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Delayed DNA joining at 3′ mismatches by human DNA ligases

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ABSTRACT

Repair synthesis catalysed by DNA polymerase β at 1 nt gaps occurs in the main pathway of mammalian base excision repair. DNA polymerase β has no exonucleolytic proof-reading ability, and exhibits high error frequency during DNA synthesis. Consequently, continuous correction of endogenous DNA damage by short-patch repair synthesis might lead to a high spontaneous mutation rate, unless subsequent steps in the repair pathway allow for selective removal of incorporation errors. We show here that both human DNA ligase I and III discriminate strongly between a correctly paired versus a mispaired residue at the 3′ position of a nick in DNA, when assayed in the presence of physiological concentrations of KCl. The resulting delay in joining after misincorporation by DNA polymerase β during gap filling could allow for removal of the mismatched terminal residue by a distinct 3′ exonuclease.

INTRODUCTION

DNA undergoes slow but significant turnover in mammalian cells due to the continuous repair of endogenous lesions generated by hydrolysis, active oxygen, and accidental damage inflicted by reactive metabolites and coenzymes (1,2). These lesions are largely removed by the base excision repair (BER) pathway, in which single-strand breaks are introduced at abasic sites by a specific endonuclease, usually followed by the filling-in of a 1 nt gap by repair replication, removal of the abasic sugar-phosphate residue either before or after repair synthesis, and finally ligation. In mammalian cells, DNA polymerase β (Pol β) catalyses the short-patch repair replication step, and this is the only known function of Pol β (3). Recent experiments with a variety of repair-defective mammalian cell lines indicate strongly that the subsequent joining step is catalysed by DNA ligase III *in vivo* (4,5), whereas biochemical data have suggested that either DNA ligase III or DNA ligase I could be active (6,7).

Pol β lacks 3′ exonuclease activity, and consequently is unable to perform proof-reading during DNA synthesis to remove occasional misincorporated residues by an editing process. By a variety of experimental approaches (8–12), Pol β has been estimated to make about 1 incorporation error per 1900–4500 synthesis events generating pyrimidine–pyrimidine and pyrimidine–purine mispairs, whereas purine–purine mispairs are much less frequent. The actual frequencies observed vary between different base pairs and experimental approaches, and there is also some controversy as to whether repair synthesis of a single nucleotide gap is more accurate than filling-in of longer stretches (10,12,13).

Loss of purine bases from the genome by non-enzymatic hydrolytic depurination occurs at a frequency of 9000–10 000 events per day in a mammalian cell (14,15), and other forms of endogenous events, including oxygen damage, are also relevant. As a consequence, BER of endogenous DNA damage with a Pol β misincorporation frequency of 1 error in ~3000 gaps filled would yield several premutagenic events per day for actively growing as well as non-proliferating cells. In order to avoid a high spontaneous mutation rate in mammalian cells, an error avoidance mechanism subsequent to the action of Pol β during BER appears to be required. The 3′ exonuclease DNase III was shown recently to be able to fulfil the requirement for an editing function during BER, using a reconstituted system with Pol β, DNA ligase III and a nicked DNA substrate containing a 3′-terminal mismatch (16). This enzyme, which is homologous to the *Escherichia coli* DnaQ/MutD editing exonuclease, acts in a non-processive way and preferentially removes single-stranded or mismatched regions at DNA 3′-ends (16,17). Alternatively, the human homologue of the *Saccharomyces cerevisiae* RAD17/*Schizosaccharomyces pombe* RAD1/*Ustilago maydis* REC1 protein (18) might be able to perform exonucleolytic DNA editing. A DNA ligase needs to contribute to the predicted proof-reading mechanism. If the ligase were unable to join a nick with a 3′ mismatched residue produced by erroneous 1 nt gap filling by Pol β, or could only join it slowly by comparison with the sealing of nicks with correct base pairs, an autonomous exonuclease would have a window of time to remove the mismatched residue. This would resolve the paradox that repair of endogenous DNA damage might be the cause of a high spontaneous mutation rate.

