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Fen-1 Facilitates Homologous Recombination by Removing Divergent Sequences at DNA Break Ends†

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Homologous recombination (HR) requires nuclease activities at multiple steps, but the contribution of individual nucleases to the processing of double-strand DNA ends at different stages of HR has not been clearly defined. We used chicken DT40 cells to investigate the role of flap endonuclease 1 (Fen-1) in HR. *FEN-1*-deficient cells exhibited a significant decrease in the efficiency of immunoglobulin gene conversion while being proficient in recombination between sister chromatids, suggesting that Fen-1 may play a role in HR between sequences of considerable divergence. To clarify whether sequence divergence at DNA ends is truly the reason for the observed HR defect in *FEN-1*$^{-/-}$ cells we inserted a unique I-SceI restriction site in the genome and tested various donor and recipient HR substrates. We found that the efficiency of HR-mediated DNA repair was indeed greatly diminished when divergent sequences were present at the DNA break site. We conclude that Fen-1 eliminates heterologous sequences at DNA damage site and facilitates DNA repair by HR.

Homologous recombination (HR) plays a critical role in genome maintenance by repairing double-strand breaks (DSBs) induced by exogenous agents or occurring during DNA replication. In chicken B lymphocyte precursors, HR mediates the diversification of the immunoglobulin (Ig) variable region, a process called gene conversion (7, 31, 33, 45). HR is also essential when transfected DNA is integrated in the genome at specific sites of homology during gene targeting.

HR is initiated by DNA damage, including DSBs and single-strand breaks. It involves interactions between damaged DNA and intact homologous sequences and results in the transfer of genetic information from the intact donor to the damaged recipient. HR-dependent DSB repair has been well studied in the budding yeast by use of the HO restriction enzyme. The initial step involves processing of DNA ends to produce a 3′ single-strand overhang, which is covered by Rad51. The resulting nucleoprotein filament is responsible for homology search, homologous pairing, and strand invasion (D loop formation) followed by DNA synthesis from the 3′ end of the invading strand (46). This HR-dependent repair of HO-induced DSBs requires a number of nucleases (13). Firstly, the 3′ overhang formation at DSBs appears to be carried out by Mre11 and Exo1 and perhaps by other unknown nucleases in mitotic cells (12, 20, 24, 40, 41). Secondly, nonhomologous tails from the 3′ overhang including the HO site should be eliminated. These overhangs interfere with subsequent steps of HR by destabilizing the D loop and precluding 3′-OH-end extensions (26). In higher eukaryotic cells, the end processing of DNA during the initial step of HR is more controversial. For example, *mre11*-deficient DT40 cells exhibit normal kinetics of Rad51 focus formation after irradiation (IR) (48), indicating that induced DSBs are processed normally in the absence of Mre11. Likewise, *exo1*-deficient mouse embryonic stem cells show defective mismatch repair but normal HR capability (44).

Fen-1 is a structure-specific nuclease that cleaves 5′ flaps of the branched DNA structures and possesses double-strand-specific 5′-to-3′ exonuclease activity (14, 27). Recently Zheng et al. (51) reported that Fen-1 cleaves DNA bubble structures by 5′ and 3′ incision in vitro. The endonuclease activity of Fen-1 is required for processing the 5′ ends of Okazaki fragments in lagging strand DNA synthesis (42, 43). Fen-1 also contributes to base excision repair (BER) by removing 5′ flap structures formed during gap-filling DNA synthesis (28). This notion is supported by the phenotype of *FEN-1*-deficient DT40 cells, which are hypersensitive to killing by alkylating agents such as methylmethane sulfonate and hydroxyperoxide (22). Consistent with the important role for Fen-1 in DNA replication and BER, *FEN-1*$^{-/-}$ mice are lethal during early embryogenesis (19), and even mice heterozygous for *FEN-1* display a high incidence of tumorigenesis, presumably due to genome instability (18).

Although mammalian mutants deficient in *FEN-1* are not viable, *FEN-1*$^{-/-}$ DT40 cells are able to proliferate with slightly elongated cell cycle time (22). Thus, *FEN-1*$^{-/-}$ DT40

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cells provide a novel opportunity to analyze in vivo functions of vertebrate Fen-1. DT40 cells are useful for comprehensive analysis of a variety of HR reactions, because a number of phenotypic assays have been developed (15, 31, 50). These assays include the measurement of the rate of Ig gene conversion, sister chromatid exchange (SCE), gene targeting, repair of DSBs created in artificial constructs, and repair of DSBs induced by ionizing radiation (IR) at the late S to G_2 phase (1, 2, 4, 32, 34, 35, 36, 37). Using these assays we found in this study that Fen-1 is required for HR between homologous sequences with nonhomologous tails at the DNA break ends while it is dispensable for HR between perfect homologies.

