- Nairn,R.S., Adair,G.M., Christmann,C.B. and Humphrey,R.M. (1991) Ultraviolet stimulation of intermolecular homologous recombination in Chinese hamster ovary cells. *Mol. Carcinog.*, **4**, 519–526.
- Negritto,M.T., Wu,X., Kuo,T., Chu,S. and Bailis,A.M. (1997) Influence of DNA sequence identity on efficiency of targeted gene replacement. *Mol. Cell. Biol.*, **17**, 278–286.
- Nicholson,A., Hendrix,M., Jinks-Robertson,S. and Crouse,G.F. (2000) Regulation of mitotic homeologous recombination in yeast. Functions of mismatch repair and nucleotide excision repair genes. *Genetics*, **154**, 133–146.
- Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) Yeast transformation: a model system for the study of recombination. *Proc. Natl Acad. Sci. USA*, **78**, 6354–6358.
- Paques,F. and Haber,J.E. (1997) Two pathways for removal of nonhomologous DNA ends during double-strand break repair in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **17**, 6765–6771.
- Paques,F. and Haber,J.E. (1999) Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.*, **63**, 349–404.
- Prado,F. and Aguilera,A. (1995) Role of reciprocal exchange, one-ended invasion crossover and single-strand annealing on inverted and direct repeat recombination in yeast: different requirements for the *RAD1*, *RAD10* and *RAD52* genes. *Genetics*, **139**, 109–123.
- Richardson,C., Moynahan,M.E. and Jasin,M. (1999) Homologous recombination between heterologs during repair of a double-strand break. Suppression of translocations in normal cells. *Ann. N. Y. Acad. Sci.*, **886**, 183–186.
- Rodel,C., Kirchhoff,S. and Schmidt,H. (1992) The protein sequence and some intron positions are conserved between the switching gene swi10 of *Schizosaccharomyces pombe* and the human excision repair gene ERCC1. *Nucleic Acids Res.*, **20**, 6347–6353.
- Rothstein,R.J. (1983) One-step gene disruption in yeast. *Methods Enzymol.*, **101**, 202–211.
- Rouet,P., Smih,F. and Jasin,M. (1994) Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Mol. Cell. Biol.*, **14**, 8096–8106.
- Saparbav,M., Prakash,L. and Prakash,S. (1996) Requirement of mismatch repair genes *MSH2* and *MSH3* in the *RAD1*–*RAD10* pathway of mitotic recombination in *Saccharomyces cerevisiae*. *Genetics*, **142**, 727–736.
- Sargent,R.G., Rolig,R.L., Kilburn,A.E., Adair,G.M., Wilson,J.H. and Nairn,R.S. (1997) Recombination-dependent deletion formation in mammalian cells deficient in the nucleotide excision repair gene ERCC1. *Proc. Natl Acad. Sci. USA*, **94**, 13122–13127.
- Sargent,R.G., Meservy,J.L., Perkins,B.D., Kilburn,A.E., Intody,Z., Adair,G.M., Nairn,R.S. and Wilson,J.H. (2000) Role of the nucleotide excision repair gene ERCC1 in formation of recombination-dependent rearrangements in mammalian cells. *Nucleic Acids Res.*, **28**, 3771–3778.
- Schiestl,R.H. and Prakash,S. (1988) *RAD1*, an excision repair gene of *Saccharomyces cerevisiae*, is also involved in recombination. *Mol. Cell. Biol.*, **8**, 3619–3626.
- Schiestl,R.H. and Prakash,S. (1990) *RAD10*, an excision repair gene of *Saccharomyces cerevisiae*, is involved in the *RAD1* pathway of mitotic recombination. *Mol. Cell. Biol.*, **10**, 2485–2491.
- Selfridge,J., Pow,A.M., McWhir,J., Magin,T.M. and Melton,D.W. (1992) Gene targeting using a mouse HPRT minigene/HPRT-deficient embryonic stem cell system: inactivation of mouse *ERCC-1* gene. *Somat. Cell Mol. Genet.*, **18**, 325–336.