In apparent disagreement with this model, several reports have described efficient joining at nicks with mismatches by a number of eukaryotic DNA ligases (19–22). We have reinvestigated the apparent ability of human DNA ligases I and III to join substrates containing 3′ mismatches and have found that

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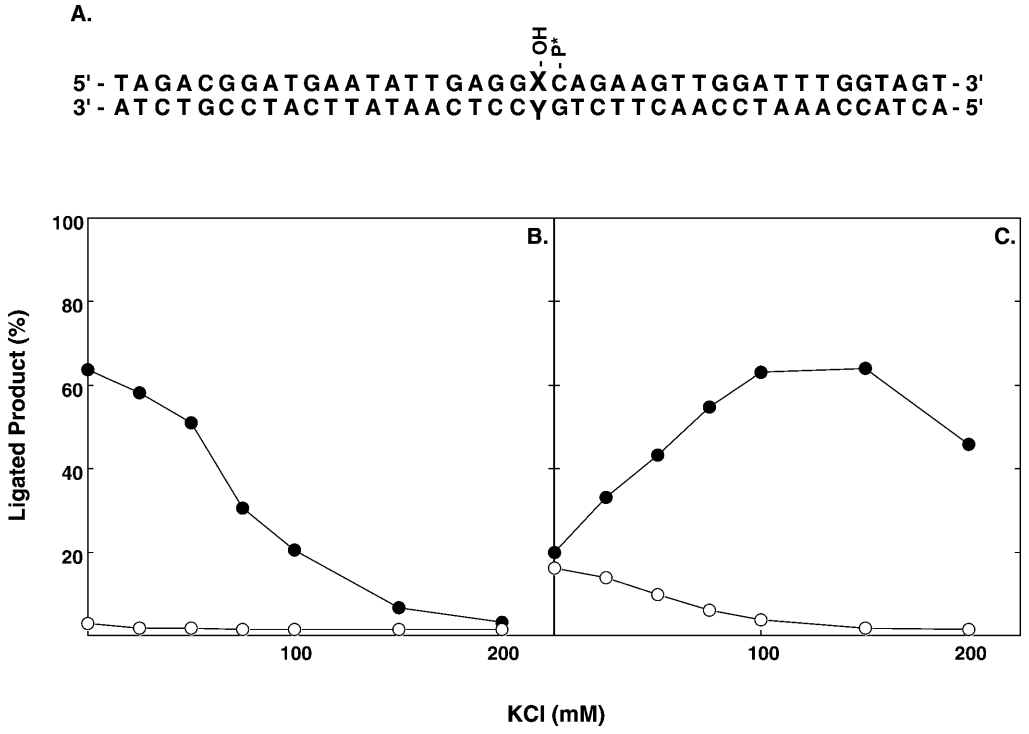

Figure 1. Joining of matched versus 3′ mismatched substrates by human DNA ligases at different KCl concentrations. (**A**) Duplex [^{32}P]DNA substrates containing a single-strand break with a matched or mismatched 3′-OH terminus are designated 3′-X/Y, where X and Y represent any one of the four common nucleotides. DNA ligation reactions (20 µl) contained 360 fmol of DNA ligase I (**B**) or 180 fmol of DNA ligase III (**C**) and 180 fmol of either [^{32}P]3′-A/T (closed circles) or [^{32}P]3′-G/T (open circles) substrates. Each reaction mixture was supplemented with KCl as indicated. After incubation at 37°C for 5 min, reaction products were analysed by denaturing polyacrylamide gel electrophoresis, the amount of radiolabeled unreacted substrate (20mer) and product (41mer) were quantitated by phosphorimage analysis, and the percentage of ligated product was calculated for each reaction mixture.

the results depend strongly on experimental conditions. Our data indicate that human DNA ligase III requires physiological salt concentrations to discriminate effectively between paired and mispaired 3′ residues at nicks, whereas DNA ligase I is efficient in this regard at all tested salt concentrations.

MATERIALS AND METHODS

Oligonucleotide substrates

Substrates were a 41-bp duplex DNA containing a centrally located nick with a matched or mismatched base pair (X/Y) positioned at the 3′-OH terminus of the nick (Fig. 1A). The following DNA oligonucleotides were made: a 20mer (CAGAAGTTGGATTTGGTAGT), four 21mers (TAGACGGATGAATATTGAGG**X**, where X = C, T, G or A) and four 41mers (ACTACCAAATCCAACTTCTG**Y**CCTCAATATTCATCCGTCTA, where Y = C, T, G or A). All oligonucleotides were synthesized on an Applied Biosystems 394 DNA Synthesizer. The 20mer oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis and 5′-^{32}P-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). To prepare the double-stranded nicked DNA substrates, the 20mer (5 pmol) and a 21mer (10 pmol) were hybridised to a 41mer (10 pmol) in mixtures (50 µl) containing 20 mM Tris–HCl, pH 7.5, and 2 mM $MgCl_2$. Substrates were annealed by incubating at 90°C for 2 min and cooling slowly to room temperature (~4 h).