MATERIALS AND METHODS

Plasmid constructs. Two XPG disruption constructs, *XPG-hisD* and *XPG-bsr*, were generated from genomic PCR products combined with *hisD*- and *bsr*-selection marker cassettes (see Fig. S2 in the supplemental material). Genomic DNA sequences were amplified using the primers 5′-TCTGATACATGAACT GACAGATAAGCACAG-3′ and 5′-CGGGATCCGTCTGCTAAAGTAACTC ACCACCACAAGC-3′ (for the left arm of the disruption construct) and 5′-CT CGGATCCTTTTCCCAGTACCAGCTTAGGGGTTTGC-3′ and 5′-GAGGG TACCTGAAGCATTTCCTGCTCAGCAGAAAGGTC-3′ (for the right arm of the disruption construct). Amplified PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen). The 2.1-kb NotI-BamHI fragment from the left arm and the 2.5-kb BamHI-KpnI fragment from the right arm were cloned into NotI and KpnI sites of pBluescript KS, respectively. The BamHI site between the two arms was used to clone marker gene cassettes. For the probe used in Southern blot analysis, the genomic DNA was amplified using the primers 5′-GCTACTT CTGTAACAGGACAAATGTTCTTG-3′ and 5′-ATAACCAAACATCACTA TCATCAGTGATTG-3′ and was digested with EcoRV to obtain a 1-kb fragment. The expression vector pCR3-loxP-*XPG*/IRES-EGFP-loxP, in which the *XPG* and enhanced green fluorescent protein (EGFP) genes are flanked by two *loxP* sequences, was constructed by inserting an *XPG* SalI-BamHI cDNA fragment between the SalI and BamHI sites of pCR3-loxP-MCS-loxP (49). Gene targeting substrates and heterologous sequences inserted into S2neo reporter gene were amplified with primers listed elsewhere (see Table S1 in the supplemental material). Expression vectors of chicken *FEN-1* and nuclease-dead mutant of *FEN-1* were given by Keizo Tano (Research Reactor Institute, Kyoto University, Osaka, Japan).

Cell culture, DNA transfection, and γ irradiation. Cells were cultured in RPMI 1640 supplemented with 10^{-5} M β-mercaptoethanol, 10% fetal calf serum, and 1% chicken serum (Sigma, St Louis, MO) at 39.5°C. Methods of DNA transfection for producing stable transfectants and genotoxic treatments were as described previously (34). Cell synchronization was achieved by elutriation as described previously (34). ^{137}Cs (Gammacell 40, Nordion, Kanata, Ontario, Canada) (0.02 Gy/s) was used for γ irradiation.

Measurement of SCE levels. SCE levels were measured as described previously (49).

Analysis of Ig gene conversion. *FEN-1*$^{-/-}$ cells were established from CL18, a subclone of DT40 cells that is negative for surface IgM (sIgM) (3, 22). We confirmed that *FEN-1*$^{-/-}$ cells retained the same frameshift mutation as do wild-type CL18 cells by sequencing the Ig V_λ region. The rate of Ig gene conversion was assessed by measuring the gain of sIgM expression during a 3-week period as described previously (3).

I-SceI-induced gene conversion and gene targeting. A total of 10^7 cells were suspended in 0.1 ml Nucleofector Solution T (Amaxa biosystems) and electroporated using an Amaxa system (Amaxa biosystems) at program B-23. For the gene conversion assay, 5 μg of circular I-SceI expression vector (pcBASce) with or without nuclease expression vector was transfected into the cells. For the gene targeting assay, 2 μg of substrate DNA and 4 μg of pcBASce with or without nuclease expression vector was transfected. pBluescript II KS+ was used as a negative control. At 24 h after electroporation, the number of live cells were counted by fluorescence-activated cell sorting (FACS) and the cells were transferred to 96-well cluster trays with or without 2.0 mg of G418 per ml. Cells were grown for 7 to 10 days, and HR frequencies were calculated by the following equation: HR frequency (colonies/cell) = number of G418-resistant colonies/(plating efficiency of transfected cells in the absence of G418 × number of live cells determined by FACS 24 h after electroporation).

