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Eukaryotic Mismatch Repair in Relation to DNA Replication

Thomas A. Kunkel[1] and **Dorothy A. Erie**[2]

[1]Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, North Carolina 27709

[2]Department of Chemistry and Curriculum in Applied Sciences and Engineering, University of North Carolina, Chapel Hill, North Carolina 27599-3290

Abstract

Three processes act in series to accurately replicate the eukaryotic nuclear genome. The major replicative DNA polymerases strongly prevent mismatch formation, occasional mismatches that do form are proofread during replication, and rare mismatches that escape proofreading are corrected by mismatch repair (MMR). This review focuses on MMR in light of increasing knowledge about nuclear DNA replication enzymology and the rate and specificity with which mismatches are generated during leading- and lagging-strand replication. We consider differences in MMR efficiency in relation to mismatch recognition, signaling to direct MMR to the nascent strand, mismatch removal, and the timing of MMR. These studies are refining our understanding of relationships between generating and repairing replication errors to achieve accurate replication of both DNA strands of the nuclear genome.

Keywords

DNA mismatch repair; replication fidelity; genome instability; mutation rate; mutator

INTRODUCTION

Building on seminal studies of mismatch repair (MMR) in *Escherichia coli* (see 94 and references therein), examination of eukaryotic MMR began more than 25 years ago. It quickly emerged that MMR of nuclear DNA replication errors involves a set of evolutionarily conserved core proteins that recognize mismatches, identify a signal to direct MMR to the newly replicated DNA strand that contains the error, remove the DNA containing the mismatch, and correctly resynthesize the DNA and ligate the nick to complete repair. Mutations in the genes encoding MMR proteins destabilize the nuclear genome and can increase cancer susceptibility, thus revealing the importance of MMR. MMR proteins also modulate cellular responses to environmental stress, prevent recombination between diverged sequences, modulate development of the immune system, influence the stability of

Correspondence to: Thomas A. Kunkel.

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trinucleotide repeat sequences associated with degenerative diseases, and participate in meiosis. All of these subjects continue to garner widespread interest, as evidenced by the large number of review articles on the functions of MMR proteins published this year alone (4, 12, 20, 24, 33, 41, 43, 44, 53, 59, 72, 74–77, 86, 108, 109, 113a, 127, 150). The broad range of topics covered in these reviews allows us to focus this review on relationships between MMR and nuclear DNA replication.

In *E. coli*, MMR is directed to the nascent strand by transient undermethylation of adenines in GATC sequences (113, 143). These adenines are quickly methylated after replication (87), after which processing of the mismatch is no longer strand specific and therefore does not enhance genome stability. This observation indicates that replication and MMR are coordinated, and the conservation between bacterial and eukaryotic MMR suggests that eukaryotic MMR and nuclear DNA replication are also coordinated. Indeed, both transactions use several common proteins, including the matchmaker protein PCNA (proliferating cell nuclear antigen) sliding clamp, which has multiple roles in both MMR and replication. Despite the importance of understanding relationships between generating and correcting replication errors, studying this subject has been challenging. One reason is that studies of eukaryotic MMR in vitro typically use preformed mismatches rather than mismatches actually generated by the replication machinery. Furthermore, until recently, genetic studies did not identify the replicase that generated the mismatch, the nascent strand in which the mismatch was located, or the base composition of the mismatch. However, in the past few years, an increased understanding of replicase-specific and strand-specific generation of mismatches has allowed the study of strand- and mismatch-specific MMR in vivo. This review briefly describes recent advances in understanding nuclear DNA replication enzymology, including the rates at which mismatches are generated and repaired during leading-and lagging-strand replication. We then consider how this information relates to the efficiency, mechanisms, and timing of eukaryotic MMR.

LEADING- AND LAGGING-STRAND REPLICATION OF NUCLEAR DNA

In *E. coli*, a single MutS-dependent MMR pathway corrects mismatches generated by proofreading-proficient DNA polymerase III, the major replicase for both DNA strands. The situation is more complex in eukaryotes (Figure 1), in which replication errors are generated by three different Family B DNA polymerases (a.k.a. replicases), and in which there are multiple opportunities for MMR (discussed below) that likely involve different DNA ends and enzymology because of different relationships to the replication fork. Nuclear DNA replication (101) is initiated at replication origins when a primase associated with DNA polymerase α (Pol α) synthesizes an RNA chain that is subsequently extended by limited DNA synthesis by Pol α. Pol-α primase also initiates the formation of Okazaki fragments during replication of the nascent lagging strand. This initiation occurs at intervals of several hundred bases, indicating that a few percent of nuclear DNA may initially be synthesized by Pol α. The RNA primers are removed during Okazaki fragment maturation (OFM). RNases H1 and H2 (9) are capable of removing all but the final 5′ ribonucleotide of these RNA primers, but they are not essential for OFM. The primary OFM pathway involves strand-displacement synthesis by Pol δ and subsequent cleavage by flap endonuclease 1 (Fen1). In

the absence of Fen1, other nucleases participate in OFM (152), including Exo1 and Dna2, the latter of which participates in a long-flap pathway (3).

Using the DNA primers synthesized by Pol α, the majority of nuclear DNA replication is catalyzed by two multi-subunit polymerases, Pols δ and ε. Unlike Pol α, the polymerase catalytic subunits of Pols δ and ε contain a 3′-exonuclease activity that can proofread replication errors. Pols δ and ε differ in structure, subunit composition, protein partnerships, processivity, and fidelity (see 51, 68 and references therein). At the time of our previous MMR review in 2005 (69), several models were proposed for the roles of Pols δ and ε in leading- and lagging-strand replication (see 67, 107 and references therein). Among these, it now appears likely that Pol ε and Pol δ are the primary leading- and lagging-strand replicases, respectively (Figure 1). This conclusion is supported by genetic studies of two types of replication errors seen in repair-deficient cells. In yeast strains deficient in MMR, variant derivatives of Pols α, δ, and ε generate single-base mismatches whose strand specificity in relation to replication origins implicates Pol ε primarily in leading-strand replication and Pol δ primarily in lagging-strand replication (see 82 and references therein). This interpretation likely extends to mammals, as evidenced by the evolutionary conservation of all three major replicases and by a recent study of base substitution patterns in human cells harboring mutations in the proofreading exonuclease domain of Pol ε (125). This division of replicase labor is also supported by studies of yeast strains defective in ribonucleotide excision repair (RER) (11, 13, 58, 116). These studies show that Pol ε primarily incorporates ribonucleotides into the nascent leading strand, whereas Pols α and δ primarily incorporate ribonucleotides into the nascent lagging strand. The primary strand-specific roles of Pols δ and ε are also supported by other methods of analysis (e.g., see 148 and references therein), including biochemical studies of replication reactions reconstituted using purified proteins (see 27 and references therein). The latter study indicates that an 11-protein CMG helicase complex composed of Cdc45, Mcm2–7, and GINS selectively recruits Pol ε over Pol δ for leading-strand replication, whereas PCNA selectively recruits Pol δ over Pol ε for lagging-strand replication. These processes may be highly relevant to proofreading during replication as well as to events occurring after replication, including mismatch removal. Although the fork depicted in Figure 1 likely reflects the norm, considerable evidence indicates that replication enzymology is pliable and may change depending on distance from origins, replication timing, and chromosomal location (e.g., in telomeres and at fragile sites); upon encounters with transcription complexes; or under environmental stress.

