CAF-I-dependent control of degradation of the discontinuous strands during mismatch repair

Lyudmila Y. Kadyrova, Elena Rodriges Blanko, and Farid A. Kadyrov1

Department of Biochemistry and Molecular Biology, Southern Illinois University School of Medicine, Carbondale, IL 62901

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DNA mismatch repair (MMR) is a multifunctional process that promotes genetic stability and suppresses carcinogenesis. Correction of DNA replication errors is its major function. Despite the importance of MMR, its functioning in eukaryotes is not well understood. Here we report that human mismatch correction reactions in cell-free extracts occur during concomitant nick-dependent nucleosome assembly shaped by the replication histone chaperone CAF-I. Concomitant nucleosome assembly protects the discontinuous mismatch-containing strands from excessive degradation by MMR machinery. Such protection is also demonstrated in a defined purified system that supports both mismatch correction and CAF-I-dependent histone H3–H4 deposition reactions. In addition, we find that the mismatch recognition factor MutSα suppresses CAF-I-dependent histone H3–H4 deposition in a mismatch-dependent manner. We suggest that there is active crosstalk between MMR and replication-dependent nucleosome assembly during the correction of DNA replication errors and, as a result, the nascent mismatch-containing strands are degraded in a controlled manner.

DNA mismatch repair (MMR) is a strongly conserved process that guards genetic stability and suppresses carcinogenesis by correcting replication errors, removing mismatches during homologous recombination, suppressing homologous recombination, and initiating cell signaling and apoptotic responses upon detection of DNA damage of several classes (reviewed in refs. 1–3). It removes both single base–base mismatches and small insertions/deletions.

MMR is best understood in Escherichia coli, where the sequence of biochemical events that lead to the correction of DNA replication errors has been identified (3). Significant progress has also been made in understanding the correction of DNA replication errors in eukaryotes. Naturally occurring strand breaks (nicks and gaps) in the daughter strands suffice to direct human MMR to remove a DNA replication error (4, 5). The current understanding of eukaryotic MMR suggests that it consists of mismatch recognition, incision, excision, and DNA synthesis steps (6). Eukaryotic correction of a DNA replication error is initiated by recognition of the mismatch by the primary mismatch recognition factor MutSα. In the next step, MutSα, the PCNA clamp, and the RFC clamp loader activate MutLα endonuclease to incise the daughter strand in the vicinity of the error in an ATP-dependent reaction (7). Function of RFC in the activation of MutLα endonuclease is limited by loading PCNA on the nicked DNA, whereas the loaded PCNA directs MutLα endonuclease to incise the discontinuous strand (8). An incision introduced by MutLα 5’ to the error is used by MutSα-activated Exo1 as the entry site for a 5’–to-3’-directed excision that removes the error along with a stretch of surrounding DNA in a reaction modulated by the RPA ssDNA-binding protein (7, 9). The resulting gap is then filled by DNA polymerase δ holoenzyme (10, 11). In the absence of Exo1, eukaryotic correction of DNA replication errors can proceed without the excision step by relying on the strand-displacement activity of DNA polymerase δ, which removes a mismatch in a reaction initiated from a 5’ MutLα incision (12). Although the described view of eukaryotic correction of replication errors is supported by both biochemical and genetic data, more research is needed to advance this view and to investigate whether there are complementary and alternative mechanisms of mismatch correction.

Eukaryotic MMR operates in the nucleosomal environment, but the impact of the nucleosomal organization on eukaryotic MMR is not understood. Nuclear DNA is packaged into nucleosomes by various histone chaperones during diverse DNA transactions such as replication and transcription, but nucleosome assembly follows the same path: (H3–H4)2 tetramer is initially loaded onto DNA and two H2A–H2B dimers are then added to the (H3–H4)2 tetramer (13). The correction of DNA replication errors occurs on newly replicated DNA that is packaged into nucleosomes by the histone chaperone CAF-I (14–16). In this work, we present evidence that CAF-I-dependent nucleosome assembly controls the mismatch-provoked degradation of the discontinuous strands during the correction of DNA replication errors.

Results

CAF-I-Dependent Control of Degradation of the Discontinuous Strands During Mismatch Repair in Cell-Free Extracts. A biochemical analysis revealed that mismatch correction reactions with 90–180-μg HeLa cytosolic extract or 90–μg HeLa nuclear extract are accompanied by mismatch-provoked degradation of the discontinuous strands, but that this degradation is suppressed in reactions with 135–180-μg HeLa nuclear extract (Fig. 1 A–C). For example, the reactions with 135-μg HeLa cytosolic extract correct 20% of available mispaired G bases (Fig. 1 C) and provoked 16% degradation of the indirectly labeled discontinuous strands (bracketed area of Fig. 1 A, and Fig. 1B), whereas the 135-μg HeLa nuclear extract reactions repair 43% of mispairs and degrade only 5% of the discontinuous strands. Comparing these results suggests that mismatch correction in HeLa cytosolic extracts is accompanied by excessive degradation of the discontinuous mismatch-containing strands, and such degradation is suppressed in reactions with larger amounts of HeLa nuclear extract.