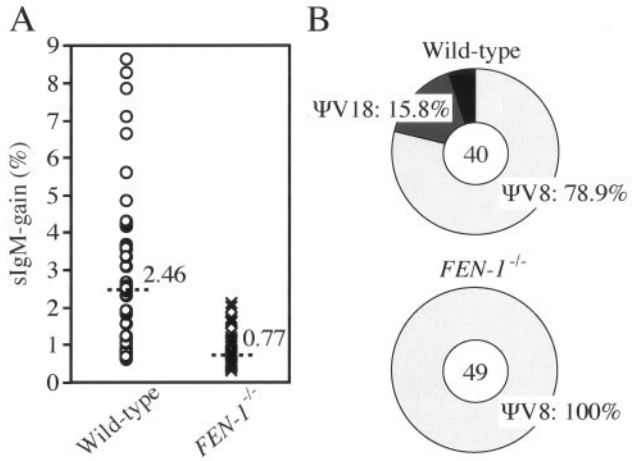


FIG. 1. Reduced frequency of Ig gene conversion in *FEN-1*$^{-/-}$ cells. (A) Fluctuation analysis of appearance of sIgM-gain revertants. The abundance of sIgM-gain revertants was determined in parallel cultures derived from sIgM$^-$ single cells after 3-week clonal expansion; median percentages are noted above each data set and are indicated by the line. (B) Preference of pseudo-V gene usage as a donor for Ig gene conversion among isolated sIgM-gain populations. The total number of V_λ sequences analyzed is indicated in the center of the charts.

Measurement of targeted integration frequencies. To analyze the targeted integration events at the *Ovalbumin* (4), *RAD54* (2), and β-*ACTIN* loci, each disruption construct was transfected into cells, and Southern blot analysis was performed following selection of clones against appropriate antibiotics. For the *CENP-H* locus, a *CENP-H*-EGFP knock-in construct (10) was used, and the targeted events were scored by FACS analysis.

Nucleotide sequence accession number. The chicken *XPG* cDNA sequences have been submitted to the GenBank database under accession number AB063480.

RESULTS

Reduced kinetics of the Ig gene conversion in *FEN-1*$^{-/-}$ cells. We have screened nucleases that are required for HR by analyzing the rate of Ig gene conversion in nuclease gene-disrupted DT40 clones. In this assay, intragenic gene conversion between two diverged homologous sequences, i.e., donor pseudo-V (ΨV) and recipient VJ_λ segments, allows determination of gene conversion events as well as the identification of aberrant events (29). To assess the kinetics of Ig gene conversion, we measured the gain of surface IgM (sIgM) expression, which may reflect elimination of a given frameshift mutation at the recipient VJ_λ by superimposed gene conversion events (3, 30). Interestingly, *FEN-1*$^{-/-}$ cells exhibited a 3.2-fold reduction of sIgM gain (Fig. 1A), suggesting a role for Fen-1 in Ig gene conversion. In contrast, gene conversion was not impaired in cells deficient in *XPG* (data not shown), which belongs to the same nuclease group as Fen-1 (21). Since a defect in Ig gene conversion is often accompanied by the alteration of the usage of donor ΨV segments, we determined nucleotide sequences of the Ig V_λ segment in the cells that acquired sIgM expression. We found that the usage of ΨV segments was different between wild-type and *FEN-1*$^{-/-}$ cells, while no significant alternation was found in their gene conversion tract length. The ΨV8 segment was used in 78.9% of the gene conversion events in wild-type cells, while all analyzed 49 gene conversion events exclusively involved ΨV8 in *FEN-1*$^{-/-}$ cells

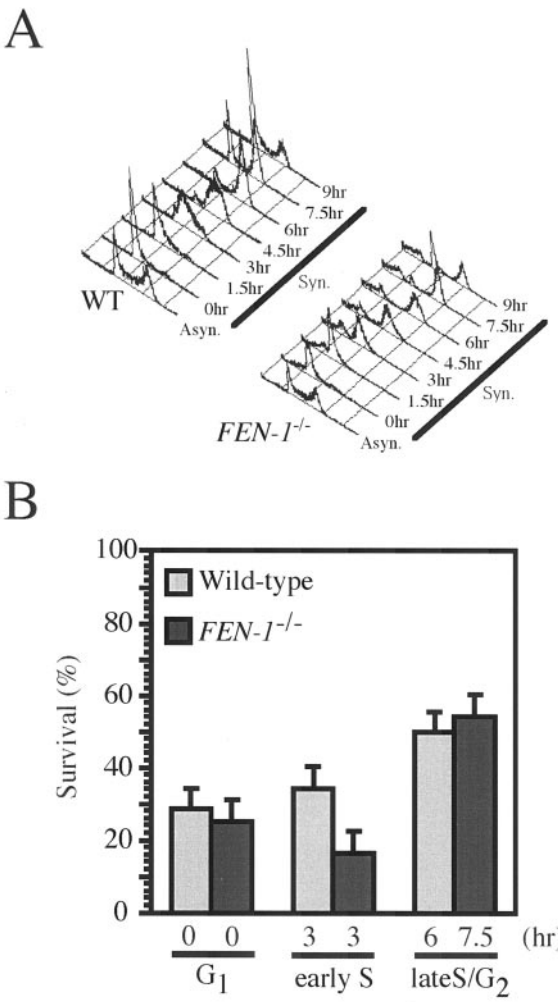


FIG. 2. γ-Ray sensitivity of cells at the G_1, early S, and late S/G_2 phases. (A) Cells of the indicated genotypes were synchronized at the G_1 phase with elutriation and released into culture at 0 h. WT, wild type. (B) Wild-type or *FEN-1*$^{-/-}$ cells were exposed to 2-Gy γ-rays at the indicated cell cycle phase. The number of colonies which appeared after irradiation was divided by that of nonirradiated controls; results are shown as % survival.

