Cleavage of Model Replication Forks by Fission Yeast Mus81-Eme1 and Budding Yeast Mus81-Mms4

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The blockage of replication forks can result in the disassembly of the replicative apparatus and reversal of the fork to form a DNA junction that must be processed in order for replication to restart and sister chromatids to segregate at mitosis. Fission yeast Mus81-Eme1 and budding yeast Mus81-Mms4 are endonucleases that have been implicated in the processing of aberrant DNA junctions formed at stalled replication forks. Here we have investigated the activity of purified Mus81-Eme1 and Mus81-Mms4 on substrates that resemble DNA junctions that are expected to form when a replication fork reverses. Both enzymes cleave Holliday junctions and substrates that resemble normal replication forks poorly or not at all. However, forks where the equivalents of either both the leading and lagging strands or just the lagging strand are juxtaposed at the junction point, or where either the leading or lagging strand has been unwound to produce a fork with a single-stranded tail, are cleaved well. Cleavage sites map predominantly between 3 and 6 bp 5’ of the junction point. For most substrates the leading strand template is cleaved. The sole exception is a fork with a 5’ single-stranded tail, which is cleaved in the lagging strand template.

DNA replication complexes (replicosomes) routinely encounter blockages in the DNA that they are copying (1). Obstructions include transcription complexes, DNA repair complexes and bulky DNA lesions (e.g. pyrimidine dimers). It is imagined that the cellular response to each type of blockage differs according to parameters that have yet to be clearly defined. In principle if the blockade is another protein complex it would make sense for the replicosome to suspend synthesis until the obstruction has moved out the way. However, simply waiting for a blocking lesion to be removed may not be an option for the replicosome since it may itself be blocking repair complexes from accessing the damaged DNA. In such circumstances polymerases that are able to function on damaged templates can be recruited (2). In *Escherichia coli* these polymerases are error-prone and therefore appear to be used only when there is an excess of DNA damage (3). However, in eukaryotes some of these polymerases are not inherently mutagenic and therefore they may be used more readily (2). If the lesion is in the lagging template strand then synthesis of the relevant Okazaki fragment may be skipped resulting in a lesion-containing DNA gap that is dealt with by post-replication repair (4). Alternatively lesions in either the leading or lagging template strand may provoke the partial or complete disassembly of the replicosome followed by the spontaneous or enzyme-driven reversal of the replication fork (5–9). Fork reversal (also referred to as fork regression) involves the unwinding of nascent strands and re-pairing of parental strands. This may serve both to provide room for lesion repair and/or a means of unfecting nascent strands to act in a template switching mechanism for the bypass of the lesion (10). Depending on the extent of fork reversal either one or both nascent strands are extruded from the fork. In the latter case this will result in the formation of a Holliday junction when the strands anneal. In order for replication to restart reversed forks have to be reset. This can be achieved either by the removal of the extruded DNA by a nuclease or reverse branch migration of the junction point by a DNA helicase (6, 11–14). Alternatively the reversed fork can be cleaved to detach either the leading or lagging strand arm of the fork (6, 15). The exposed DNA end is then targeted by recombination enzymes that both repair the break and facilitate the reassembly of the replisome (16, 17).

In the fission yeast *Schizosaccharomyces pombe* the Rqh1 DNA helicase and Mus81-Eme1 endonuclease have both been implicated in processing DNA junctions at stalled replication forks (11, 18, 19). Rqh1 is a member of the RecQ family of DNA helicases that includes Sgs1 in *Saccharomyces cerevisiae* and the Bloom’s (BLM), Werner’s (WRN), and Rothmund-Thomson’s Syndrome helicases in humans (20). At least some RecQ helicases, including Rqh1, appear to limit recombination at stalled replication forks (11, 21, 22). Both BLM and WRN can bind and branch migrate Holliday junctions in vitro prompting the suggestion that they could catalyze fork resetting and thereby limit recombination caused by fork cleavage (12, 14). A similar function has been ascribed to Rqh1. This is based on theobservation that an *rqh1* mutant’s chromosome segregation defects, which are induced by replication fork stalling, are partially suppressed by the *E. coli* Holliday junction resolvasse RusA (11). It would appear that in the absence of Rqh1 at least some Holliday junctions at stalled replication forks remain unprocessed and so impede sister chromatid segregation.