The excessive mismatch-dependent degradation of discontinuous strands in the mismatch-correction reactions in HeLa cytosolic extract (Fig. 1 A and B) is similar to that observed in the reconstituted four-protein incision system consisting of MutLα endonuclease, MutSα, the PCNA clamp, and the RFC clamp loader (7). This commonality suggested to us that both the cytosolic extract and the reconstituted system might lack a nuclear factor that protects the discontinuous mismatch-containing strands from uncontrolled degradation. We considered that tight binding of such a factor to DNA in the vicinity of a mismatch might prevent an incision complex containing MutLα from sliding away and from subsequent incision of the discontinuous strand at remote...
Mismatch correction and nucleosome assembly in HeLa cell-free extracts. Mismatch-correction reactions with 3′ G·T or control 3′ A·T DNA and the indicated amounts of HeLa nuclear or cytosolic extract were performed and analyzed as described in Materials and Methods. (A) Recovered DNA products of the indicated mismatch correction reactions were cleaved with Clal, separated under denaturing conditions in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled probe complementary to the discontinuous strand position 2505–2526. The diagram on the left of the image shows the relative positions of the 3′ strand break, the G·T mispair (sharp bump), a 32P-labeled probe (dashed asterisk), and the unique Clal site in the 3′ G·T DNA. The asterisk specifies the position of the indirectly labeled Clal fragment of the discontinuous strand detected in the control reaction carried out in the absence of any extract or purified proteins. In the extract reactions, the discontinuous strand is subjected to mismatch correction and/or ligation; ligation generates the 6.44-kb ligated product. The mismatch-correction reaction proceeds via mismatch-provoked degradation of the discontinuous strand that generates smaller fragments located in the area indicated by the bracket. Degradation of the discontinuous strand in a particular reaction expressed as a percentage of the total label and representing the mean of three experiments is indicated below the reaction lane. (B) Degradation of the discontinuous strand of 3′ G·T ( and □) or 3′ A·T (● and ◦) DNA as a function of the concentration of HeLa nuclear (□) or cytosolic (●) extract. Data are from images like the one shown in A. (C) Dependence of mismatch correction of 3′ G·T DNA in HeLa nuclear (□) or cytosolic (●) extract on the amount of extract used in the reactions. The data in B and C are presented as the means ± 1 SD, n = 3. (D) Assembly of nucleosomes on 3′ G·T or cc G·T DNA during mismatch correction in HeLa nuclear and cytosolic extracts. DNA products of the indicated mismatch correction reactions were subjected to micrococcal nuclease cleavage and separation in a 1.5% native gel, and Southern hybridization with a 32P-labeled probe complementary to f1MR59 minus strand position 5756 (35). 32P hybrids were visualized with a GE Storm PhosphorImager system. (E) Detection of CAF-I in HeLa nuclear and cytosolic extracts by Western analysis. Proteins of HeLa nuclear and cytosolic extracts (20 µg each) were separated in a denaturing SDS-gel, transferred to a PVDF membrane and incubated with rabbit CAF-I p150 antibodies (Santa Cruz), followed by detection of the immune complexes with an ECL Plus kit (GE HealthCare).

Fig. 1. Mismatch correction and nucleosome assembly in HeLa cell-free extracts. Mismatch-correction reactions with 3′ G·T or control 3′ A·T DNA and the indicated amounts of HeLa nuclear or cytosolic extract were performed and analyzed as described in Materials and Methods. (A) Recovered DNA products of the indicated mismatch correction reactions were cleaved with Clal, separated under denaturing conditions in a 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a 32P-labeled probe complementary to the discontinuous strand position 2505–2526. The diagram on the left of the image shows the relative positions of the 3′ strand break, the G·T mispair (sharp bump), a 32P-labeled probe (dashed asterisk), and the unique Clal site in the 3′ G·T DNA. The asterisk specifies the position of the indirectly labeled Clal fragment of the discontinuous strand detected in the control reaction carried out in the absence of any extract or purified proteins. In the extract reactions, the discontinuous strand is subjected to mismatch correction and/or ligation; ligation generates the 6.44-kb ligated product. The mismatch-correction reaction proceeds via mismatch-provoked degradation of the discontinuous strand that generates smaller fragments located in the area indicated by the bracket. Degradation of the discontinuous strand in a particular reaction expressed as a percentage of the total label and representing the mean of three experiments is indicated below the reaction lane. (B) Degradation of the discontinuous strand of 3′ G·T (● and ◦) or 3′ A·T (● and ◦) DNA as a function of the concentration of HeLa nuclear (□) or cytosolic (●) extract. Data are from images like the one shown in A. (C) Dependence of mismatch correction of 3′ G·T DNA in HeLa nuclear (□) or cytosolic (●) extract on the amount of extract used in the reactions. The data in B and C are presented as the means ± 1 SD, n = 3. (D) Assembly of nucleosomes on 3′ G·T or cc G·T DNA during mismatch correction in HeLa nuclear and cytosolic extracts. DNA products of the indicated mismatch correction reactions were subjected to micrococcal nuclease cleavage and separation in a 1.5% native gel, and Southern hybridization with a 32P-labeled probe complementary to f1MR59 minus strand position 5753–5756 (35). 32P hybrids were visualized with a GE Storm PhosphorImager system. (E) Detection of CAF-I in HeLa nuclear and cytosolic extracts by Western analysis. Proteins of HeLa nuclear and cytosolic extracts (20 µg each) were separated in a denaturing SDS-gel, transferred to a PVDF membrane and incubated with rabbit CAF-I p150 antibodies (Santa Cruz), followed by detection of the immune complexes with an ECL Plus kit (GE HealthCare).
extract efficiently complements the MMR deficiency (20) (Fig. S3). To extend our observations obtained with HeLa cytosolic extract, we conducted mismatch-correction reactions in 293T cytosolic extracts in the presence or absence of purified CAF-I. Our control reactions showed that the indirectly end-labeled region of the 3′ G-T discontinuous strands is degraded by only 5% in the absence of purified MutLα and that this MMR-independent degradation is not affected by the presence of 7.5-nM CAF-I (Fig. S3A, lanes 14–15). Consistent with our previous observations (Fig. 2 and Fig. S2), considerable MutLα-dependent degradation of the indirectly end-labeled discontinuous strands is observed in 293T cytosolic reactions lacking purified CAF-I, and the addition of CAF-I strongly suppresses this degradation (Fig. S3A and B). Furthermore, we observed that CAF-I supplementation increases 3′ and 5′ nick-directed mismatch correction up to twofold (Fig. S3 C–E) and is necessary for nick-directed assembly of nucleosomes (Fig. S3F). The combined results of these experiments further support our hypothesis that CAF-I-dependent nucleosome assembly is involved in the control of mismatch-provoked degradation of discontinuous strands.