- Sharples,G.J., Corbett,L.M. and Graham,I.R. (1998) λ Rap protein is a structure-specific endonuclease involved in phage recombination. *Proc. Natl Acad. Sci. USA*, **95**, 13507–13512.
- Sijbers,A.M. et al. (1996) Xeroderma pigmentosum group F caused by a defect in a structure-specific DNA repair endonuclease. *Cell*, **86**, 811–822.
- Sladek,F., Munn,M., Rupp,W. and Howard-Flanders,P. (1989) *In vitro* repair of psoralen-DNA cross-links by RecA, UvrABC and the 5'-exonuclease of DNA polymerase I. *J. Biol. Chem.*, **264**, 6755–6765.
- Smih,F., Rouet,P., Romanienko,P.J. and Jasin,M. (1995) Double-strand breaks at the target locus stimulate gene targeting in embryonic stem cells. *Nucleic Acids Res.*, **23**, 5012–5019.
- Sonoda,E., Sasaki,M.S., Morrison,C., Yamaguchi-Iwai,Y., Takata,M. and Takeda,S. (1999) Sister chromatid exchanges are mediated by homologous recombination in vertebrate cells. *Mol. Cell. Biol.*, **19**, 5166–5169.
- Surosky,R.T. and Tye,B.K. (1985) Construction of telocentric chromosomes in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **82**, 2106–2110.
- Szostak,J.W., Orr-Weaver,T.L., Rothstein,R.J. and Stahl,F.W. (1983) The double-strand-break repair model for recombination. *Cell*, **33**, 25–35.
- Thomas,K.R., Deng,C. and Capecchi,M.R. (1992) High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol. Cell. Biol.*, **12**, 2919–2923.
- Tomkinson,A.E., Bardwell,A.J., Bardwell,L., Tappe,N.J. and Friedberg,E.C. (1993) Yeast DNA repair and recombination proteins Rad1 and Rad10 constitute a single-stranded-DNA endonuclease. *Nature*, **362**, 860–862.
- van Duin,M., de Wit,J., Odijk,H., Westerveld,A., Yasui,A., Koken,M.H.M., Hoeijmakers,J.H.J. and Bootsma,D. (1986) Molecular characterization of the human excision repair gene ERCC-1: cDNA cloning and amino acid homology with the yeast DNA repair gene RAD10. *Cell*, **44**, 913–923.
- van Duin,M. et al. (1989) The cloned human DNA excision repair gene ERCC-1 fails to correct xeroderma pigmentosum complementation groups A through I. *Mutat. Res.*, **217**, 83–92.
- van Vuuren,A.J., Appeldoorn,E., Odijk,H., Yasui,A., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers,J.H.J. (1993) Evidence for a repair

- enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F. *EMBO J.*, **12**, 3693–3701.
- van Vuuren,A.J., Appeldoorn,E., Odijk,H., Humbert,S., Moncollin,V., Eker,A.P.M., Jaspers,N.G.J., Egly,J.M. and Hoeijmakers,J.H.J. (1995) Partial characterization of the DNA repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities. *Mutat. Res.*, **337**, 25–39.
- Weeda,G. *et al.* (1997) Disruption of mouse ERCC1 results in a novel repair syndrome with growth failure, nuclear abnormalities and senescence. *Curr. Biol.*, **7**, 427–439.
- Westerveld,A., Hoeijmakers,J.H.J., van Duin,M., de Wit,J., Odijk,H., Pastink,A., Wood,R.D. and Bootsma,D. (1984) Molecular cloning of a human DNA repair gene. *Nature*, **310**, 425–429.
- Zehfus,B.R., McWilliams,A.D., Lin,Y.-H., Hoekstra,M.F. and Keil,R.L. (1990) Genetic control of RNA polymerase I-stimulated recombination in yeast. *Genetics*, **126**, 41–52.
- Zheng,H., Hast,P., Brennenman,M.A., Grompe,M., Gibbs,R.A., Wilson,J.H. and Bradley,A. (1991) Fidelity of targeted recombination in human fibroblasts and murine embryonic stem cells. *Proc. Natl Acad. Sci. USA*, **88**, 8067–8071.

*Received July 3, 2001; revised September 11, 2001;
accepted September 19, 2001*