DNA ligation reactions

Recombinant human DNA ligases I and III were purified as described previously (23,24). Reaction mixtures contained 60 mM Tris–HCl pH 7.5, 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 50 µg/ml nuclease-free bovine serum albumin, 9 nM nicked [^{32}P]DNA substrate, and also KCl and DNA ligase I or III as indicated in the figure legends. Incubations were at 37°C for the times indicated and reactions were terminated by the addition of an equal volume of denaturing gel loading buffer (95% formamide, 10 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) followed by heating at 95°C for 3 min. Ligation products were analysed by gel electrophoresis using denaturing 12.5% polyacrylamide gels. The gels were dried under vacuum and radioactive DNA bands were quantitated using a Storm 860 Phosphorimager (Molecular Dynamics) and ImageQuant software.

RESULTS

Ligation specificity at different KCl concentrations

The ability of human DNA ligases I and III, as well as several other ATP-dependent DNA ligases isolated from a variety of

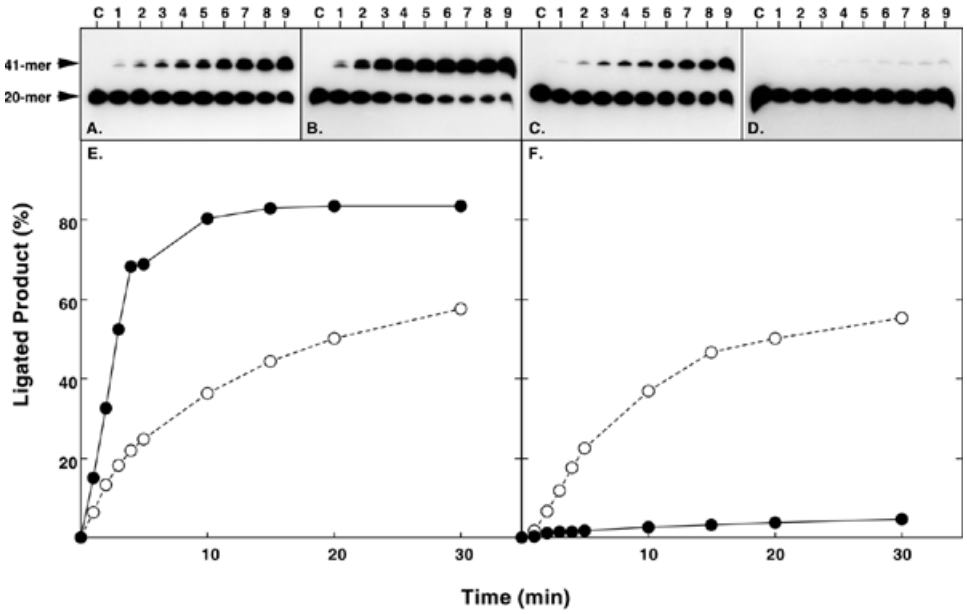

Figure 2. Effect of KCl concentration on the rates of joining of different substrates by DNA ligase III. DNA ligase reactions (100 µl) contained 900 fmol of DNA ligase III and either 900 fmol of [^{32}P]3′-A/T substrate supplemented with 0 (**A**) or 150 mM (**B**) KCl or 900 fmol of [^{32}P]3′-G/T substrate supplemented with 0 (**C**) or 150 mM (**D**) KCl. Incubations were at 37°C for 1, 2, 3, 4, 5, 10, 15, 20 and 30 min (lanes 1–9, respectively). Control reactions (lane C) containing 90 fmol of the appropriate [^{32}P]DNA substrate were incubated at 37°C for 30 min without enzyme. Samples (10 µl) were removed at the indicated time points and subjected to denaturing polyacrylamide gel electrophoresis. Arrows indicate the location on the autoradiogram of unreacted substrate (20mer) and product (41mer). The overall extent of ligation after various incubation times was determined by phosphorimager analysis (see Fig. 1) and plotted as a function of time for [^{32}P]3′-A/T (**E**) and [^{32}P]3′-G/T (**F**) substrates at 0 (open circles) and 150 mM (closed circles) KCl, respectively.

biological sources, to join nicks containing mismatches have been investigated previously using low salt reaction conditions (19–22). Generally, these enzymes efficiently sealed strand interruptions with a mismatch located at either the 5′-phosphate or 3′-OH terminus of the nick, except for a poor ability to join at 3′ purine–purine mispairs. It is unclear whether 5′ mismatches at nicks would ever occur to a significant extent *in vivo*. In contrast, the fates of 3′ mismatches are clearly important because they can be generated by misincorporation during DNA gap filling. For this reason, we have only been concerned with 3′ mismatches in the present investigation.