**MutSα Represses CAF-I-Dependent Histone H3–H4 Deposition in a Mismatch-Dependent Manner.** In order to understand how CAF-I affects the mismatch-provoked degradation of discontinuous strands, we carried out reconstitution studies. Deposition of a histone (H3-H4)_2 tetramer protects a 73–146-bp DNA fragment from micrococcal nuclease cleavage (21), enabling us to use this feature to detect the deposition. Because PCNA recruits CAF-I to newly replicated DNA (22) and RFC loads PCNA onto nicked DNA, we investigated whether CAF-I-dependent histone (H3-H4)_2 deposition occurs in a purified system that contains purified human CAF-I, histone (H3-H4)_2 tetramer, PCNA, RFC, and RPA proteins and nicked DNA. In these experiments, 73–146-bp DNA fragments that resist micrococcal nuclease cleavage due to the histone deposition were visualized with Southern hybridization (Fig. 3). The results of these experiments suggest that CAF-I-dependent histone (H3-H4)_2 tetramer deposition on a nicked DNA occurs in reactions also containing PCNA and RFC (Fig. 3, lanes 2 and 5). The omission/substitution experiments suggest that the observed histone deposition requires the presence of CAF-I, histone (H3-H4)_2 tetramer, RFC, and a DNA strand break and largely depends on PCNA (Fig. 3 and Table S1).

Because the correction of DNA replication errors and CAF-I-dependent nucleosome assembly are two postreplicative processes that are likely to co-occur on newly synthesized DNA, we tested whether the mismatch-recognition factor MutSα impacts reconstituted histone (H3-H4)_2 tetramer deposition in a defined system. Histone (H3-H4)_2 tetramer deposition reactions and their analysis were performed as described in Materials and Methods. The reactions contained the specified protein components and 3′ A-T DNA (lanes 1–7) or cc A-T DNA (lanes 8–9). DNA products generated by micrococcal nuclease cleavage were visualized with a 32P-labeled probe complementary to the minus strand of the two DNAs at position 5225–5248. The brackets indicate positions of DNA species protected from micrococcal nuclease by histone (H3-H4)_2 tetramer deposition.
CAF-I-Dependent Control of Mismatch-Provoked Degradation of the Discontinuous Strands in a Purified System. The availability of the minimal histone-deposition (Fig. 3) and mismatch-correction reactions (11) permitted us to investigate whether CAF-I-dependent histone H3–H4 deposition, the first step in replication-dependent nucleosome assembly, can control the mismatch-provoked degradation of the discontinuous strands in a defined system. In accordance with earlier studies (11, 12), we find that the seven-protein MMR system consisting of MutSα, MutLα, RFC, PCNA, Exo1, RPA, and DNA polymerase δ is proficient in 3′-nick-directed mismatch correction (Fig. 5D). In addition, we observe a strong MutSα-dependent degradation of the discontinuous mismatch-containing strands in the reconstituted reaction (Fig. 5A and B and Fig. S4A, lanes 8 and 9). This is a result of the enzymatic actions of MutLα endonuclease and Exo1 (7, 9, 23). The addition of CAF-I to the seven-protein system has no effect on the degradation of the discontinuous strands (Fig. 5A and B, lanes 3 and 9, and Fig. 5C) or on mismatch correction (Fig. 5D). Supplementation of the seven-protein MMR system with both CAF-I and the (H3-H4)2 tetramer protects the discontinuous mismatch-containing strands from excessive degradation (Fig. 5A–C) and weakly stimulates mismatch correction (Fig. 5D). These two effects require the presence of both CAF-I and the (H3-H4)2 tetramer and depend on the concentration of the histone tetramer (Fig. 5). The results of these reconstitution experiments indicate that the controlled degradation of the discontinuous strands observed in the cytosolic extracts supplemented with purified CAF-I (Fig. 2 and Figs. S2 and S3) can be mediated by CAF-I-dependent histone H3-H4 deposition.