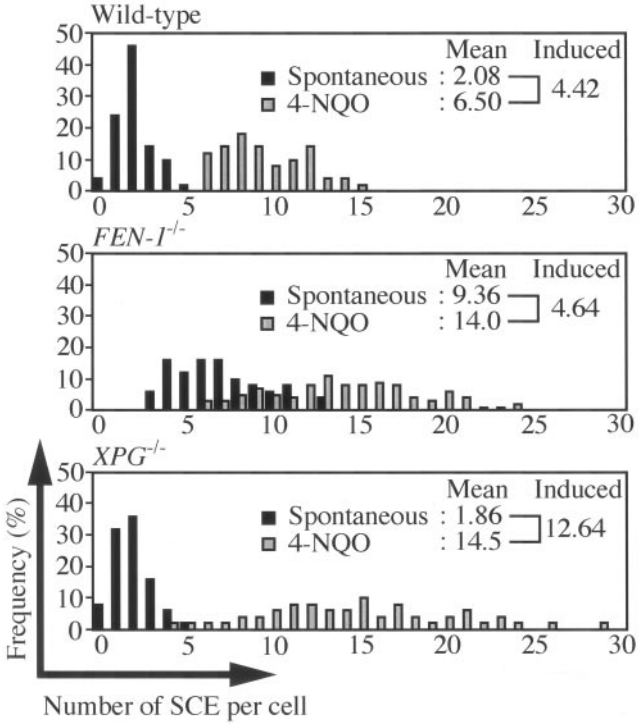


FIG. 3. The level of induced SCE was indistinguishable between wild-type and *FEN-1*$^{-/-}$ cells. The histogram indicates the number of SCE per cell in the wild-type, *FEN-1*$^{-/-}$, and *XPG*$^{-/-}$ cells. The values of induced SCE were calculated by subtracting the mean value of nontreated cells from that of 4NQO-treated cells; results are shown at the top of each panel. Black and gray bars indicate spontaneous SCE as well as SCE induced by 0.2 ng/ml 4NQO treatment, respectively.

(Fig. 1B). The ΨV8 donor segment shares the highest homology with the VJ_λ recipient segment among the ΨV segments (3). Thus, we conclude that deletion of *FEN-1* reduced the frequency of Ig gene conversion involving ΨV8 by 2.5-fold and completely abolished Ig gene conversion with more-diverged ΨV donor segments. These observations imply that Fen-1 may be involved in HR, particularly between diverged homologous sequences.

The reduced kinetics of Ig gene conversion in *FEN-1*$^{-/-}$ cells led us to perform other phenotypic assays of HR reactions. We previously showed that HR-deficient clones such as *RAD54*$^{-/-}$ cells exhibit elevated IR sensitivity specifically in the late S to G_2 phase, when sister chromatids are available to provide exactly matching repair templates for HR (2, 34). We analyzed the sensitivity of synchronized populations of cells to killing by γ radiation using a colony formation assay. Unlike *RAD54*$^{-/-}$ cells, *FEN-1*$^{-/-}$ cells showed elevated IR sensitivity in the early S phase but not in the late S to G_2 phase (Fig. 2B), indicating that Fen-1 is dispensable for HR between sister chromatids. We next determined the level of microscopically visible SCE events, which display gene conversion associated with crossover between two sister chromatids (32). Consistent with the hyper-recombination phenotype of the budding yeast *rad27*Δ strain (39), *FEN-1*$^{-/-}$ DT40 cells showed about 4.5-fold-higher spontaneous SCE levels than did wild-type cells (Fig. 3). When we induced SCE by exposing the cells to 4-nitroquinoline 1-oxide (4-NQO), which mimics UV damage (9), the levels of induced SCE were very similar between wild-type and *FEN-1*$^{-/-}$ cells (Fig. 3). These data suggest that Fen-1 is not required for HR between identical sister chromatids.