In order to determine the influence of salt concentration on the ligation efficiency and fidelity of DNA ligases I and III at nicks containing correctly paired or mispaired 3′-OH termini, ligation experiments were conducted with increasing concentrations of KCl (Fig. 1). The joining activity of DNA ligase I at a nick containing a 3′-A/T base pair was greatest when no KCl was added to the reaction mixture and steadily decreased at higher salt concentrations (Fig. 1B). A similar amount of enzyme failed to efficiently promote strand joining at nicks containing a 3′-G/T mispair at all KCl concentrations, although some misjoining was observed in the absence of added KCl (Fig. 1B). A similar experiment with DNA ligase III revealed that, unlike DNA ligase I, strand joining activity towards a 3′-A/T nicked substrate was optimal between 100 and 150 mM KCl, whereas efficient joining activity at a mispaired 3′-G/T substrate was observed at low salt concentrations but was suppressed by increasing KCl (Fig. 1C). The data for DNA ligase III in the absence of added KCl were similar to those previously published (19). Thus, at physiological salt concentrations (100–150 mM), a 17- to 36-fold increase in the amount of ligated product (ligation specificity) by DNA ligase III was achieved for a 3′-A/T base pair relative to that of a 3′-G/T mispair. Similar results were obtained with DNA ligase III at different salt concentrations both in the presence and absence of an equimolar concentration of the scaffold protein XRCC1, with only a marginal improvement in ligation specificity in the presence of XRCC1 (data not shown); while the two proteins interact tightly through their C-terminal regions, the interaction domain does not overlap with the catalytic domain of DNA ligase III (24). Due to the low level of activity towards 3′ mispairs by DNA ligase I, the ligation specificity at different salt concentrations could not be quantitated accurately under these experimental conditions but there was a >20-fold difference in the rate of joining of paired versus mispaired substrates (Fig. 1B). As reported by Husain *et al*. (19) and also observed here, DNA ligase I is more discriminatory than DNA ligase III in joining at nicks containing mispaired 3′-OH termini.

Ligation specificity at different 3′ mismatches

Increased discrimination against DNA ligation at 3′ mismatches is obtained when ionic reaction conditions mimic those present *in vivo* (Fig. 1). To further address the effect of physiological salt concentrations on substrate specificity, the rate of ligation of a 3′-A/T base pair relative to a 3′-G/T mispair was examined for DNA ligase III (Fig. 2). Analysis of