MRR of DNA in Nucleosomes. Because newly replicated DNA subjected to mismatch correction is likely to contain naked regions and subnucleosomal assemblies surrounded by assembled nucleosomes (24, 25), it is important to understand whether MMR can function on DNA in nucleosomes. Mismatch-containing nucleosomal DNAs were reconstituted by salt dialysis (26) of 5′ G-T DNA and purified HeLa chromatin histones (Fig. S1B) mixed at different molar ratios. A 1:31 molar ratio of 5′ G-T DNA to HeLa histone octamers saturates the DNA with nucleosomes. Probing the dialyzed DNA–protein complexes with micrococcal nuclease confirms that nucleosomes were reconstituted (Fig. S6A). Next, we used the assembled nucleosomal DNAs as substrates in mismatch-correction reactions in 293T cytosolic extracts supplemented with purified MutLα. The results of these experiments suggest that MMR can function in the presence of subsaturating amounts of nucleosomes, whereas saturation of mismatch-containing DNA with nucleosomes represses mismatch correction (Fig. S6B).

Discussion

CAF-I was discovered as a factor that assembles newly replicated DNA into nucleosomes in cytosolic extracts of human 293 cells (14). CAF-I is essential for the viability of human cells (16) but not Saccharomyces cerevisiae (27). Given that the core histones are indispensable for eukaryotic organisms, the absence of a growth defect in S. cerevisiae cac1-3 mutants encoding the three CAF-I subunits suggests that these yeast mutants can assemble nucleosomes during replication, probably by relying on the mechanisms that normally function during transcription (13). Although yeast CAF-I is not an essential protein, it is required for the suppression of gross chromosome rearrangements (28). In this work, we provide several lines of evidence that human CAF-I-dependent nucleosome assembly is responsible for controlling the degradation of the discontinuous mismatch-containing strands during mismatch correction. First, significant mismatch-dependent degradation of discontinuous strands is observed in HeLa cytosolic extract containing only trace amounts of CAF-I (Fig. 1). Second, supplementation of the cytosolic extracts with purified CAF-I abolishes most mismatch-provoked degradation of the discontinuous strands but does not suppress mismatch correction (Fig. 2 and Figs. S2 and S3), suggesting that the degradation occurring in the absence of CAF-I is uncontrolled and unnecessary. Third, the absence of significant degradation of discontinuous mismatch-containing strands in HeLa nuclear extract correlates with the presence of both CAF-I protein and nick-directed nucleosome assembly (Fig. 1), the latter being known to depend on CAF-I (17). Fourth, CAF-I-dependent histone (H3-H4)2 tetramer deposition protects the discontinuous mismatch-containing strand from excessive degradation by MMR in a defined purified system (Fig. 5). In addition to controlling degradation, CAF-I-dependent nucleosome assembly modestly stimulates mismatch correction (up to twofold) (Fig. 2 and Figs. S2 and S3). However, we believe that this effect might be of limited importance for MMR because the stimula-
reaction with tracts of HeLa and H6 cells in which MutLThis incision pattern differs from that observed in nuclear ex-
derated along its complete 6.44-kb length (7, 29) (Fig. 5
discontinuous mismatch-containing strands at numerous sites scat-
Claendonuclease activity in the nuclear extracts may be
αthe basis for the difference in the incision pattern of MutL
αing freely along mismatch-containing DNA, which is necessary
and the indicated proteins were performed and analyzed as described in Materials and Methods. (A–C) CAF-I-dependent histone H3–H4 deposition protects the discontinuous mismatch-containing strands from excessive degradation by MMR. Recovered products of the mismatch correction reactions were cleaved with \( \text{Cla} \), separated in a denaturing 9.8% agarose gel, transferred to a nylon membrane, and hybridized with \(^{32}P\)-labeled probe v2505 (A) or \( v2531 \) (B), complementary to discontinuous-strand positions 2505–2526 and 2531–2552, respectively. (C) The effect of the presence of CAF-I and H3–H4 tetramer on degradation of discontinuous mismatch-containing strands. Data are from images like those in A and Fig. S5. Degradation occurring in the reconstituted reactions with (● and ○) or without (□ and ◯) 15-nM CAF-I was determined by Southern analyses with \(^{32}P\)-labeled probe v2505 (● and ○) or \( v2531 \) (● and ○). (D) Mismatch correction in the defined system in the presence of CAF-I and H3–H4 tetramer. The reactions containing MutS\( \alpha \), MutL\( \alpha \), RFC, PCNA, Exo1, Exo1, RFC, Pol\( \delta \), and the indicated proteins were performed and analyzed as described in Materials and Methods.

Our current hypothesis to explain the impact of CAF-I on mis-
match correction is that nucleosomes assembled by CAF-I serve
as roadblocks that prevent the MMR incision complex from slid-
ing freely along mismatch-containing DNA, which is necessary for
MutL\( \alpha \) to be able to cleave the discontinuous strand at sites
located far from the mismatch (Figs. 2 and 5 and Figs. S2 and S3).
Because Exo1 excision and DNA synthesis by polymerase \( \delta \) initiated from remote MutL\( \alpha \) incision sites require more time for completion than those initiated from nearby sites, and because the remote incisions compete with the near incisions as starting points of Exo1 excision and subsequent DNA polymerization, it is hardly surprising that protection of discontinuous mismatch-
containing strands by CAF-I-dependent nucleosome assembly weakly stimulates mismatch correction.