GENERATING AND PROOFREADING MISMATCHES DURING DNA REPLICATION

The replication machinery generates replication errors at different rates depending on the DNA polymerase, the mismatch, and the local DNA sequence. Before considering MMR itself, we briefly review information on the mechanisms and rates at which the substrates for MMR are generated during replication.

Error Prevention and Proofreading In Vitro

If DNA polymerases merely acted as zippers to polymerize DNA based on free energy differences between correct and incorrect base pairs (ΔΔG), then mismatches would be generated at a rate of approximately 10^{-2} to 10^{-3} (80). Fortunately for genome stability, Pols α, δ, and ε all impose high selectivity to the polymerization reaction and on average generate only around one mismatch for every 10^4 to 10^5 correct bases incorporated in vitro (66). Importantly, the probability that any particular mismatch will initially be made by a replicase varies from extremely rare misinsertion of dCTP opposite template C by Pol α [$\leq 10^{-7}$ (92)] to much more frequent formation of single-base deletion mismatches in long homonucleotide runs [$\geq 10^{-3}$ (25)]. Polymerases can also be tricked into generating damaged mismatches that are subject to MMR. A prime example is preferential insertion of adenine rather than cytosine opposite 8-oxo-guanine by Pols α, δ, and ε (36, 119, 124).

The accuracies of Pols ε and δ are enhanced by the 3′-exonuclease activities encoded in a separate domain of their catalytic subunits (115). Proofreading occurs when the abnormal geometry of mismatches slows polymerization, promotes fraying, and allows excision of the incorrect base. From this logic, it follows that altering the relative rates of 5′-to-3′ polymerization and 3′-to-5′ excision will influence proofreading efficiency, which can vary by more than 100-fold, depending on several parameters. One important variable is base composition, perhaps best exemplified by little if any proofreading of 8-oxoG-dA mismatches, a Hoogsteen base pair whose geometry mimics that of correct base pairs. Another example is inefficient proofreading of insertion and deletion (indel) mismatches generated by strand slippage in long repetitive sequences (62). In this case, the unpaired base(s) can be embedded in the duplex-primer template far upstream of the polymerase active site, thereby reducing fraying and favoring extension. Notably, this latter effect is not constant among replicases because even in the same repetitive sequence, proofreading-proficient Pol δ generates single-base deletion mismatches in vitro at higher rates than does proofreading-proficient Pol ε (25). This result further indicates that proofreading is not restricted to excising only primer terminal mismatches but can extend over some distance. For example, a T-dG mismatch located seven base pairs upstream of the active site of yeast Pol δ still elicits excision by its 3′ exonuclease, even when dNTPs are present to allow polymerization (90). Proofreading can also be performed by an exonuclease separate from the polymerase that generated the mismatch. This extrinsic proofreading is the norm in *E. coli*, where replication errors generated by the polymerase subunit of DNA polymerase III are proofread by a 3′ exonuclease in a different subunit. In a similar fashion, biochemical (110) and genetic evidence (105) suggest that the exonuclease activity of Pol δ, but not that of Pol ε, can proofread errors made by Pol α. Theoretically, Pols ε and δ may also proofread errors made by their counterpart. Given that 14 of 17 human DNA polymerases lack intrinsic 3′-exonuclease activity, extrinsic proofreading may occur during other DNA transactions, e.g., during DNA repair or translesion synthesis (99).

Mismatches Are Rarely Generated During Normal Replication In Vivo

When measured at specific loci, the spontaneous mutation rate in eukaryotic genomes is approximately 10^{-10} mutations per base pair per generation (μ_{bp}) (79). Many studies (e.g., see 37 and references therein) show that rates at specific loci are strongly increased by loss

of MMR. More recent genome-wide measurements in yeast provide an even broader view of the rates, types, and locations of mismatches that escape the replication fork in MMR-deficient cells. Three recent studies of yeast that are completely MMR-deficient report genomic mutation rates (μ_g) of 0.36 (123), 0.38 (82), and 1.7 (71) point mutations per genome per generation. Although rates in the absence of MMR could be higher in mammalian genomes containing higher proportions of long repetitive sequences, the yeast studies indicate that only about one of the approximately 600 replication forks in yeast generates a mismatch. This is a tiny (albeit incredibly important) workload compared with three other postreplication events in yeast: (*a*) removal of more than 10,000 ribonucleotides incorporated per replication cycle; (*b*) Okazaki fragment maturation; and (*c*) histone deposition for assembly into nucleosomes. The latter two processes occur approximately 60,000 times per replication cycle. Interestingly, PCNA participates in all these processes and has multiple roles in MMR.

Rates of Generating Mismatches During Replication In Vivo Vary by a Millionfold

Although the average rate at which replication errors are generated and escape proofreading is very low, rates for individual base-base and indel mismatches vary by more than a millionfold (Figure 2*a*). The highest rates in MMR-deficient yeast strains are for single-base indels in long repetitive sequences. These high rates reflect increased strand slippage during replication and diminished proofreading of single-base indel mismatches in long repetitive sequences. The rates per base pair for deleting or adding a single base in a homonucleotide run are much greater than are the rates of adding or deleting repeats of two or more bases from repetitive sequences of equivalent length (e.g., see 82, 126), which may provide the selective pressure to evolve two eukaryotic MutS heterodimers that can repair single-base indel mismatches (see below).

Replication in MMR-defective yeast strains also generates a variety of single base-base mismatches that result in base substitutions. As anticipated by studies in vitro, the rates for these substitutions differ over a wide range. The replicase that made the errors, the nascent strand containing the errors, and the base composition of the mismatches can now be deduced from studies of yeast strains whose replicases have been engineered to preferentially generate nascent leading-and lagging-strand mismatches (see 82 and references therein). These mutator derivatives of Pols α, δ, and ε have single amino acid substitutions in the nascent base-pair binding pocket of the polymerase active site that reduce nucleotide selectivity. The Pol ε and Pol δ variants are also promiscuous for mismatch extension, thereby reducing their proofreading efficiency despite having normal exonuclease active sites. Recent studies of MMR-defective strains containing these mutant replicases, and of strains encoding wild-type replicases, reveal an amazing variety of challenges to MMR that depend on the replicase, the DNA strand, the mismatch composition, and the local sequence context (for global views of these differences, see 82). Among the 12 single base-base mismatches, substitutions resulting from C-dC mismatches are rare, whereas the three mismatches generated at the highest rates are T-dG, G-dT, and C-dT. The first two mismatches, but not the third, have long been thought to be common replication errors. Interestingly, the latter two mismatches result from misincorporation of dTTP, the precursor present at the highest concentration in the dNTP pools in yeast (100).