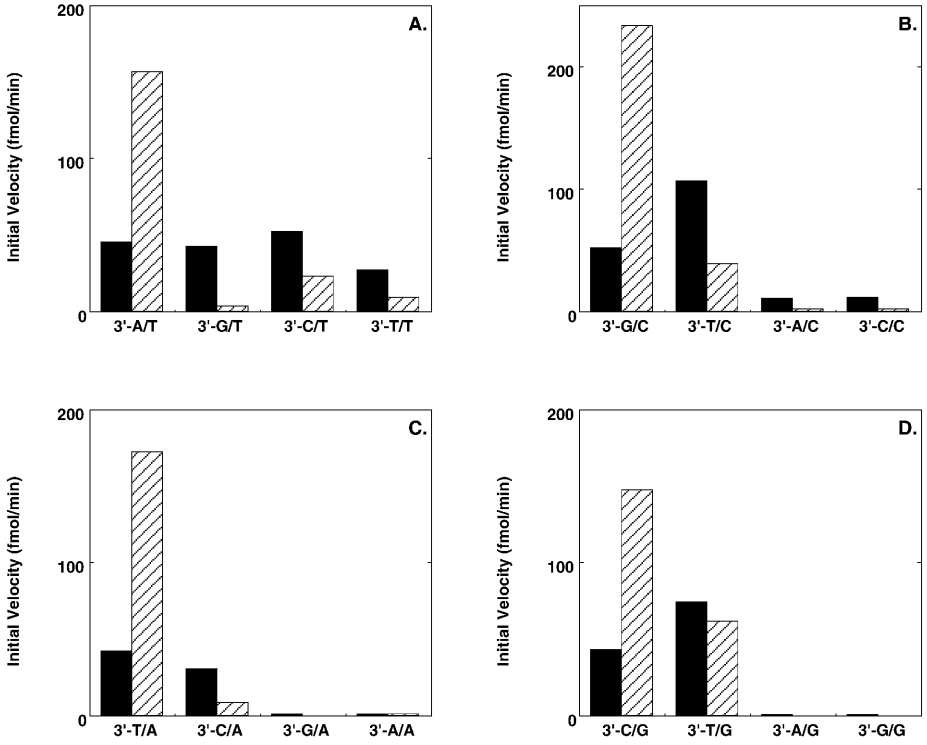

Figure 3. Kinetics of ligation at different 3′ mismatched base pairs by DNA ligase III. Sixteen DNA ligase reaction mixtures (100 µl) containing 900 fmol of DNA ligase III and 900 fmol of each [^{32}P]DNA substrate with the indicated base pair configuration at the 3′-OH terminus of the nick were incubated at 37°C and aliquots removed at 1, 2, 3 and 4 min as in Figure 2. Following denaturing polyacrylamide gel electrophoresis and phosphorimager analysis, the amount of [^{32}P]41mer product was measured in order to obtain the initial velocity (fmol/min) of the ligation reaction for [^{32}P]DNA substrates containing base-paired and mispaired nucleotides opposite a template T (**A**), C (**B**), A (**C**) or G (**D**). The results are plotted for each substrate in reaction mixtures containing 0 (black bars) and 150 mM (striped bars) KCl, respectively.

ligation products from the first 4 min of the reaction with a nick containing a 3′-A/T base pair at 0 (Fig. 2A) and 150 mM (Fig. 2B) KCl showed initial velocity values of 45 and 157 fmol product formed per min, respectively, under the experimental conditions used. As expected, similar KCl-dependent stimulation of the initial rate of ligation (3.4- to 4.5-fold) was observed for all other nicks containing properly base paired 3′-OH termini (Fig. 3). In comparison, the initial rate of strand joining at nicks with a 3′-G/T mispair at 0 mM KCl (Fig. 2C) was significant (42 fmol/min) but decreased dramatically (3 fmol/min) in the presence of 150 mM KCl (Fig. 2D–F). The kinetics of ligation of all 12 possible 3′ mismatches were compared at 0 and 150 mM KCl. DNA ligase III was fairly permissive with most nicked DNA substrates containing 3′-OH mismatches in the absence of KCl, except for 3′ purine–purine mispairs which were largely refractory to strand joining (<1% product formed) (Fig. 3). In particular, nicks with mispaired bases opposite a template T were good substrates for DNA ligase III. However, addition of 150 mM KCl resulted in substantial decreases in the initial velocity of the ligation reaction for 3′ mispairs (Fig. 3). Discrimination of greater than one order of magnitude reduction in the initial velocity, when compared to the base-paired control, was observed with 3′-A/C, 3′-C/C, 3′-G/T, 3′-T/T and 3′-C/A substrates, while less discrimination was noted with 3′-C/T, 3′-T/C and 3′-T/G substrates. Thus, the ability of DNA ligase III to distinguish between correctly paired and mispaired bases at the 3′-terminus of a nick is enhanced by the presence of physiological salt concentrations and favours ligation of the properly base-paired strand interruption.

DISCUSSION

Proof-reading during DNA synthesis by 3′ exonucleolytic removal of a newly misincorporated 3′ residue is important in achieving high replication fidelity (25). During DNA replication, a reduced rate of addition of a second nucleotide to a 3′ mismatch by a DNA polymerase allows time for excision of the mismatched nucleotide (26,27). This means of proof-reading is not applicable to the main pathway of BER in mammalian cells, because gap filling by Pol β primarily involves the replacement of a single nucleotide prior to ligation. The concept of proof-reading during such DNA repair, however, could still be valid if the DNA ligase were able to discriminate against 3′ mismatched residues.