In the reconstituted systems, MutL\( \alpha \) endonuclease cleaves the discontinuous mismatch-containing strand at numerous sites scattered along its complete 6.44-kb length (7, 29) (Fig. 5 A and B). This incision pattern differs from that observed in nuclear extracts of HeLa and H6 cells in which MutL\( \alpha \) endonuclease incises the discontinuous strand within a small, approximately 600-bp region surrounding a mismatch (7). The basis of this different cleavage pattern is not understood, but protein factors that reg-
ulate MutL\( \alpha \) endonuclease activity in the nuclear extracts may be
missing from the purified system. Results obtained in this study provide evidence that CAF-I-dependent nucleosome assembly is the basis for the difference in the incision pattern of MutL\( \alpha \) endonuclease (Fig. 5).

Previous investigations of replication-dependent nucleosome assembly have implicated the histone H3–H4 chaperone CAF-I and the PCNA clamp in packaging the newly synthesized DNA into nucleosomes (14, 19, 22). We have now shown that, in addi-
tion to CAF-I, PCNA, and histone (H3–H4)_2 tetramer, CAF-I-
dependent tetramer deposition also requires RFC, which loads PCNA onto DNA (Fig. 3 and Table S1). These requirements define a minimal set of proteins for this reaction. Surprisingly, we observed that a low level of tetramer deposition takes place in the absence of PCNA. Previous work showed that PCNA is essential for CAF-I-dependent nucleosome assembly (19). Thus, we think that the weak PCNA-independent histone deposition is normally suppressed by the cellular environment lacking in our reconstituted system and/or is not sufficient to support nucleo-
some assembly during DNA replication. Our results showing that MutS\( \alpha \) prevents CAF-I-dependent histone (H3–H4)_2 tetramer deposition in the vicinity of a mismatch (Fig. 4 and Fig. S4) sug-
gest the existence of crosstalk between MMR and replication-
dependent nucleosome assembly. The simplest interpretation of this observation is that MutS\( \alpha \) clamps bound to and/or moving away from a mismatch (30, 31) sterically occlude DNA surrounding the mismatch from being assembled into nucleosomes by CAF-I.

We determined that the presence of the saturating amounts of preassembled nucleosomes suppresses nick-directed mismatch correction in a cell-free extract (Fig. S6). This observation is not very surprising, given that nucleosomes inhibit DNA sliding (32, 33) and mismatch recognition (32) by MutS\( \alpha \). We suggest that one of the roles of the nucleosomal organization is to pro-
mote genetic stability by preventing MMR-dependent strand breakage during and after S phase.

In summary, our results suggest that postreplicative mismatch correction and CAF-I-dependent nucleosome assembly co-occur on the newly replicated DNA and that there is active communic-
tion between the two processes. As a result, the mismatch-
containing daughter strands are degraded in a controlled manner. The abilities of MutS\( \alpha \) to suppress CAF-I-dependent nucleosome assembly in the vicinity of mismatch (Fig. 4 and Fig. S4) and to passively disassemble a nucleosome in a mismatch-dependent
manner (34) are likely to contribute to MMR functioning during concomitant CAF-I-dependent nucleosome assembly.

Materials and Methods
Circular 6.44-kb DNA substrates for the mismatch-correction reactions, referred to as 3′-GT, 3′-AT, 5′-GT, 5′-AT, cc G-T, and cc A-T DNAs, were prepared on the basis of f1 phages as described (7, 38). The 3′-GT and 3′-AT DNAs contain a strand break 141 base pairs 3′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively. A strand break in the 5′-GT and 5′-AT substrates is located 128 base pairs 5′ to a G-T mispair and an A-T pair, respectively.

Mismatch-correction reactions were carried out according to the previously developed procedures (4, 7, 12) at 37 °C for 10 min. Mismatch-correction reactions including cell-free extracts were performed in 80-μl mixtures containing 20 mM HEPES-NaOH (pH 7.4), 91 mM KCl, 19 mM NaCl, 5 mM MgCl2, 5 mM potassium phosphate (pH 7.5), 3 mM ATP, 2 mM dithiothreitol (DTT), 0.2 mg/ml bovine serum albumin (BSA), 0.3 mM S′- or S′-3′-nicked DNA (0.1 μg), 0.1 mM each of the four dNTPs (dATP, dGTP, dCTP, and dTTP), and the specified amount of cell-free extract. When indicated, the cell-free-extract reactions were supplemented with purified CAF-I and the reactions containing 293T cytosolic extract were also supplemented with purified MutLα. Mismatch-correction reactions in the reconstituted system were performed in 40-μl mixtures containing 20 mM HEPES-NaOH (pH 7.4), 121 mM KCl, 19 mM NaCl, 5 mM MgCl2, 3 mM ATP, 2 mM DTT, 0.4 mg/ml BSA, 1% (w/v) glycerol, 0.6 mM S′-3′-nicked DNA (0.1 μg), 0.1 mM each of the four dNTPs, 12.5 mM MutSα, 2.5 mM MutLα, 15 mM PCNA, 2 mM RCF, 25 mM RPA, 0.6 mM Exo1, 0.6 mM DNA polymerase δ, 15 mM CAF-I, and 1–15 mM (H3-H4)2 tetramer. In control reconstituted reactions, one or several indicated nucleases were omitted. Mismatch-correction reactions in the extract and reconstituted systems were stopped, processed, and analyzed as described in SI Text.