This specificity is consistent with studies in vitro demonstrating that imbalanced dNTP pools promote misinsertion, and high dNTP concentrations promote mismatch extension at the expense of proofreading. Both mechanisms are apparent in recent studies of yeast strains encoding mutations in ribonucleotide reductase that create dNTP imbalances (e.g., see 64 and references therein). The resulting mismatches are subject to MMR but with variable efficiencies (6). It remains for future studies to quantify the range of specific rates at which mismatches are generated during nuclear DNA replication in mammals, especially at the genome-wide level. Such information will be useful for interpreting the patterns of mutations present in the genomes of tumors from humans with defects in proofreading and MMR (8, 10, 22, 41, 104, 109, 125, 127, 147).

VARIATIONS IN MISMATCH REPAIR EFFICIENCY

Biochemical studies of MutSα-MutLα-dependent MMR (Figure 3) indicate that among the various base-base and indel mismatches examined to date, the efficiency of MMR varies by at least tenfold, which is the approximate dynamic range of the most often used in vitro assays for MMR activity. More sensitive genetic studies that compare point mutation rates in MMR-deficient and MMR-proficient cells indicate that the efficiency with which the MutSα-MutLα-dependent pathway corrects replication errors varies by more than 100,000-fold (Figure 2*b*). This amazing range varies from little apparent MMR of a particular T-dT mismatch generated by a variant of yeast Pol ε (84) to greater than 99.999% repair of a single-base deletion mismatch in a run of 10 consecutive G-C base pairs (38). In addition to the effects of base composition of the mismatch and the local sequence on MMR efficiency, variables such as genomic location, the timing of MMR, and base damage are also likely to be relevant to MMR efficiency. For example, some of the variation in Figure 2*b* could reflect a small fraction of mismatches generated in MMR-proficient cells but outside the context of normal replication in S phase, e.g., during lesion bypass in G2 or during synthesis associated with repairing DNA damage. Additional possibilities for variations in MMR efficiency that may be related to the mechanisms of MMR are discussed below.

RECIPROCITY BETWEEN GENERATING AND CORRECTING REPLICATION ERRORS

Early studies in *E. coli* (e.g., see 121 and references therein) led to the idea that MMR most efficiently corrects the mismatches generated at the highest rates during replication. A growing number of studies now indicate similar reciprocity between replication and MMR in eukaryotic cells. A striking example in yeast involves single-base indel mismatches in long homonucleotide runs (e.g., see 38, 71, 82, 85, 123, 139, 149). Because these mismatches are generated at high rates during replication and are inefficiently proofread (Figure 2*a*), MMR is the major guardian of genome stability against these errors, as indicated by their incredibly efficient correction by MMR (Figure 2*b*). The same logic applies in mammalian cells (65), and it explains why microsatellite instability is diagnostic for MMR-defective tumors (see 57, 109 and references therein). The high rates at which single-base indel mismatches are generated in nuclear genomes loaded with such repeats may explain the evolution of two MutS heterodimers, MutSα and MutSβ, that can both

correct single-base indel mismatches. Another striking example of reciprocity involves 8-oxo-dG-A. Pols α, δ, and ε preferentially insert adenine opposite 8-oxo-G, and the resulting 8-oxo-dG-dA is not efficiently proofread. However, this mismatch is corrected by MMR (18, 36, 96), with an efficiency so high in one study as to lead to the suggestion that recognizing mismatches opposite damaged bases may be more important than correcting undamaged mismatches (18). MMR also corrects mismatches resulting from misincorporation of damaged dNTPs in mammalian cells (see 118 and references therein), a fact that has implications for chemotherapy (4, 26).

On the other end of the reciprocity gradient, mismatches generated at lower rates, e.g., C-dC mismatches or T-dT mismatches generated by Pol ε during leading-strand replication, are corrected less efficiently (Figure 2*b*). Within this wide range, the average rate at which mismatches are generated correlates with an average MMR efficiency of 99% (Figure 2*b*) for correcting mismatches in both nascent strands. Notably, a recent genome-wide study (82) indicates that lagging-strand replication is approximately twofold less accurate than leading-strand replication and that MMR of lagging-strand mismatches is twofold more efficient than MMR of leading-strand mismatches. This genome-wide reciprocity is consistent with an earlier study suggesting more efficient MMR of a lagging-strand 8-oxo-G-dA mismatch in the yeast *URA3* gene (106) and with another study (61) indicating preferential action of MutSα on the lagging strand. Reciprocity is also observed for mismatches of different composition generated by the same replicase; e.g., compare G-dT and T-dT mismatches generated by Pol ε (Figure 2*b*) and see other examples in Reference 82. The reciprocal relationship between generating and correcting replication errors implies that all three major replication fidelity processes have coevolved to accurately replicate both DNA strands. Given that defects in MMR (41, 109, 127) and proofreading (8, 10, 22, 41, 104, 109, 125, 127, 147) are associated with increased cancer risk, it will be interesting to determine whether reciprocity exists between MMR and proofreading, the latter of which is not yet well quantified in vivo.

MECHANISMS OF MISMATCH REPAIR IN RELATION TO REPLICATION

In the context of the challenges posed by the replication fork, we now briefly describe the major MMR pathway. We then consider several processes that could be relevant to the wide variations in MMR efficiency observed in vivo.

The Major MutSα-MutLα-Dependent Mismatch Repair Pathway

Most of our knowledge of the mechanisms of eukaryotic MMR involves the MutSα-MutLα pathway, whose role is to repair the vast majority of replication errors. This pathway (Figure 3) is initiated when a MutSα heterodimer comprising Msh2 and Msh6 binds to a mismatch. MutSα is primarily responsible for repairing the most common replication errors, which are single base-base and indel mismatches. MutSα contains two ATPase active sites that are essential for MMR (33, 43). ATP and mismatch binding induce a conformational change in MutSα, such that it forms a clamp that can move along the DNA (74). This ATP-activated state of MutSα allows its interaction (76) with MutLα, a heterodimer comprising Mlh1 and Pms2 (or Mlh1 and Pms1 in yeast). Subsequently, the PCNA sliding clamp, which is loaded

onto DNA by replication factor C (RFC) and is a component of the replication apparatus, activates MutLα to incise the nascent strand in an ATP-dependent manner (54, 56). These nicks can then be used for removing the replication error (see below), after which repair is completed by correct DNA synthesis by DNA polymerase δ (81), or possibly by Pol ε (138), followed by ligation.