Defective I-SceI restriction enzyme-induced gene conversion in *FEN-1*$^{-/-}$ cells. It is believed that Ig gene conversion is initiated by single-strand damage, which is generated as the consequence of AID deaminase-dependent formation of uracil at the Ig V gene (1, 6). To test whether Fen-1 is required not only for Ig gene conversion that is induced by single-strand DNA damage but also for DSB-induced HR, we used an artificial gene conversion substrate DNA, SCneo (16). In the SCneo construct, two mutated neomycin-resistance genes (*neo*^{R}) that are complementary to each other are localized in tandem. The recipient *neo*^{R} coding region is disrupted by the 18-bp I-SceI cleavage site including a stop codon (S2neo; Fig. 4A), while the other promoterless intact *neo*^{R} gene serves as a

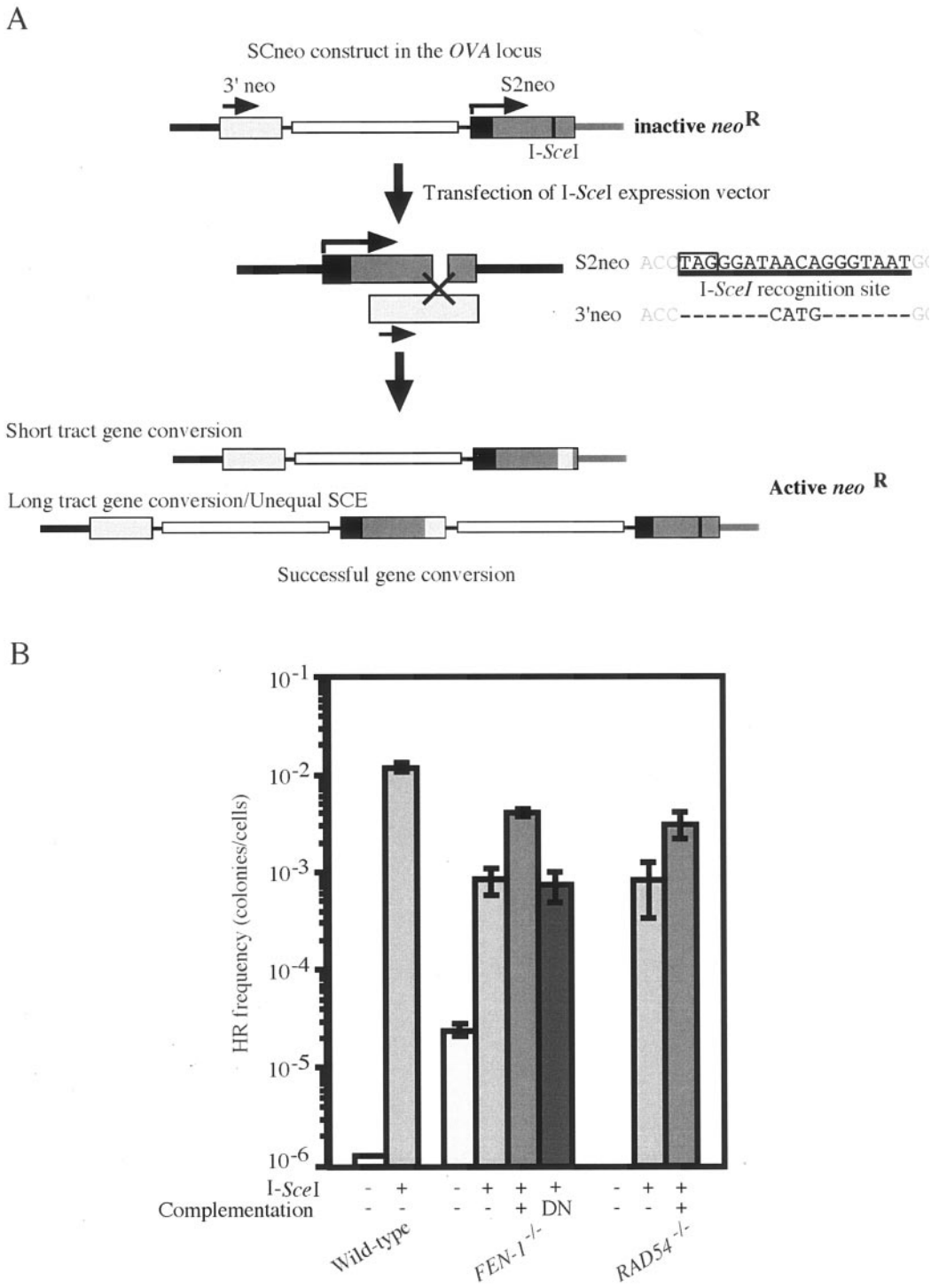


FIG. 4. The reduction of I-SceI-induced gene conversion in *FEN-1*$^{-/-}$ cells. (A) Experimental method of measuring the frequency of gene conversion by counting G418-resistant colonies. The expression vector encoding I-SceI is introduced into cells carrying SCneo in the *Ovalbumin* locus. Black and gray boxes in S2neo represent the 5′ untranslated and coding regions of the *neo*^{R} gene, respectively. The figure is not drawn to scale. Successful gene conversion would reconstitute functional *neo*^{R} gene. (B) The recombination frequency in the SCneo reporter construct in each genotype is shown as the number of G418-resistant colonies derived from 10^{7} cells transfected with the indicated plasmid. Complementation denotes cotransfection of the I-SceI expression plasmid with expression vector of the disrupted gene in the indicated transfected cells. DN, an expression plasmid for nuclease-dead mutant of chicken *FEN-1*. The experiments were done more than four times.