Analysis of CAF-I-dependent nucleosome assembly in mismatch-correction reactions in cell-free extracts was performed as described in SI Text. CAF-I-dependent histone H3-H4 deposition reactions were performed at 37 °C in 40-μl mixtures containing 20 mM HEPES-NaOH (pH 7.4), 121 mM KCl, 19 mM NaCl, 5 mM MgCl2, 3 mM ATP, 2 mM DTT, 0.4 mg/ml BSA, 1% (w/v) glycerol, 0.6 mM S′-3′-nicked DNA or cc A-T DNA, 15 mM CAF-I, 15 mM (H3-H4)2 tetramer, 15 mM PCNA, 2 mM RCF, 25 mM RPA. When indicated, the deposition reactions were also supplemented with 6–25 mM MutSα. In 10 min of the incubation, 35-μl fractions of the histone-deposition reactions were supplemented with a 5-μl mixture containing micrococcal nuclease and CaCl2 so that their final concentrations were 0.3 μg/ml and 2.5 mM, respectively, and the reaction temperature was switched to 21–23 °C. Micrococcal nuclease cleavage was carried out for 5 min and stopped by the addition of a 4-μl mixture containing 0.5% SDS, 70 mM EDTA, 2.5 mg/ml Proteinase K, and 40% glycerol, followed by incubation at 50 °C for 15 min. DNAs of the stopped reactions were separated in 1% agarose gels in 1xTAE, followed by Southern hybridization analyses performed as described in SI Text.

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Supporting Information

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SI Materials and Methods.

Preparation of Cell-Free Extracts, Proteins, and Nucleosomal DNA.

293T cells were grown as an attached culture in DMEM/high glucose with 10% fetal bovine serum. HeLa cells were cultured in suspension in RPMI-1640 with 5% fetal bovine serum. Both media were supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml L-glutamine. 293T cytosolic extract was prepared as described (1–3), and HeLa cytosolic extract was prepared in the same way except that the hypotonic buffer contained 75 mM KCl instead of 5 mM KCl. To prepare HeLa nuclear extract, the nuclear pellet (~4 ml) obtained during the last stage of cytosolic extract preparation (3) was immediately resuspended in 6 ml of hypotonic buffer supplemented with 200 mM KCl, 1 mM DTT, 2 μg/ml aprotinin, 4 μg/ml leupeptin, 1 μg/ml E64, and 1.4 μg/ml pepstatin so that the final concentration of KCl was 130 mM, and the extraction was carried out on ice for 30 min. The insoluble part was then removed by centrifugation at 27,000 g for 15 min. The supernatant, nuclear extract, was subdivided, frozen in liquid N₂, and stored at −80°C.

Recombinant human MutLα, MutSα, Exo1, DNA polymerase δ, PCNA, RFC, and RPA were obtained in a nearly homogenous form as described (3–6). The first four proteins were produced in insect SF9 cells and the latter three in Escherichia coli. Histone H2A-H2B heterodimers and (H3-H4)₂ tetramers were purified to apparent homogeneity from HeLa chromatin by chromatography on hydroxyapatite (BioRad) and MonoS (GE HealthCare) columns.

To prepare recombinant human CAF-I protein, its p150, p60, and p48 genes (7, 8) were amplified with primer pairs #1 (5′-TTTGGCGGCCGCGCCACCATGGATTGCAAAGATAG-3′), #2 (5′-TTTGGCGGCCGCGCCACCATGAAGATCATCATTG-G3′) and 5′-CCCGTCGAGATCATCATTGCGAAATGGTTCGTTACATCCTG-3′), and #3 (5′-TTTGCGGCCGCGCCACCATGGATTGCAAAGATAG-3′) and 5′-CCCGTCGAGATCATCATTGCGAAATGGTTCGTTACATCCTG-3′), and #3 (5′-TTTGCGGCCGCGCCACCATGGATTGCAAAGATAG-3′) and 5′-CCCGTCGAGATCATCATTGCGAAATGGTTCGTTACATCCTG-3′), respectively, and cloned separately into the pFASTBac1 vector (Invitrogen). Cloning details are available upon request. The genomes of the Bac-to-Bac vectors were confirmed by DNA sequencing. The recombinant baculoviruses were propagated in Sf9 cells and CAF-I was produced according to the Bac-to-Bac protocol (Invitrogen). To produce CAF-I, insect Sf9 cells were infected with the three baculoviruses encoding CAF-I p150, p48, and p60 subunits, and infection was for 48 h. The recombinant CAF-I was purified by chromatography on M2 anti-Flag beads (Sigma) and a MonoQ column (GE HealthCare). A CAF-I peak fraction (98% pure) obtained after MonoQ chromatography was subdivided and stored at −80°C.

Analysis of CAF-I-Dependent Nucleosome Assembly. To analyze CAF-I-dependent nucleosome assembly in mismatch-correction reactions with cell-free extracts, 35-μl fractions of the reactions, carried out as described in Materials and Methods for 10 min, were supplemented with a 5-μl mixture containing micrococcal nuclease, RNAses A and C, and 25 °C, so that their final concentrations were 0.5 U/μl, 25 ng/ml and 8 mM, respectively, and the reaction temperature was switched to 21–23°C. The micrococcal nuclease cleavage of the HeLa and 293T reactions was for 5 min and 10 min, respectively, and was stopped by the addition of a 10-μl mixture containing 0.5% SDS, 70 mM EDTA, 2 mg/ml Proteinase K, 25 ng HindIII-cleaved pAH1A DNA (used as a gel loading control), and 40% glycerol, followed by incubation of the reactions at 50°C for 15–30 min. The cleaved DNAs were separated in a 1.5% agarose gel in 1xTAE buffer, followed by alkaline transfer and a Southern analysis of the separated DNAs with an appropriate probe.