Mus81-Eme1 is related to the XPF-ERCC1 and Rad1-Rad10 family of heteromeric structure-specific endonucleases that function in nucleotide excision repair, DNA interstrand cross-link repair and recombination (23, 24). *mus81* and *eme1* mutants are hypersensitive to ultraviolet light, the ribonucleotide reductase inhibitor hydroxyurea and the alkylating agent methylmethane sulfonate that cause replication fork stalling, and are inviable in the absence of *rqh1* (18, 19, 24). These phenotypes are suppressed by RusA suggesting that Mus81-Eme1 cleaves Holliday junctions, and thereby provides an alternative to Rqh1 for processing junctions formed at stalled replication forks (18). Indeed both *S. pombe* and human Mus81, purified from their endogenous cells, cleave Holliday junctions...
in vitro (19, 25). However, when purified from E. coli cells, Mus81-Eme1 and its homologue from S. cerevisiae Mus81-Mms4 exhibit only low levels of Holliday junction cleavage activity, whereas they cleave fork substrates very well (18, 26). A strong preference for fork substrates is also observed for Mus81 from HeLa cells (27). These observations have prompted the suggestion that Mus81-Eme1 and Mus81-Mms4 may cleave stalled replication forks before they have fully reversed to form a Holliday junction (18, 26, 27).

To gain a better understanding of Mus81-Eme1’s potential for processing DNA junctions at stalled replication forks, we have tested its ability to cleave a variety of fork substrates made from oligonucleotides and DNA molecules. Cleavage sites have also been mapped and a direct comparison with Mus81-Mms4 made. From this study we show that both Mus81-Eme1 and Mus81-Mms4 cleave structures that are expected to form when a replication fork reverses. Cleavage always occurs between 3 and 6 bp 5′ to the junction point and in most cases is targeted to the equivalent of the leading strand template. The one exception to this rule is a substrate that mimics a replication fork that has reversed to expose only its nascent lagging strand, which is cleaved in the equivalent of the latching strand template. The potential significance of these data for fork processing in vitro is discussed.

**EXPERIMENTAL PROCEDURES**

**Proteins—** Mus81-Eme1 and NLS-RusA-GFP were purified as described previously (18). S. cerevisiae Mus81-Mms4 was purified as described below. Amounts of protein were estimated by a modified Bradford assay using a Bio-Rad protein assay kit and bovine serum albumin as the standard. The concentration of NLS-RusA-GFP is expressed as moles of monomers. Mus81-Eme1 and Mus81-Mms4 are assumed to be heterodimers and therefore their concentrations represent moles of heterodimers.

**Plasmids—** S. cerevisiae MUS81 and MMS4 were amplified by PCR from a genomic DNA template using primers that incorporate suitable restriction sites 5′ and 3′ of their open reading frames to facilitate cloning. MUS81 was cloned as an NdeI-BamHI fragment into pT7-7 to make pMW566, and MMS4 was cloned as an NdeI-EcoRI fragment into pET14b (Novagen) to make pMW567. A fragment containing the T7 phase Ø10 promoter and MUS81 gene was then amplified by PCR from pMW566 using primers that incorporate terminal SphI sites, and cloned into a unique SphI site upstream of the T7 phase Ø10 promoter in pMW567 to make pMW571.

**Oligonucleotides—** Oligonucleotides (19, 25) were used for nicking and purification of Mus81-Mms4—100 ml of E. coli BL21-RIL cells (Stratagene) containing pMW571 were grown with aerated LB broth containing 125 μg/ml carbenicillin and 50 μg/ml chloramphenicol. At a cell density corresponding to an OD600 of 0.5 Mms4, containing an N-terminal His6 tag, and Mus81 were induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. After a 2.5 h induction the cells were harvested by centrifugation, resuspended in 10 ml of Buffer H (50 mM potassium phosphate, pH 8.0, 0.3 M NaCl, 10% glycerol) and frozen and stored at −80 °C until required. Frozen cells were thawed at room temperature and then mixed on ice with protease inhibitors, 10 mM n-mercaptoethanol, and 1% Triton X-100 before lysis by passage through a French pressure cell. The lysate was loaded directly onto a 1-ml Ni-nitrilotriacetic acid superflow column (Qiagen), which was washed with 30 ml of Buffer H + 50 mM imidazole before eluting bound Mus81-Mms4 with 2 ml of Buffer H + 200 mM imidazole. Muscle81-Mms4 was then stored as aliquots at −80 °C.