Previously published data on joining at mismatches by eukaryotic DNA ligases have offered little support for this model, because the enzymes apparently could join efficiently at most 3′ mismatches. Husain *et al*. (19) showed that both mammalian DNA ligase I and DNA ligase III joined strand

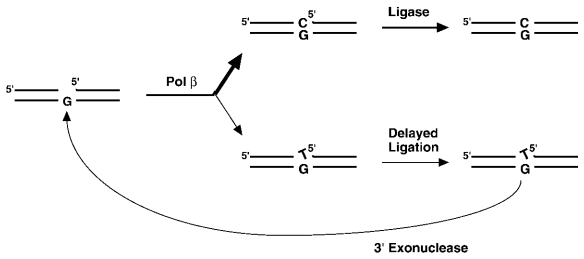

Figure 4. Model for proof-reading in the BER pathway of mammalian cells. Removal of a base, nicking and excision of the sugar-phosphate residue at the abasic site result in the formation of DNA with a 1 nt gap as an intermediate (shown to the left). The gap is filled-in by Pol β. Alternatively, Pol β may incorporate a nucleotide at a nicked abasic site and then also be active in removal of the displaced abasic sugar-phosphate residue. In either case, the final step in the repair process is DNA joining (shown in the upper branch). The last steps probably occur in a concerted fashion, with the scaffold protein XRCC1 bringing Pol β and DNA ligase III together at the site of the lesion. Since Pol β does not possess an innate proof-reading 3′ exonuclease activity, frequent misincorporation events occur (lower branch). Subsequent ligation at such mismatches is suppressed by the reduced ability of the DNA ligase to join at a 3′ mismatch. This allows for removal of the misincorporated nucleotide by a separate nuclear 3′ exonuclease such as DNase III, and a renewed attempt by Pol β at correct gap filling.

breaks with C/T or G/T mismatches at the 3′ side of the nick, with the T residue in the continuous strand. Similar efficient ligation of nicks with an adjacent mismatched base pair has been described for vaccinia virus DNA ligase (20), for a *Chlorella* DNA ligase (21), and for the *S.cerevisiae* CDC9 gene product (22). The major finding in the present study is that those *in vitro* results, although correct, are misleading because they were obtained at non-physiologically low salt concentrations. When reaction conditions were employed here with KCl levels that are likely to be close to those occurring intracellularly, significant discrimination against joining at 3′ mismatches was observed. It is probably also physiologically relevant that kinetically favorable nucleotide misincorporation events catalysed by Pol β, i.e. dGMP/T, dCMP/A and dAMP/C (10), correspond to strong discrimination events during ligation by DNA ligase III (Fig. 3).

The importance of correct salt concentrations in DNA ligation reactions was highlighted recently by Ramsden and Gellert (28), who showed that efficient joining of DNA double-strand breaks by purified mammalian DNA ligases only occurs at low, non-physiological salt concentrations. End-joining under physiological ionic conditions is strongly stimulated by Ku protein, which consequently has a direct role in the sealing of DNA double-strand breaks. An earlier study by Wu and Wallace (29) on the joining specificity of T4 DNA ligase noted that mismatch ligation could be suppressed by including NaCl or (less effectively) spermidine in reaction mixtures, but these results seem to have been overlooked in more recent work on eukaryotic ligases. Detailed investigations of DNA–protein interactions in well-characterised systems such as the binding of *lac* repressor to DNA have shown that large changes in equilibrium and rate constants can be induced by relatively small changes in ionic conditions; the large dependence of non-specific electrostatic binding on salt concentration results in increased specificity with increasing ionic strength (30,31). The suppression of mismatch ligation by physiological salt concentrations is, therefore, likely to be valid for all DNA ligases, not just for T4 DNA ligase (29) and the human DNA ligase III investigated in the present study.

In conclusion, discrimination against ligation of 3′ mismatches at single-strand interruptions provides a means of proof-reading of a single nucleotide residue incorporated at a 1 nt gap during BER in mammalian cells (Fig. 4). A related general model has been proposed for *Thermus thermophilus*, where an NAD-dependent high fidelity DNA ligase may have evolved to compensate for the absence of proof-reading activity of DNA polymerases (32). It is not yet known if delayed ligation is the only cellular strategy for prevention of joining at incorporated errors during DNA BER in mammalian cells. Suppression of DNA ligation at mismatches is one possible surveillance mechanism for reducing spontaneous mutation rates, and a cellular mutator phenotype might be observed if this particular mechanism were inactivated.

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