Analysis of Mismatch-Correction Reactions. Mismatch-correction reactions with cell-free extracts were terminated by the addition of 30 μl of 0.7% SDS, 0.33 mg/ml Proteinase K, 0.8 M NaCl, 0.33 mg/ml glycerol, and 26 mM EDTA, followed by incubation of the stopped reactions at 50°C for 15–30 min. The reconstituted reactions were stopped by the addition of 30 μl of 0.35% SDS, 0.17 mg/ml Proteinase K, 0.4 M NaCl, 0.33 mg/ml glycerol, and 13 mM EDTA, followed by incubation of the stopped reactions at 50°C for 15–30 min. The mismatch-correction products of the extract and reconstituted reactions were extracted with phenol/chloroform, precipitated with isopropanol, washed with 75% ethanol, and dissolved in 10 mM Tris-Cl (pH 7.5), 1 mM EDTA. To score MMR, halves of the recovered DNAs were cleaved with 1 U each of HindIII and ClaI or of HindIII and AlwNI for 1–2 h at 37°C, followed by separation of the products in a 1% agarose gel in 1xTAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) and visualization of the ethidium bromide-stained products with a cooled charge-coupled device camera. Mismatch-correction in an analyzed reaction was calculated according to the percentage formula: mismatch correction (%) = 100 x A/(A + B), where A is a combined area of the peaks of the repaired DNA bands and B is an area of the peak of the mismatch-containing DNA band.

To perform Southern hybridization analysis of mismatch-correction products, the other halves of the recovered DNAs were cleaved with ClaI, followed by separation of the cleaved DNAs in a 0.7–0.8% agarose gel under denaturing conditions (40 mM NaOH/2 mM EDTA). The separated DNA products were then transferred to a nylon membrane in 0.2 M NaOH and hybridized with appropriate 32P-labeled oligonucleotide probes. The indirectly labeled DNA species were visualized and quantified with a Molecular Dynamics PhosphorImager system (GE HealthCare). To reprobe a membrane with a different 32P-labeled oligonucleotide probe, the previous probe was stripped off with 0.2 M NaOH as described (6).

Assembly of Nucleosomal DNA. Nucleosomal DNA was prepared according to a previously described salt dialysis reconstitution method (9). Briefly, 60-μl mixtures containing 2 M NaCl, 20 mM 5'-G-T DNA, 125-1,379 nM purified HeLa histone octamers, and 2 mM DTT were dialyzed sequentially against buffer A (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.2 mM DTT) containing 1 M NaCl for 3 h, buffer A containing 0.75 M NaCl for 3 h, and buffer A containing 5 mM NaCl for 16 h. Reconstitution with 627-nm HeLa histone octamers and 20 nM 5'-G-T DNA saturates the DNA with nucleosomes because a nucleosome contains 146 bp of DNA and the linker DNA is 59–60 nt.