Additional MutS and MutL Heterodimers

In addition to MutSα and MutLα, other MutS and MutL heterodimers participate in MMR processes (recently reviewed in 50; also see 7 and references therein). For example, the MutSβ heterodimer comprising Msh2 and Msh3 participates in repairing large as well as one- and two-base indel mismatches (37, 38, 60, 61, 126, 130), with a bias toward repairing single-base deletion as compared with single-base addition mismatches (117). MutSβ can also participate in repairing a subset of base-base mismatches (30, 39). In addition to MutLα, two other MutL heterodimers, Mlh1–Mlh2 and Mlh1–Mlh3, also contribute to the repair of indel mismatches (50). However, the mutator phenotypes conferred by defects in *MSH3*, *MLH2*, and *MLH3* are much smaller than those conferred by defects in *MSH2*, *MSH6*, *MLH1*, or *PMS2* (*yPMS1*). It is partly for this reason that relatively less is known about exactly when, where, and how the subsets of mismatches repaired by the more specialized MMR heterodimers are generated in vivo. Nonetheless, repair of indel mismatches has been reconstituted in vitro with MutSβ (151), and structural, biochemical, and genetic studies indicate that the mechanisms of mismatch recognition and signaling for strand-specific repair may differ for MutSα and MutSβ (16, 34, 103, 132). Although MutSα and MutSβ binding to DNA both induce significant DNA bending, the extents of bending and the protein-DNA interactions that promote bending are different for MutSα and MutSβ (34, 145). In addition, the ATP-binding and hydrolysis properties of MutSβ differ from those of MutSα, with the steady-state ATPase activity of MutSα increasing upon mismatch binding and that of MutSβ decreasing upon indel mismatch binding (2, 88, 103, 135). Finally, whereas MutSα can simultaneously interact with PCNA and MutLα, MutLα and PCNA compete for the same binding site on MutSβ, and PCNA can inhibit MutSβ-MutLα ternary complex formation with an indel mismatch (49). The differential interactions of PCNA with MutSα and MutSβ might regulate processing of small indel mismatches (49). Together, these data imply that the mechanisms by which MutSα and MutSβ signal repair may not be equivalent.

Timing Between Replication and Mismatch Repair

As mentioned above, the signal that directs MMR to the nascent strand in *E. coli* quickly disappears after replication. That timing is also important for eukaryotic MMR is indicated by a recent study (46) in which the availability of MutSα for MMR was restricted by fusing *MSH6* to cyclins expressed in either the S phase or the G2/M phase of the cell cycle. The *MSH6*-S phase cyclin fusion suppressed mutations at three loci that replicate in mid-S phase, whereas the *MSH6*-G2/M phase cyclin fusion did not; however, it did suppress mutations in a region of the genome that replicates very late. These results led to the suggestion that replication and MMR are temporally coupled in a manner that may be related to the regulation or appearance of the signals used for MMR. Stochastic or genetically determined variations in coupling between replication and MMR could render

some replication errors unavailable to the MMR machinery. For example, MMR might not be available to correct mismatches generated during translesion DNA synthesis or during DNA synthesis associated with certain types DNA repair or recombination. These possibilities and others might be relevant to (*a*) some of the variation in MMR efficiency depicted in Figure 2*b*, (*b*) evidence that MMR may be less efficient late in S phase (40, 82), and (*c*) evidence that MMR of leading-strand replication errors is slightly less efficient at interorigin midpoints than at replication origins where the replication machinery is assembled (82). Another important parameter under current investigation is coordination between MMR and histone deposition and the assembly and modifications of nucleosomes behind the replication fork. These are the subjects of numerous studies recently reviewed by others (44, 53, 74, 75).

Expression of Mismatch Repair Proteins

One variable that could influence the timing and efficiency of MMR is the availability of MMR proteins. There are only a few studies examining the expression of the MMR repair proteins during the cell cycle (19). These studies suggest that the MMR proteins are expressed in G1, with expression being increased in S and G2. An early study reported that the expression of Msh6 is approximately tenfold higher than Msh2, whereas a recent study of mice found that the expression of Msh3 is higher than expression of Msh6 in most tissues, with similar levels of Msh2 and Msh6 in testis (137). The number of MMR proteins has been measured in yeast using quantitative western blots of TAP (tandem affinity purification) tagged and untagged MMR proteins (28, 63). The number of proteins found in *Saccharomyces cerevisiae* is ~1,300 for Msh2, 1,600–5,000 for Msh6, ~740 for Msh3, ~320 for Mlh1, and ~520 for Pms1. If at any one moment in S phase, 200 origins give rise to 400 operational replication forks, these numbers suggest that (*a*) at least one MutS and MutL heterodimer could be available at each fork and (*b*) the concentration of MutLα in the cell may limit the extent of MMR under conditions that promote a high mutation load. Consistent with the latter possibility, studies in *E. coli* found that saturation of MMR could be overcome by overexpression of MutL (122). Moreover, if the MMR proteins are not localized at the fork where the error occurs, then they might not arrive at the error in time to correct it. Putative problems related to concentration and localization may be offset if replication forks are grouped into replication factories containing ~14 replication forks (91).

Mismatch Binding and Conformational Changes

We lack a complete understanding of how mismatch recognition by MutSα results in the ATP-dependent recruitment of MutLα. In crystal structures (reviewed in 33), bacterial MutS and human MutSα induce a well-defined kink in the DNA at the mismatch. Although DNA bending has been suggested to serve important roles in mismatch identification and specificity (69, 102, 144, 145), DNA kinking and the majority of contacts are remarkably similar in all MutS(α) structures, independent of the DNA substrate or the presence of nucleotide cofactors (70, 95, 102, 145). This similarity leaves open the question of why different mismatches, or even the same mismatch in a different sequence context, are repaired with different efficiencies. One possibility is that the stability of interaction of MutSα with mismatches varies by mismatch and/or with sequence context, as supported by the fact that binding affinities of MutS homologs depend on the type of mismatch and the

sequence context (32, 89, 129). Nonetheless, although early studies in *E. coli* revealed a general trend between the efficiency of repair and the binding affinity of MutS for a mismatch, the trend is not absolute, and binding alone is not sufficient to induce repair (131). A difficulty in correlating the crystal structures and binding affinities with repair efficiencies is that most studies are done in the absence of ATP. Recent studies examining the binding of MutSα, MutSβ, and *E. coli* MutS to end-blocked and unblocked DNAs, with different mismatches in different sequence contexts, found that the relative affinities of MutS for the different mismatches are different in the presence and absence of ATP (32, 129). It is not surprising that the binding affinity does not correlate with repair efficiency because MutS homologs undergo at least one mismatch-and ATP-dependent conformational change to interact with MutL homologs to initiate repair.