**DNA Substrates—** Oligonucleotides 2, 5–8, 10–11, and 14 used to make fork substrates have been described previously (18, 28). The sequence of oligonucleotide 15 is 5′-TGCAGCATCTACAGCTGCAGTGTG-3′. Oligonucleotides were supplied by Sigma-Genosys Ltd. and were purified by electrophoresis through a 15% denaturing gel containing 7 M urea. For native gels, deproteinized reactions were mixed with loading dye and loaded directly onto the gel. For denaturing gels, reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the DNA was precipitated with ethanol, resuspended in gel-loading buffer (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, 10 mM EDTA, pH 7.5, 97.5% [v/v] formamide), and denatured by boiling for 2 min before loading onto the gel. To map cleavage sites reaction products were run alongside Maxam-Gilbert sequence ladders of the appropriate labeled oligonucleotide. A 1.5-base allowance was made to compensate for the nucleoside eliminated in the sequencing reaction.

**FIG. 1. Cleavage of DNA by Mus81-Eme1.** A, schematic diagram of χ DNA showing the Holliday junction situated within a central homologous core (gray lines). The restriction sites used to generate the structure from a figure-of-eight DNA molecule, the polarity of the DNA strands, and the sizes (in kb) of each duplex arm are indicated. B, agarose gel showing cleavage of the Holliday junction in χ by Mus81-Eme1 and NLS-RusA-GFP to generate nicked duplex species of the sizes indicated (in kb). The band or smear running ahead of the 1.8-kb product band in lanes d and f is probably generated by a pair of cleavages in strands of unlike polarity so that one duplex arm is detached. The reactions in lanes b–d contain 2, 10, and 20 nM Mus81-Eme1, whereas lane f contains 20 nM NLS-RusA-GFP.

Enzyme Assays—χ, χMET, and χVFS cleavage reactions (10 μl) contained DNA and enzyme as indicated in reaction buffer (2.5% SDS, 200 mM EDTA, 10 μg/ml bovine serum albumin, 0% glycerol, 10 mM [MgCl2]). Reactions were incubated at 30 °C for 30 min. They were stopped by the addition of one-fifth volume of stop mixture (2.5% SDS, 200 mM EDTA, 10 μg/ml protease K) followed by a further 15 min at 30 °C to deproteinize the mixture. Reaction products were then analyzed by electrophoresis through a 0.8% agarose gel in Trisborate/EDTA (TBE) buffer at 100 V for 80 min. The cleavage reactions (30 μl) in Figs. 3 and 5 contained 10 nM of either Mus81-Eme1 or Mus81-Mms4 as indicated, and between 0.4 and 3.6 nM of junction DNA (depending on the oligonucleotide that is labeled), in the same reaction buffer as above. Reactions were incubated and stopped as above, and the products analyzed by electrophoresis through 10% native polyacrylamide gels in TBE buffer at 200 V for 2 h and 15% denaturing gels containing 7 M urea. For native gels, deproteinized reactions were mixed with loading dye and loaded directly onto the gel. For denaturing gels, reactions were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol, resuspended in gel-loading buffer (0.05% [w/v] bromophenol blue, 0.05% [w/v] xylene cyanol, 10 mM EDTA, pH 7.5, 97.5% [v/v] formamide), and denatured by boiling for 2 min before loading onto the gel. To map cleavage sites reaction products were run alongside Maxam-Gilbert sequence ladders of the appropriate labeled oligonucleotide. A 1.5-base allowance was made to compensate for the nucleoside eliminated in the sequencing reaction.
RESULTS

Mus81-Eme1 Cleaves \(\chi\) DNA Poorly—Previously we have shown that Mus81-Eme1 cleaves small synthetic Holliday junctions (X-12) relatively poorly compared with fork substrates (18). X-12 contains only a 12-bp homologous core in which the junction crossover point branch migrates, and therefore could in theory be cleaved inefficiently by Holliday junction resolvases that are affected by sequence context. To see if Mus81-Eme1 can cleave more efficiently Holliday junctions that are contained within larger regions of homology, \(\chi\) DNA, with a 312-bp homologous core, was used (Fig. 1A). The E. coli Holliday junction resolvase RusA efficiently resolves this structure generating four nicked duplex DNA species (Fig. 1B, lane f). In comparison Mus81-Eme1 resolves \(\chi\) DNA relatively inefficiently converting less than 5% of the available substrate into duplex products at the highest concentration of enzyme used (Fig. 1B, lane d).