**Fig. S1.** Electrophoretic analysis of CAF-I and core histones used in this work. Recombinant human histone chaperone CAF-I and native core histones (H2A, H2B, H3, and H4) were purified from insect cells and the chromatin fraction of HeLa cells, respectively, as described in SI Materials and Methods. One μg of CAF-I (A), 1 μg (B, lane 1), and 2 μg (B, lane 2) of core histones, approximately 2 μg of H2A-H2B dimer (B, lane 3), and approximately 2 μg of (H3-H4)2 tetramer (B, lane 4) obtained at the final purification steps were separated in denaturing polyacrylamide SDS-gels and visualized with Coomassie-R250 staining. The images were captured by a cooled CCD camera.
Fig. S2. CAF-I-dependent control of degradation of the 5'-G-T discontinuous strands in HeLa cytosolic extract mismatch-correction reactions. Mismatch-correction and nucleosome-assembly reactions in HeLa cytosolic extract (90 μg), and their analyses, were performed as described in the legend to Fig. 2. (A) CAF-I-dependent assembly of nucleosomes on 5'-G-T DNA. Formation of nucleosomal DNA in mismatch-correction reactions was detected by micrococcal nuclease cleavage in combination with Southern hybridization with a 32p-labeled probe complementary to the discontinuous-strand position 5491–5514. (B–D) The products of the mismatch-correction reactions, after denaturing gel-electrophoresis separation, were analyzed by Southern hybridization with a 32p-labeled probe complementary to the discontinuous-strand position 2505–2526 (B) or 2531–2552 (C). (D) Degradation of the 5'-G-T discontinuous strands as a function of CAF-I concentration. Data were obtained from the images like those shown in (B) and (C) and are presented as the means ± 1 SD, n ≥ 2.
Fig. S3. The effects of CAF-I on mismatch-correction reactions in 293T cytosolic extract. Mismatch-correction reactions with MLH1−/− 293T cytosolic extract (60 μg) and the indicated concentrations of purified MutLα, and analyses of these reactions, were carried out as described in the legend to Fig. 2. (A) CAF-I protects the discontinuous mismatch-containing strands from MutLα-dependent degradation. The mismatch-correction reactions were carried out in the presence or absence of purified CAF-I (7.5 nM). Recovered DNA products were separated in a denaturing agarose gel and analyzed by Southern hybridization with a 32P-labeled probe complementary to the discontinuous-strand position 2505–2526. (B) Graphic representation of the results obtained from the images like the one shown in (A). (C–D) Dependence of the mismatch-correction of 3′ G-T DNA on the presence of CAF-I (7.5 nM) and the indicated MutLα concentrations. (C) Products of the reactions were cleaved with Clal and HindIII, separated in a 1% native agarose gel, and quantified. Mismatch-correction expressed as a percentage of the total DNA in a particular reaction is shown below the reaction lane. (D) Mismatch-correction of 3′ G-T (● and □) or 5′ G-T (○ and △) occurring in the presence (● and ○) or absence (□ and △) of purified 7.5-nM CAF-I as a function of MutLα concentration. Data are from gels like that shown in (C). (E) The effects of different CAF-I concentrations on the mismatch-correction of 3′ G-T DNA (●) or 5′ G-T DNA (○) in 293T extract supplemented with 1.3 nM MutLα. Data in B, D, and E are presented as the means ± 1 SD, n ≥ 2. (F) CAF-I- and nick-dependent nucleosome-assembly in 293T cytosolic extract. 293T mismatch-correction reactions contained cc A-T, 5′ A-T, 5′ G-T, or cc G-T DNA, 1.3 nM purified MutLα, and the specified concentration of purified CAF-I. The DNAs of these reactions were subjected to micrococcal nuclease cleavage followed by Southern hybridization analysis with a 32P-labeled probe complementary to the f1MR1 minus strand position 5491–5514. Arrows with small and large arrowheads indicate the gel positions of micrococcal nuclease cleavage products that were free of and within nucleosomes, respectively.
Fig. S4. MutSα and mismatch-dependent suppression of CAF-I-mediated histone H3–H4 tetramer deposition. Graphical representation of the data obtained from quantifications of images like the one in Fig. 4A (A), Fig. 4B (B), or Fig. 4C (C). To score CAF-I-dependent histone deposition, the background value of nonspecific binding of histone (H3-H4)$_2$ tetramer to DNA, determined in the reactions lacking the CAF-I (Fig. 4, lane 2), was subtracted from the value of chaperone CAF-I-dependent histone H3-H4 tetramer deposition observed in an analyzed reaction. CAF-I-dependent histone (H3-H4)$_2$ tetramer depositions are expressed as the percentages ± 1 SD, n = 2, where 100% is CAF-I-dependent histone (H3-H4)$_2$ tetramer deposition observed in the reactions lacking MutSα (Fig. 4, lane 3).

Fig. S5. CAF-I-dependent control of 3′ G-T discontinuous-strand degradation in a defined system. Reconstituted reactions containing 3′ G-T DNA and the indicated proteins were performed and analyzed as described in Fig. 5. (A) Recovered products of the mismatch-correction reactions were cleaved with ClaI, separated in a denaturing 0.8% agarose gel, transferred to a nylon membrane, and hybridized with $^{32}$P-labeled probe complementary to discontinuous-strand position 5629–5652. (B) Graphic representation of the results obtained from images like the one in (A). Data are given as the means ± 1 SD, n ≥ 2.

Fig. S6. Mismatch-correction in the presence of preassembled nucleosomes. (A) 5′ G-T DNA and the HeLa core histones mixed at the saturating octamer:5′ G-T DNA molar ratio of 31:1 were assembled into nucleosomes by salt dialysis as described in SI Materials and Methods. The assembled DNA was cleaved with micrococcal nuclease and the cleavage products were visualized by Southern hybridization with a $^{32}$P-labeled probe complementary to discontinuous-strand position 5629–5652. (B) Mismatch-correction reactions carried out for 15 min contained 60 μg 293T cytosolic extract, 20 nM MutLα, and 5′ G-T DNA that was naked or assembled into nucleosomes at the indicated octamer:5′ G-T DNA ratios. Data are presented as the means ± 1 SD, n ≥ 2.
Table S1. Dependence of CAF-I-mediated histone H3–H4 tetramer deposition on the presence of PCNA, RFC, RPA, and a strand break

<table>
<thead>
<tr>
<th>Reaction</th>
<th>3′ A-T DNA (%)</th>
<th>5′ A-T DNA (%)</th>
<th>ccDNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>100</td>
<td>100</td>
<td>2±3</td>
</tr>
<tr>
<td>- PCNA</td>
<td>9 ± 4</td>
<td>19 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>- RFC</td>
<td>&lt;1</td>
<td>7 ± 10</td>
<td>ND</td>
</tr>
<tr>
<td>- RPA</td>
<td>58 ± 7</td>
<td>35 ± 28</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are from images like the one shown in Fig. 3. The reaction containing CAF-I, (H3-H4)₂ tetramer, PCNA, RFC, and RPA is designated as complete. The other reactions contained the indicated omissions. CAF-I-dependent histone depositions on 5′ A-T DNA were visualized by Southern hybridizations with a 32P-labeled probe complementary to the discontinuous-strand position 5491–5514. Results were quantified as described in Fig. S4 and are presented as the averages ± 1 SD, n ≥ 2. ND—not determined.