genetic donor for recombinational repair of the I-SceI-induced DSB. To accurately compare data of wild-type and *FEN-1*$^{-/-}$ cells, we integrated the SCneo substrate at the *Ovalbumin* locus in each genotype (11). DSBs induced by transient expression of I-SceI are repaired by gene conversion either from the upstream donor homologous sequences (intragenic gene conversion) or the other sister chromatid (unequal sister recombination) (16). Of note, the 18 nucleotides comprising the I-SceI site in the SCneo recipient sequence should be eliminated during gene conversion-dependent DSB repair to reconstitute a functional *neo*^{R} gene in the cells. Accordingly, we found that HR-mediated DSB-repair induced by transient expression of I-SceI was reduced more than 10-fold in the *FEN-1*$^{-/-}$ cells while there was no significant decrease in colony number in *XPG*$^{-/-}$ cells (Fig. 4B and data not shown). Notably, the reduction in I-SceI gene conversion efficiency was partially reversed by transfection of chicken *FEN-1* cDNA into *FEN-1*$^{-/-}$ cells but not by a nuclease-dead mutant of chicken *FEN-1* (Fig. 4B). Overexpression of chicken *FEN-1* in wild-type cells enhanced I-SceI-induced gene conversion by 2.87-fold (see Fig. S1 in the supplemental material). These data indicate that Fen-1 is required for efficient DSB-induced gene conversion (Fig. 4B) as well as single-strand-damage-induced Ig gene conversion (Fig. 1), and the nuclease activity of Fen-1 is important for this process.

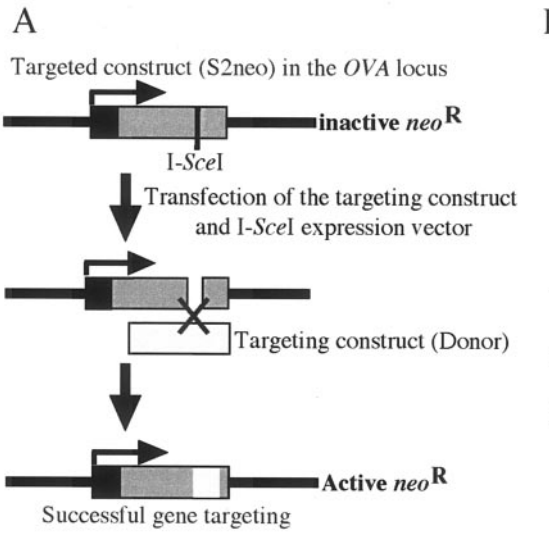
Fen-1 is involved in elimination of heterologous sequences at the I-SceI cleavage site. To investigate whether the sequence divergence between donor and recipient was indeed responsible for the reduction of HR frequency in the absence of Fen-1, we examined I-SceI-induced gene targeting as previously studied (8). Wild-type or *FEN-1*$^{-/-}$ cells carrying S2neo targeted sequence at the *Ovalbumin* locus were transiently transfected with the donor construct and the I-SceI expression vector (Fig. 5A). We used a series of donor constructs carrying different degrees of sequence divergence at I-SceI site (Fig. 5B). Among these modified targeting constructs, Mneo-1 had the smallest sequence divergence from the intact I-SceI site, differing only at two residues. When successfully targeted, these modified sequences will replace the stop codon in I-SceI site and be translated into four extra (Mneo-1, Mneo-2, Mneo-3), one extra (Mneo-4), one fewer (Mneo-5), or two fewer (Mneo-6) amino acid residue(s) in the *neo*^{R} gene product compared to the wild-type *neo*^{R} gene. Using this series of targeting constructs we found that Mneo-1 gave a similar number of colonies when transfected into *FEN-1*$^{-/-}$ cells and wild-type cells carrying S2neo. Conversely, the gene targeting efficiency in *FEN-1*$^{-/-}$ cells, compared to wild-type cells, decreased with the extent of heterologous sequence at the site of DNA breaks (Fig. 5C). These data support our notion that Fen-1 facilitates HR by eliminating heterologous sequences at the I-SceI site.