It has been known for more than two decades that after mismatch recognition, MutSα undergoes an ATP-dependent conformational change (or changes) (43) to a mobile-clamp state that can move along the DNA (74). It is also known that the ATPase activity of MutSα is required for its interaction with MutLα that initiates repair (76). Nonetheless, the point(s) at which MutLα interacts with MutSα and the functions of the mobile clamp remain uncertain. The observation that MutLα can interact with an ATPase-site mutant of MutSα that does not form a mobile clamp (42) suggests that formation of the MutSα mobile clamp is not required for interaction with MutLα, and that MutSα may undergo multiple conformational changes before becoming a mobile clamp. Studying conformational changes during dynamic assembly processes, such as the mismatch-dependent assembly of MutSα and MutLα on DNA, remains challenging, but single molecule techniques are providing opportunities to examine such complicated processes (reviewed in 24, 73). Single molecule fluorescence studies (114) of *Taq* MutS indicate that it is conformationally dynamic when scanning homoduplex DNA but that its conformation is restricted upon mismatch binding. The transition to the mobile clamp occurs via two sequential conformational changes that persist for seconds, providing ample opportunity for interaction with MutL. Not all complexes that recognize a mismatch are competent to form a mobile clamp (114), and their fate as they proceed from mismatch recognition to forming a clamp depends on MutS-DNA complex conformations and the ligation states of their ATPase sites (114, 134). Compared with bacterial MutS, the recognition mechanism for eukaryotic MutSα is less certain, but preliminary studies of MutSα-DNA complexes suggest that they also can adopt multiple conformations (14). In addition, studies of the ATP-induced dissociation kinetics of MutS-DNA complexes reveal multiple populations of complexes, some that dissociate rapidly and others that dissociate slowly upon the addition of ATP (5, 43).

Mechanisms and Signals for Strand Discrimination

For many years, the strand-discrimination signal in eukaryotes remained a mystery. When human proteins are used to repair a mismatch in a nicked plasmid DNA, repair is preferentially directed to the nicked strand. In both the reconstituted system and in extracts, if the nick is 5′ to the mismatch, MMR does not require MutLα. However, if the nick is 3′ to the mismatch, MutLα is required. Surprisingly, MMR in vitro does not require a 3′ exonuclease even when the initial nick is 3′ to the mismatch. The mystery was clarified by the discovery (53, 54, 56) that MutLα contains a latent endonuclease activity that is

activated by PCNA to nick the DNA in a strand-specific manner, preferentially incising the strand containing the initial nick. These studies strongly suggested that the interaction of MutLα with PCNA provided the strand-discrimination signal for MMR, because RFC asymmetrically loads PCNA onto DNA at a nick. This idea was reinforced by studies in which a single-stranded bubble was placed into a covalently closed plasmid DNA, which allows RFC to load PCNA onto DNA but without strand-specific orientation. Repair of a mismatch in these bubble substrates is no longer strand specific (111, 112). Taken together, these studies imply that PCNA, which is loaded asymmetrically at replication forks, interacts with MutLα in an orientation such that its intrinsic endonuclease activity preferentially nicks the nascent strand to allow removal of the replication error. In principle, all that is needed to direct repair to the daughter strand is a nick in the daughter strand. Importantly, mutations that impair nicking by MutLα in vitro strongly elevate the mutation rate in vivo (15, 23, 56), indicating that nicks generated by MutLα are the major source of DNA ends used for mismatch removal (discussed further below).

The mechanism by which MutSα and MutLα interact following mismatch recognition by MutSα and subsequent activation of MutLα endonuclease by PCNA is now beginning to emerge. MMR studies in vitro indicate that MutLα strand specifically nicks the DNA throughout the plasmid but that it preferentially nicks in the vicinity of the mismatch and on both the 3′ and 5′ side of the mismatch (47, 54, 111, 112). Although nicking activity in the absence of MMR is minimal under physiological conditions, MutLα can nick homoduplex DNA under nonphysiological conditions, which allowed the examination of the effect of PCNA (and RFC) on the nicking activity of MutLα in the absence of a mismatch or MutSα. On nicked homoduplex plasmid DNA, MutLα nicks both DNA strands equally in the absence of RFC and PCNA; however, addition of RFC and PCNA greatly enhances MutLα nicking activity on the initially nicked strand but has no effect on the covalently closed strand (56). These results indicate that a mismatch and MutSα are not required for PCNA activation of the MutLα nicking activity, and they suggest that the role of the MutSα-MutLα interaction in this early stage of repair may be to localize MutLα near the mismatch, so that PCNA activates MutLα to nick DNA in proximity to the mismatch.

Several disparate models have been proposed for MutSα-MutLα-mismatch complex formation and the subsequent signaling for repair. One model posits that MutLα joins MutSα to form MutSα-MutLα sliding clamps that diffuse along the DNA to interact with the strand-discrimination signal (74). Other models include trapping of MutSα clamps near the mismatch by MutLα, and MutSα-induced polymerization of MutLα along the DNA (45, 48, 93). Importantly, these models are not necessarily mutually exclusive. Every model needs to take into account the observation that PCNA can activate MutLα in a MutSα-MutLα-mismatch complex to nick the DNA in a strand-specific fashion, either proximal or distal to the mismatch and in its vicinity or hundreds of base pairs away. Although the sliding clamp model provides an explanation for the nicking seen across the plasmid, it is less clear how a diffusive MutSα-MutLα sliding camp would result in preferential nicking near the mismatch. The early idea that MutS may induce polymerization of MutL in an ATP- and mismatch-dependent fashion (93) has recently been reemphasized by in vivo fluorescence studies in yeast and *E. coli*, which suggest that MMR foci contain more MutL than MutS proteins (21, 45).

The properties of MutLα offer insights into the potential nature of mismatch-MutSα-MutLα complexes. MutLα dimerizes via the C-terminal domains of Mlh1 and Pms2 (Figure 4*a*), and the endonuclease active site resides in the C-terminal domain of Pms2. The N-terminal domains of both Mlh1 and Pms2 contain ATPase and DNA binding activities (33). These domains are linked to the C-terminal dimerization domains via long flexible linker arms (Figure 4*a*). Adenine nucleotides induce large asymmetric conformational changes (120) that include increases in secondary structure in the linker arms and that bring the N-terminal DNA binding domains in proximity to C-terminal domains (Figure 4*a*). Although MutLα has very weak DNA binding activity in physiological salt, studies at low salt revealed that MutLα can bind cooperatively to form long, continuous tracts of protein along duplex DNA and that it can interact simultaneously with two different strands of duplex DNA (35). Perhaps the interaction of MutLα with MutSα can activate the latent DNA binding properties of MutLα to promote the assembly of MutLα on the DNA under physiological conditions.

Models for PCNA-Activated MutLα Nicking in Mismatch Repair

Taken together, the above data allow construction of models to explain the observed nicking properties of MutLα in a reconstituted repair system (Figure 4*b*). Because nicking occurs near the mismatch, it seems likely that MutLα may interact with MutSα after it has undergone a mismatch- and ATP-dependent conformational change but before it transitions to a sliding clamp, and that this interaction traps MutSα (and MutLα) at the mismatch. This interaction may lead to additional MutLα proteins polymerizing along the DNA on one or both sides of the mismatch. In this linear polymerization model, for PCNA to activate MutLα to nick the DNA on the distal side of the nick relative to the replication fork (or site of RFC-directed PCNA loading), PCNA would need to be left behind on the DNA such that it is on the distal side of the mismatch when MutSα and MutLα assemble on the DNA. An extension of the polymerization model that could allow PCNA to induce nicking on both the proximal and distal side of the mismatch (Figure 4*b*) takes into account the observations that MutSα bends the DNA (145) and that MutLα can interact with two strands of duplex DNA simultaneously (35). In this model, one or two MutSα proteins induce bending at the mismatch. Interaction of MutSα with MutLα promotes MutLα to form short polymer tracts bringing the two DNA strands together. ATP induces a conformational change that brings the DNA bound to the N-terminal domain of Mlh1 and Pms2 into the endonuclease active site in the C-terminal domain of Pms2. Whether the DNA gets nicked on the proximal or distal side of the mismatch is determined by the orientation of MutLα binding to the two DNA strands (Figure 4*b*). This model is attractive because it provides an explanation of how PCNA could activate MutLα to nick the nascent strand both proximal and distal to the mismatch.