Mus81-Eme1 Cleaves a Replication Fork Substrate in the Leading Strand Template—To directly compare Mus81-Eme1’s ability to cleave Holliday junctions versus replication forks, \(\chi_{Kpn}\) and \(\chi_{Sma}\) substrates were generated by restriction of \(\chi\) DNA in a common duplex arm at \((\chi_{Sma})\) or close \((\chi_{Kpn})\) to the boundary with the homologous core sequence (Fig. 2, A and B). \(\chi_{Sma}\) can either resemble a Holliday junction or a replication fork depending on where its junction point branch migrates. In contrast \(\chi_{Kpn}\) retains a small heterologous region at the end of the cleaved arm, which obstructs formation of a replication fork substrate. Like \(\chi\) DNA, \(\chi_{Kpn}\) is cleaved inefficiently by Mus81-Eme1 (Fig. 2C, lanes b–d). In contrast Mus81-Eme1 cleaves \(\chi_{Sma}\) relatively well, generating 3.2- and 0.95-kb duplex products (Fig. 2C, lanes g–i). The prevalence of these cleavage products and relative absence of 2.4- and 1.75-kb duplex bands indicates that Mus81-Eme1 has a strong bias toward cleaving \(\chi_{Sma}\) in what would be the leading strand template if this were a true replication fork (Fig. 2D). To see if this cleavage bias is a conserved feature of homologues of Mus81-Eme1, we purified S. cerevisiae Mus81-Mms4 and tested it on both \(\chi_{Kpn}\) and \(\chi_{Sma}\) (Fig. 2E). Mus81-Mms4, like Mus81-Eme1, cleaves \(\chi_{Kpn}\) relatively poorly compared with \(\chi_{Sma}\) (compare lanes c and f) and generates almost exclusively 3.2- and 0.95-kb duplex products from its cleavage of \(\chi_{Sma}\) (lane f).

Mus81-Eme1 Cleaves Model Replication Forks 3′–6′ Bases 5′ of the Junction Point—To more precisely determine the sites at which Mus81-Eme1 and Mus81-Mms4 cleave fork substrates, a synthetic replication fork called F1, made from short (≈50 nucleotides) oligonucleotides, was used (Fig. 3D). Four preparations of F1 were made, each labeled in a different oligonucleotide, and tested for cleavage by Mus81-Eme1 and Mus81-Mms4 (Fig. 3A). Like \(\chi_{Sma}\) F1 is cleaved by both Mus81-Eme1 and Mus81-Mms4 in the equivalent of the leading strand template to detach one of its duplex arms. Analysis of the reaction products on a denaturing gel shows that both enzymes cut at

and 20 nM. D, schematic diagram of \(\chi_{Sma}\) showing the approximate position of the Mus81-Eme1 cleavage site. E, agarose gels showing the comparison between Mus81-Eme1 and Mus81-Mms4 for cleavage of \(\chi_{Kpn}\) and \(\chi_{Sma}\). Amounts of protein in lanes b, c, e, and f are 30 nM.
FIG. 3. Cleavage of F1 and F2 by Mus81-Eme1 and Mus81-Mms4. A, native polyacrylamide gel showing the cleavage of F1 by Mus81-Eme1 (Sp) and Mus81-Mms4 (Sc). The position of the 5' label (*) is shown in the schematics of F1. B, as for A except the substrate is F2. C, denaturing gel of some of the reactions shown in A and B run beside G + A sequencing ladders of the appropriate labeled oligonucleotide (lane a, oligonucleotide 2; lane b, oligonucleotide 8; lane l, oligonucleotide 5). D, schematic of the central region of F1 with arrows showing the position of the major Mus81-Eme1/Mms4 cleavage sites. Sp- and Sc-labeled arrows indicate cleavage sites that are predominantly used either by S. pombe Mus81-Eme1 or S. cerevisiae Mus81-Mms4 respectively. E, same as D except that the junction is F2.
the same sites (Fig. 3C, lanes c and d), which map mainly to positions between 3 and 6 bp 5′ of the junction point in oligonucleotide 2 (Fig. 3D). More minor cut sites are also detected up to 11 bp 5′ of the junction point in the same oligonucleotide (Fig. 3C, lanes c and d and data not shown). Slight differences in site preference between Mus81-Eme1 and Mus81-Mms4 are apparent, as well as some minor cut sites that appear to be exclusive to one or other of the enzymes (e.g. Fig. 3C, lanes n and o). To confirm that the cleavage bias observed using F1 is not dictated by the nucleotide sequence we constructed a further fork substrate called F2 (Fig. 3E). F2 consists of the same nucleotide sequences as F1 but differs by the strand that is discontinuous at the junction point and therefore which strand is assigned as the leading strand template. F2, like F1, is cleaved by both Mus81-Eme1 and Mus81-Mms4 in the leading strand template, the major cut sites being between 3 and 6 bp 5′ of the junction point in oligonucleotide 8 (Fig. 3B, C and E).