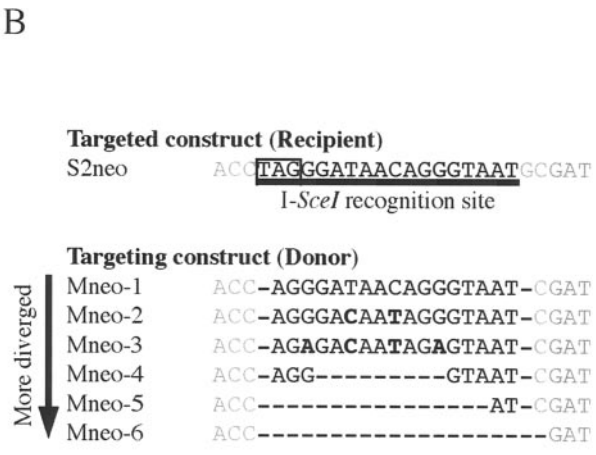
DISCUSSION

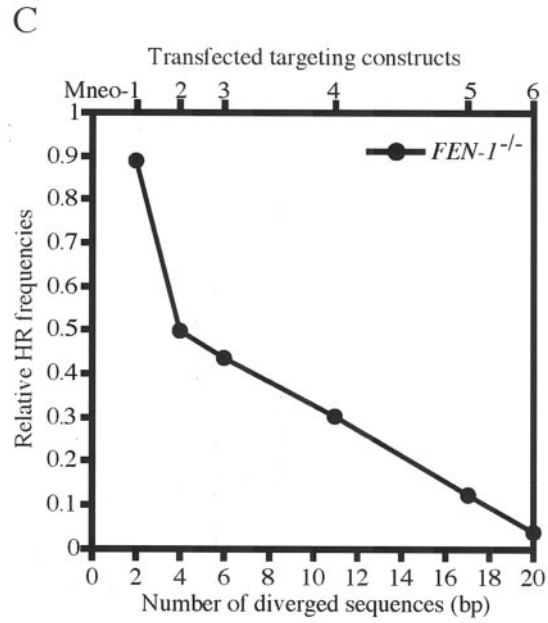
In the present study, we provide the first evidence that Fen-1 is involved in HR reactions in higher eukaryotic cells. Interestingly, only a subset of HR events is affected by loss of Fen-1. Clearly *FEN-1*$^{-/-}$ cells are not IR sensitive during the G_2 phase of the cell cycle and display wild-type levels of induced SCE. However, the HR reaction appears to be affected in *FEN-1*$^{-/-}$ cells when sequence variation between recipient and donor occurs. Thus, Ig gene conversion, which is a good example of HR between variable sequences, is significantly reduced in *FEN-1*$^{-/-}$ cells. Moreover, the mutant cells exclusively use the ΨV8 donor segment, which shares the highest homology with VJ_{λ} recipient segment.

Lastly, our S2neo reporter assay clearly points to a role of Fen-1 in removing nonhomologous sequences during the HR reaction. In these experiments, which were thoroughly controlled for variations in plating efficiency and sensitivity to the endonuclease expression, gene targeting efficiency decreased with the extent of sequence divergence only in the *FEN-1*$^{-/-}$ mutants but not in wild-type and *XPG*$^{-/-}$ cells. One could argue that the HR frequency could be affected by the enzymatic activities of reconstituted *neo*^{R} gene products. The use of various Mneo constructs may cause variation in the number of G418-resistant colonies, since the introduction of sequence divergence at the I-SceI site results in the changes of amino acids in neomycin phosphotransferase. This was not the case, since each Mneo construct showed similar HR frequencies in wild-type cells (Fig. 5C). Another point of concern is the possibility that HR frequency can be affected by nonhomologous end-joining (NHEJ) activity. As long as DSB ends are ligated precisely by NHEJ, these sites are subject to perpetual digestion due to the constitutive expression of I-SceI restriction enzyme. Either HR or imprecise ligation by NHEJ eliminates the I-SceI site and terminates the reaction. The latter case leads to the apparent decrease in HR frequency due to the improperly reconstituted *neo*^{R} gene. We excluded this possibility by a plasmid religation assay (37), in which we observed a normal NHEJ activity in *FEN-1*$^{-/-}$ cells (data not shown). Taken together, these data indicate that the HR defect in *FEN-1*$^{-/-}$ cells clearly depends on the divergence between recipient and donor sequences. Fen-1 is dispensable for the HR reactions that occur between identical or highly homologous sequences such as two sister chromatids or ΨV8 segment but is required for the recombination between DNAs that have short nonhomologous sequences at the ends. Our findings are also consistent with previous yeast genetics results (25). Fen-1 is localized together with PCNA close to the chromatin at pachytene when meiotic recombination between homologous chromosomes occurs (17). It is tempting to speculate that Fen-1 may also facilitate recombination by removal of heterologous sequences between maternal and parental chromosomes during meiosis in higher eukaryotes.