Three Models for Mismatch Removal

Biochemical and genetic studies suggest three mechanisms for mismatch removal (Figure 3). One mechanism is excision in the 5′-to-3′ direction by exonuclease 1, a reaction that has been extensively studied during MMR in vitro (recently reviewed in 53). A second mechanism also uses a 5′ DNA end and involves mismatch removal associated with strand-displacement synthesis by Pol δ or Pol ε (55). A third possibility is 3′-to-5′ excision of the

mismatch by the exonuclease activities of Pol δ or Pol ε. Although the latter pathway has yet to be supported by studies of MMR in vitro, the proofreading exonucleases of yeast Pols δ and ε can excise a mismatch embedded seven base pairs upstream of the primer terminus, even when dNTPs are present to allow polymerization (90). The 3′-exonuclease activity of Mre11 has also been implicated in MMR (142).

These removal mechanisms are supported by mutator phenotypes conferred by defects in yeast and mammalian exonuclease 1 (133, 136, 140, 146) and in yeast Rad27/Rth1 (a.k.a. human FEN1) (52). The mutator effects in these mutant cells are strong but lower than for cells lacking Msh2, consistent with only partial loss of MMR due to a defect in any one protein. Importantly, however, when a deletion of yeast exonuclease 1 is combined with a *pol32* deletion that impairs Pol δ strand-displacement activity (1) or combined with mutations that inactivate the 3′-exonuclease activity of Pol δ or Pol ε (138), mutation rates are synergistically increased to levels that indicate nearly complete loss of repair. These synergistic increases strongly suggest functional redundancy for mismatch removal, as is the case for MMR in *E. coli* (94). Additionally, mutations in MutSα that disrupt its interaction with PCNA coupled with deletion of *EXO1* also show a strong mutator phenotype (31), suggesting that PCNA plays an important role in mismatch removal in the absence of Exo1. Perhaps the interaction between PCNA and MutSα helps direct strand-displacement synthesis or the 3′-exonuclease activity of Pol δ or Pol ε toward the mismatch (Figure 3). Experiments examining Exo1-independent MMR in vitro did not detect any excision of the mismatch in the absence of dNTPs. However, addition of dNTPs led to error removal via strand-displacement synthesis without the production of single-stranded gaps (55). This strand-displacement synthesis requires the nicking activity of MutLα when the nick is 3′ to the mismatch, and MutLα greatly enhances repair activity even when the nick is 5′ to mismatch. The latter result suggests that strand-displacement synthesis is facilitated because MutLα nicking results in shorter DNA segments to be displaced and/or because having multiple nicks near the mismatch promotes loading of polymerase accessory proteins such as PCNA (55). Taken together, the biochemical data suggest that Exo1-mediated excision and strand-displacement synthesis are two major pathways for mismatch removal.

Mismatch Removal in Relation to Replication

Finally, the origins and identity of the DNA ends used for mismatch removal can be considered in light of the architecture of leading- and lagging-strand replication. A key observation here is that mutations that inactivate the endonuclease activity of MutLα elevate mutation rates in cells to levels that are consistent with complete, or nearly complete, loss of MMR (15, 23, 56, 141). This fact implies that the vast majority of mismatches (designated with a large M_1 in Figure 1) may be removed using 5′ and 3′ DNA ends generated by MutLα incision. These ends are equally available near a mismatch made during continuous leading-strand replication by Pol ε and during discontinuous lagging-strand replication by Pol δ, as well as mismatches made by Pol α, perhaps especially those most distant from the 5′ ends of Okazaki fragments. This MutLα-dependent MMR requires that PCNA be available on both daughter duplexes to activate MutLα's endonuclease activity. PCNA is regularly present during lagging-strand replication, where it promotes processive replication by Pol δ and participates in Okazaki fragment maturation. PCNA also stimulates synthesis

by Pol ε, but Pol ε's interaction with PCNA is weak compared to its interaction with the CMG helicase complex (27). These facts led to the proposal (27) that at the fork, Pol ε cycles on and off DNA-bound PCNA but holds onto CMG for stable leading-strand synthesis. This on-off action would periodically provide RFC access to the primer template for assembly of new PCNA clamps on the leading strand. These clamps would then be available to activate MutLα for incision of the continuously replicated nascent leading strand.

This mechanism could also fulfill the periodic need for PCNA on the leading strand to facilitate two much more frequent postreplication transactions, histone deposition and/or nucleosome assembly and repair of ribonucleotides (see 27, 68). In yeast, most ribonucleotides incorporated during replication are removed by RER (98, 128). RER is initiated when RNase H2 nicks the nascent DNA strand at the ribonucleotide. This repair reaction involves PCNA, which interacts with a noncatalytic subunit of RNase H2 (9). Two recent biochemical and genetic studies (29, 83) support the hypothesis (100) that these nicks, like those generated by MutLα, may function as strand-discrimination signals for MMR. Genetic evidence in yeast strains harboring wild-type (29) or variant replicases (83) suggests that this mechanism preferentially operates on replication errors present in the continuously replicated leading strand more than on replication errors present in the discontinuously replicated lagging strand, which already has DNA ends available every several hundred base pairs. Importantly, RNase H2 mutants that are defective in nicking confer mutator phenotypes characteristic of defective MMR that are much milder than observed upon complete loss of MMR in an *msh2*Δ mutant. This result implies that the contribution of nicking by RNase H2 to MMR is small compared to nicking by MutLα. It seems possible that nicks generated by RNase H2 may be particularly important for MMR of a small percentage of mismatches (designated with a small M_3 in Figure 1) that are not rapidly repaired via MutLα but are repaired later, after replication-coupled signal(s) no longer exist.