From these data we conclude that Mus81-Eme1 and Mus81-Mms4 cleave replication fork substrates in a specific manner that is directed by the structure of the substrate and seemingly independent of its nucleotide sequence.

Cleaving Substrates That Resemble Different Types of Stalled and Reversed Replication Fork—It is hypothesized that a key role for Mus81-Eme1 and Mus81-Mms4 is to cleave stalled and reversed replication forks and thereby facilitate the break-induced restart of replication (18, 19, 26). Blocked replication forks can in principle form a number of different structures depending on which template strand is blocked, whether the lagging strand polymerase can uncouple from the leading strand polymerase, and whether the fork reverses to allow the re-pairing of template strands. The occurrence of these aberrant structures in vivo has recently been confirmed by electron
microscopy (30). To test Mus81-Eme1 and Mus81-Mms4 on structures that resemble different types of stalled/reversed replication fork, substrates F3 to F6 were made (Fig. 4A). F3 and F4 resemble replication forks that have reversed to expose either a 3' (leading nascent strand) or 5' (lagging nascent strand) single-stranded tail respectively. F6 resembles a fork in

Fig. 5. Mapping the cleavage sites in F3, F4, and F6. A, denaturing gel analysis of Mus81-Mms4 cleavage of F3, F4, and F6 labeled in either oligonucleotide 2 (lanes b–g) or 5 (lanes i–n). G + A sequencing ladders of oligonucleotide 2 and 5 are in lanes a and h, respectively. B–D, schematics of the central regions of F3, F4, and F6. Arrows indicate the major cleavage sites for Mus81-Eme1 and Mus81-Mms4.
which the lagging strand polymerase continued synthesis despite the leading strand being blocked. Finally, F5 resembles a normal replication fork since, unlike the other substrates (including F1 and F2), it does not contain an equivalent of the nascent lagging strand juxtaposed at the junction point. Substrates F3, F4, and F6 are cleaved at least as well as F1 by both Mus81-Eme1 and Mus81-Mms4 (Fig. 4A and data not shown). In contrast little or no cleavage of F5 was detected (Fig. 4A, lanes n–p and data not shown). A comparison of the rates of cleavage of these substrates shows that F3 and F6 are cleaved at least 2-fold faster than either F1 or F4 (Fig. 4B).

Mapping Cleavage Sites in F3, F4, and F6—The same strategy for mapping the cleavage sites in F1 and F2 was employed for determining the cleavage sites in F3, F4, and F6. Like F1, both F3 and F6 are cleaved predominantly in oligonucleotide 2 at positions 3–6 bp 5’ of the junction point (Fig. 5A, lanes c and g, B, and C and data not shown). However, little or no cleavage in this strand is detected for F4 (Fig. 5A, lane e and data not shown). Instead F4 is cleaved predominantly in oligonucleotide 5 4 bp 5’ of the junction point, which in this substrate is the equivalent of the lagging strand template (Fig. 5A, lane l, D and data not shown).

**DISCUSSION**

Mus81-Eme1 and its homologue from *S. cerevisiae* Mus81-Mms4 have both been implicated in cleaving DNA junctions at stalled replication forks in order to facilitate some kind of break-induced replication (BIR)\(^1\) (18, 19, 26). Here we have investigated the ability of recombinant SpMus81-Eme1 and ScMus81-Mms4 to cleave various different DNA junctions that resemble those that are expected to form at stalled replication forks following complete or partial disassembly of the replisome. We confirm that these recombinant enzymes cleave Holliday junctions relatively poorly compared with three- or four-way DNA junctions that already contain some kind of strand discontinuity at the junction point. Furthermore, our side-by-side comparison of Mus81-Eme1 and Mus81-Mms4 shows that these enzymes have the same substrate specificity. They also cut at the same sites, albeit their preferred cleavage sites are in some cases slightly different. These data have enabled us to speculate on the mode of action of Mus81-Eme1/Mms4 in *vivo* (see below).