We observed an elevated level of spontaneous SCE in *FEN-1*$^{-/-}$ cells, which apparently argues against the involvement of Fen-1 in HR. However, this finding is consistent with results obtained from phenotypic analysis of the yeast *rad27*Δ strain (39), where the frequency of mitotic crossover is increased and is thought to be a consequence of defective lagging strand DNA synthesis (42, 44). The resulting defect in DNA replication appears to be replaced by HR-mediated repair, because budding yeast mutants deficient in both *rad27* and *RAD52* epistasis groups are synthetically lethal (5, 38). Likewise, our observation of increased spontaneous SCE in *FEN-1*$^{-/-}$ cells may reflect enhanced HR-dependent repair due to defective processing of Okazaki fragments and impaired BER. Accordingly, we observed increased IR sensitivity during early S phase, which could be a consequence of defective BER and subsequent replication blocking in *FEN-1*$^{-/-}$ cells.







HR donor	HR frequencies	
	Wild-type	*FEN-1*$^{-/-}$
Mneo-1	$(1.45\pm0.0425)\times10^{-4}$	$(1.29\pm0.167)\times10^{-4}$
Mneo-2	$(5.43\pm0.430)\times10^{-5}$	$(2.71\pm0.309)\times10^{-5}$
Mneo-3	$(4.00\pm0.214)\times10^{-5}$	$(1.74\pm0.196)\times10^{-5}$
Mneo-4	$(4.94\pm0.647)\times10^{-5}$	$(1.49\pm0.255)\times10^{-5}$
Mneo-5	$(1.08\pm0.0640)\times10^{-4}$	$(1.31\pm0.234)\times10^{-5}$
Mneo-6	$(1.59\pm0.0222)\times10^{-4}$	$(4.95\pm0.865)\times10^{-6}$

FIG. 5. Diverged short sequences block effective recombination in *FEN-1*$^{-/-}$ cells. (A) Experimental method of measuring the frequency of gene targeting by counting G418-resistant colonies. The expression vector encoding I-SceI is introduced together with WTneo (white box) into cells carrying S2neo in the *Ovalbumin* locus. Black and gray boxes represent the 5′ untranslated and coding regions of the *neo*^{R} gene, respectively. The figure is not drawn to scale. Successful gene targeting would reconstitute a functional *neo*^{R} gene. (B) Base sequence alignment around the I-SceI site in a series of targeting constructs (Mneo-1 to Mneo-6). Mneo-1 donor contains sequences that are two nucleotides shorter (shown by hyphen) than the corresponding sequences of S2neo recipient. Bold characters show inserted point mutations. Boxed characters show a stop codon. (C) The gene-targeting frequency of targeting constructs in *FEN-1*$^{-/-}$ cells. The indicated targeting constructs (shown at top) were transfected into cells carrying S2neo. The number of diverged sequences (shown at bottom) includes inserted point mutations and missing sequences in the targeting constructs. Relative HR frequencies on the *y* axis were calculated by dividing the HR frequency of *FEN-1*$^{-/-}$ cells which appeared after G418 selection by that of *FEN-1*$^{+/+}$ cells.

How Fen-1 eliminates nonhomology from DNA ends remains elusive. It has been shown in vitro that Fen-1 possesses 5′-to-3′ exonulease as well as structure-specific endonuclease activity. We speculate on three possibilities for the action of Fen-1 based on the biochemical evidence presented so far by others. First, Fen-1 may extend the 3′ overhang with its exonucleolytic activity until the identical sequence is exposed, so that the pairing can occur between the substrate and homologous template DNA. Secondly, the endonuclease activity of Fen-1 may eliminate nonhomologous 5′ flap structures at D-loops after the invasion of 5′ overhang into duplex DNA. This idea is consistent with biochemical evidence that Fen-1 cleaves the 5′ and not the 3′ flap structure of DNA and with the observation that Rad51 can form filaments on 5′ single-stranded DNA and perform strand exchange (23). The third of the possibilities relies on the recent observations made by

Zheng et al. (51), in which DNA bubble structures are cleaved by Fen-1 at single- and double-stranded DNA junctions on both ends. This suggests that, under some circumstances, Fen-1 could cleave the 3′ flap structure, which is generated after strand invasion by 3′ overhang. The first and third possibilities are not mutually exclusive and may in fact complement each other. Accumulating evidence has suggested a critical role of Fen-1 for processing DSB ends in the course of HR (25) and NHEJ (47) in yeast. This study sheds light on a previously unknown function of Fen-1 in higher eukaryotic cells: the elimination of imperfectly matched sequences from DSB ends for subsequent HR-mediated DSB repair. Interestingly, Fen-1 also contributes to conventional gene targeting at three different loci (see Table S2 in the supplemental material). Although the role for Fen-1 in this situation is unclear, it is tempting to speculate that Fen-1 functions in removing the heterologous sequences as it does in I-SceI-induced gene targeting. Since overexpression of Fen-1 alone enhanced I-SceI-induced gene targeting (see Fig. S1 in the supplemental material), the challenge for the future will be to seek for the way to improve gene targeting efficiency in vertebrate cells using Fen-1 and to understand how Fen-1 works in conventional gene targeting.

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