The nascent lagging strand is generated discontinuously as a series of short Okazaki fragments. Until these fragments are processed into a mature lagging strand, a 5′ and a 3′ DNA end should be available for MMR within several hundred base pairs of a mismatch. In fact, the role of Pol α in initiating Okazaki fragments predicts that the mismatches generated by Pol α will always be closer to the 5′ end of an Okazaki fragment than mismatches generated by Pol δ. Pol α lacks intrinsic proofreading activity, thereby potentially placing greater demands on MMR to correct Pol α errors at replication origins and at the 5′ DNA ends of Okazaki fragments. Two lines of evidence in yeast support the idea that the 5′ DNA ends of Okazaki fragments may serve as signals for strand discrimination and mismatch removal. First, studies involving MMR of an 8-oxo-G-A mismatch in one sequence context (106) or undamaged mismatches occurring throughout the genome (82) have reported that MMR efficiency is higher for lagging- than for leading-strand errors. Second, studies using yeast replicase variants indicate that (*a*) the efficiency of MMR is higher for errors made by Pol α than those made by Pol δ (97), (*b*) Exo1-dependent MMR is more important for correcting errors generated by Pol δ than for errors made by Pol ε (45), and (*c*) Exo1-dependent MMR is more efficient at correcting errors generated by Pol α than errors made by Pol δ (78). Together, these studies strongly support the idea that the 5′ DNA ends of Okazaki fragments are signals for strand discrimination and for removing some fraction

(designated M_2 in Figure 1) of mismatches generated during Okazaki fragment synthesis and at replication origins. The evidence for Exo1 involvement does not exclude the possible involvement of other 5′ nucleases, possibly including Fen1 (yRad27) (52, 97) and Dna2, the latter possibly during processing of long flaps (3).

CONCLUDING REMARKS

During the past decade, important insights into the production of errors during leading- and lagging-strand replication of the eukaryotic nuclear genome, and how these errors are corrected by MMR, have been uncovered. Especially notable is the identification of the nuclease activity of MutLα, which is used for strand discrimination. Such information is critical for understanding how nuclear genome stability is normally maintained and also highlights what we still need to investigate and understand about replication fidelity and how it is enhanced, or not, by DNA mismatch repair.

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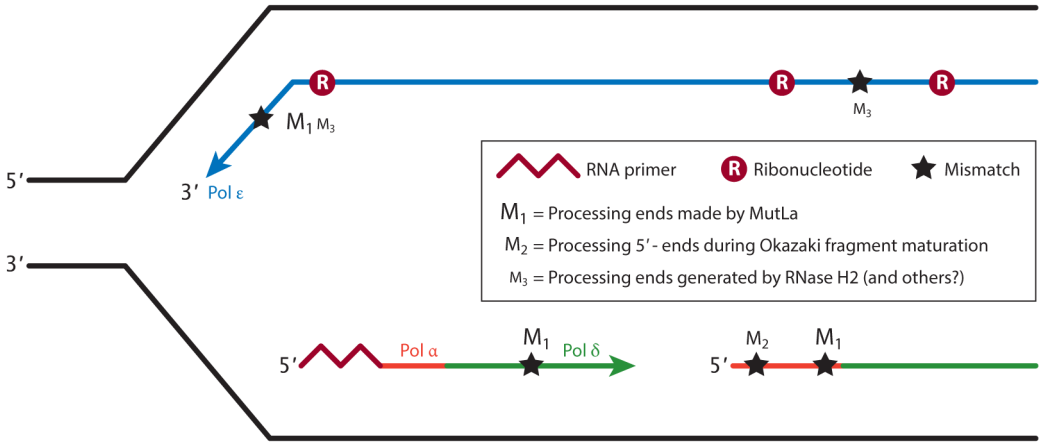

Figure 1.

The eukaryotic replication fork and opportunities for mismatch repair (MMR). The majority of replication errors are corrected by MMR that depends on DNA ends made by MutLα. Some Pol α errors are likely to be repaired using the 5′ ends of Okazaki fragments (M2), while other mismatches made during replication may be repaired using DNA ends generated by RNase H2 cleavage of ribonucleotides incorporated into DNA (M3). The different sizes of the Ms indicate their relative importance to MMR efficiency, with M1 being the most important and M3 being the least important.

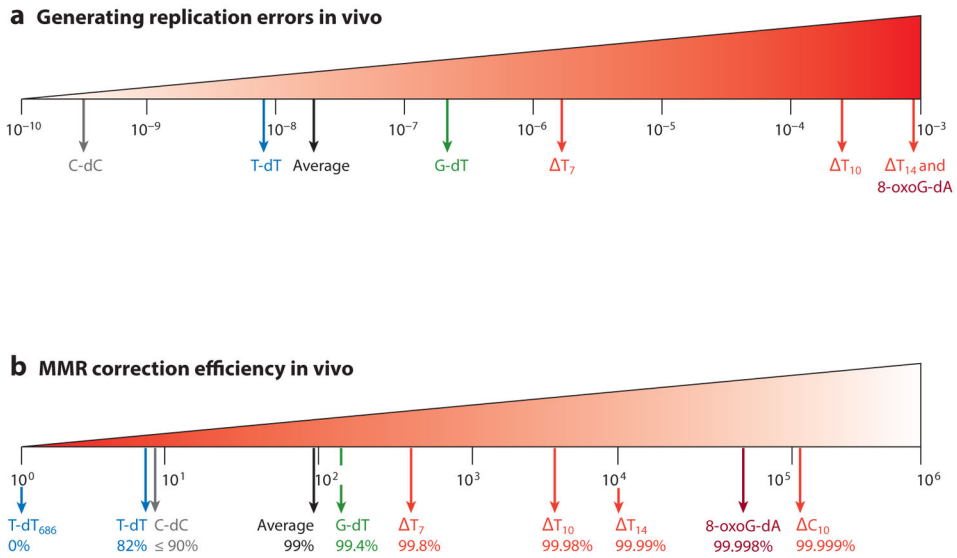

Figure 2.

Reciprocity in generating and correcting replication errors in vivo. (*a*) The rates per base pair per generation shown are from studies of budding yeast. The average rate and the rates for C-dC, T-dT, and G-dT mismatches are from a genome-wide analysis (82). The rates for deleting a T-A base pair from homonucleotide runs of length 7, 10, and 14 are from a specific locus assay (139). The rate of forming 8-oxo-G-dA mismatches in vivo has not been determined but is placed at the high end of the spectrum on the basis of evidence in vitro that Pols α, δ, and ε prefer to incorporate adenine rather than cytosine opposite 8-oxo-G (36, 119, 124). (*b*) Mismatch repair (MMR) correction efficiencies per base pair per generation (*from left to right*) are for a T-dT_{686} error made by a Pol ε variant at one base pair (84), the average of all T-dT mismatches made by this Pol ε variant (82), C-dC (82) (note that this is a ≥ value), the average of all T-dT mismatches regardless of exact location (82), $\Delta T_{7,10,14}$ (139), the average for G-dT mismatches made by a Pol δ variant (82), 8-xo-G-dA (18), and ΔC_{10} (38).