The Potential of Mus81-Eme1 and Mus81-Mms4 to Cleave Different Types of Stalled and Reversed Replication Forks—The stalling of replication forks by depletion of dNTPs or blockage in the DNA template appears to be a frequent event. What happens to the replisome when it stalls is largely unknown. In many cases its integrity and potential for further replication appears to be maintained by cell cycle checkpoint kinases such as Rad53 in *S. cerevisiae* (31, 32). In the absence of RAD53 stalled forks are converted into a range of abnormal structures, including junctions with extensive single-stranded gaps, reversed forks with extruded single-stranded tails, and reversed forks where the two nascent strands have annealed to form a Holliday junction (30). However, under certain circumstances, for example replication fork blockage by a bulky lesion in the DNA, fork reversal may be actively encouraged to provide room for DNA repair and/or a template for replicative bypass of the lesion (10). Subsequently the DNA junction formed by fork

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\(^1\)The abbreviation used is: BIR, break-induced replication.
reversal would have to be removed and a means found to restart replication. Our in vitro analysis of Mus81-Eme1 and Mus81-Mms4 substrate specificity reveals how these enzymes are well suited for processing most of the abnormal structures that can form when the replisome disassembles (Fig. 6). Reassuringly neither Mus81-Eme1 nor Mus81-Mms4 can efficiently cleave a normal replication fork structure (Fig. 6A). A blockage in the leading strand can result in the uncoupling of the two polymerases (33) to generate the structure shown in Fig. 6B, i. This can be cleaved by Mus81-Eme1/Mms4 resulting in the detachment of the leading strand arm however, here cleavage would occur in the leading strand template causing the detachment of the lagging strand arm. Mus81-Eme1/Mms4 would also be able to cleave a reversed fork with the 3′-end of the nascent leading strand exposed; however, here cleavage would occur in the leading strand template resulting in the detachment of the leading strand arm (Fig. 6C, ii-iii). However, if the fork reverses to the point at which a Holliday junction is formed then Mus81-Eme1/Mms4 may either act inefficiently or not at all (Fig. 6B, u and C, iv). This suggests that there may be a limited window of opportunity for Mus81-Eme1/Mms4 to cleave a replication fork undergoing reversal. Of course even if a Holliday junction is formed its branch migration could regenerate the substrates that are cleaved well by Mus81-Eme1/Mms4.

Why Cleave Stalled Replication Forks?—Breaking a replication fork, as envisaged in Fig. 6, B and C, is potentially hazardous to the cell in at least two ways: (i) a single non-repaired double-strand break can be lethal, and (ii) recombination, which is necessary to repair the break, can be prone to generating genomic rearrangements (34). This may be why enzymes like Rhq1 are so important for genomic stability with their potential for resetting reversed replication forks and thereby avoiding fork cleavage (11, 12, 14). Nevertheless, it is clear that in both prokaryotes and eukaryotes recombination plays an important role in restarting replication at non-origin sites (16, 17, 35). In E. coli two models have been invoked to explain how fork reversal, and the formation of a Holliday junction, can be used to load recombinases at the stalled fork. The first model says that the Holliday junction formed when the fork reverses is cleaved by RuvABC to generate a double-stranded end. This end then acts as a substrate for RecBCD, which generates a 3′-single-stranded tail for ReaC to load onto (6, 17, 35). Homologous pairing and strand invasion then ensues to make a D-loop in which the invading 3′-end primes leading strand synthesis, and where the PriA protein can bind and orchestrate primosome assembly for lagging strand synthesis (37). The second model avoids the formation of a single-end break by RecBCD processing the free DNA end formed by the reversal of the fork. Subsequent invasion of the re-annealed parental duplex can then occur before cleavage of the Holliday junction by RuvABC (6, 17, 35). In eukaryotes, which of these pathways is used may depend largely on the cell type (37). The second model would be exposed to generate the structure shown in Fig. 6B, iii. Mus81-Eme1/Mms4 would be able to cleave this structure but incisions would be redirected to the lagging strand template causing the detachment of the lagging strand arm. Mus81-Eme1/Mms4 would also be able to cleave a reversed fork with the 3′-end of the nascent leading strand exposed; however, here cleavage would occur in the leading strand template resulting in the detachment of the leading strand arm (Fig. 6C, ii-iii).

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