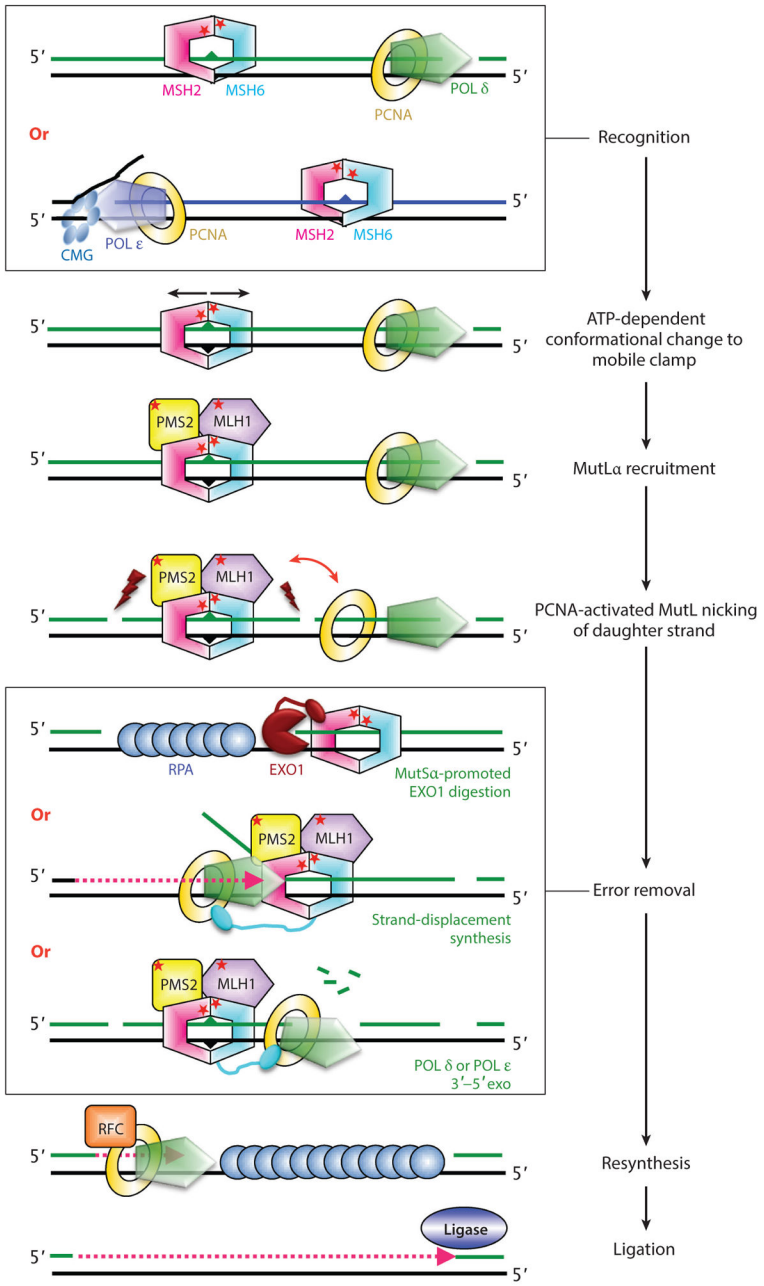

Figure 3.

Eukaryotic DNA mismatch repair (MMR). The major MMR pathway initiates when MutSα (Msh2–Msh6) binds to a mismatch. This is followed by binding of MutLα[Mlh1 and Pms2 (or yeast Pms1)]. PCNA (proliferating cell nuclear antigen) activates MutLα to incise the nascent strand and the DNA ends are used for removing the replication error. After this, repair is completed by correct DNA synthesis and ligation. Abbreviation: RFC, replication factor C.

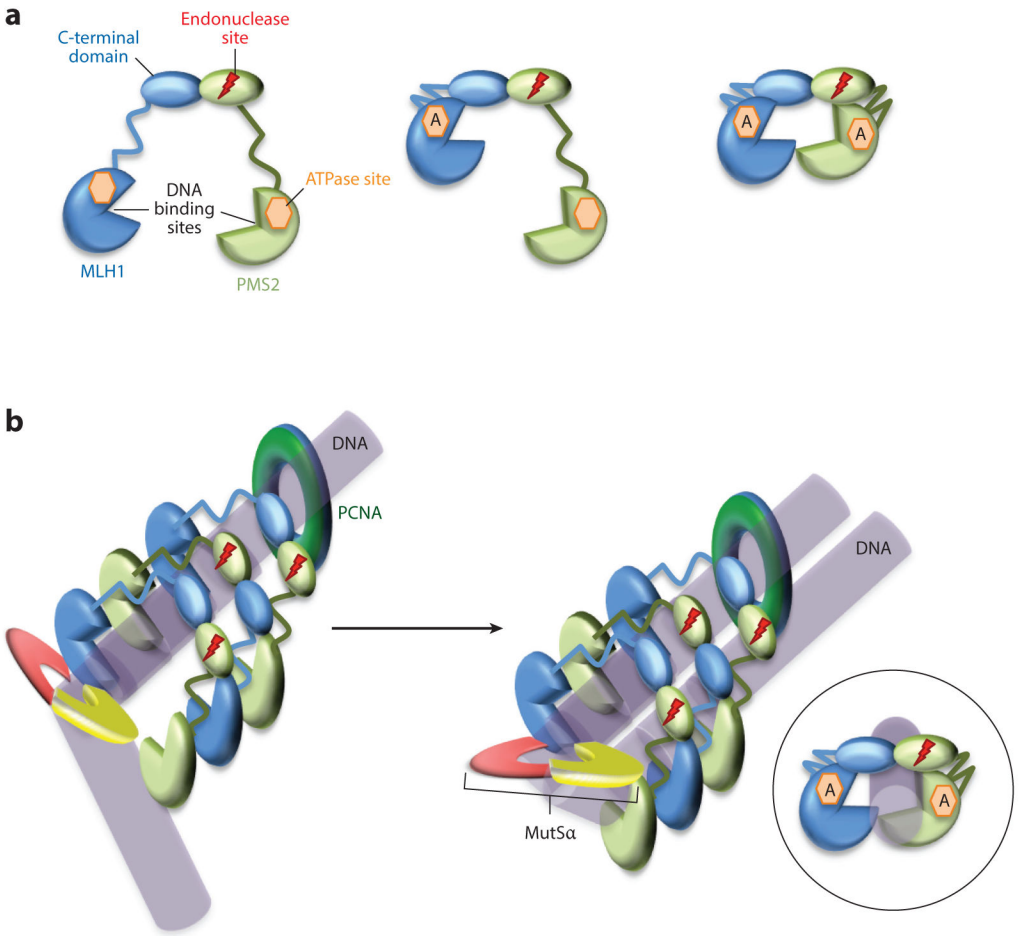

Figure 4.

MutLα conformations and models for PCNA (proliferating cell nuclear antigen) activation of MutL endonuclease activity. (*a*) MutLα is a heterodimer of MLH1 and PMS2. They dimerize by their C-terminal domains. The C-terminal domain of PMS2 contains the endonuclease active site (*lightning bolt*). Flexible linker arms connect these domains to the N-terminal domains, which each contain an ATPase active site (*hexagon*) and a DNA binding site (*represented by the wedge*). Binding of ATP (or ADP) induces conformational changes in the linker arm such that the N- and C-terminal domains move near to one another. Left: no nucleotide. Middle: nucleotide bound to MLH1. Right: nucleotide bound to both subunits. (*b*) Model of MutS-MutL complexes at a mismatch. Left: simple polymerization model (93). Right: model that takes into account the DNA binding properties of MutLα. Inset shows conformational change bringing DNA into the endonuclease site.