Identification and Characterization of the Human Mus81-Eme1 Endonuclease*

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Alberto Ciccia, Angelos Constantinou‡, and Stephen C. West§

From Cancer Research UK, London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, United Kingdom

The faithful and complete replication of DNA is necessary for the maintenance of genome stability. It is known, however, that replication forks stall at lesions in the DNA template and need to be processed so that replication restart can occur. In fission yeast, the Mus81-Eme1 endonuclease complex (Mus81-Mms4 in *Saccharomyces cerevisiae*) has been implicated in the processing of aberrant replication intermediates. In this report, we identify the human homolog of the *Schizosaccharomyces pombe* EME1 gene and have purified the human Mus81-Eme1 heterodimer. We show that Mus81-Eme1 is an endonuclease that exhibits a high specificity for synthetic replication fork structures and 3′-flaps in vitro. The nuclease cleaves Holliday junctions inefficiently (~75-fold less than flap or fork structures), although cleavage can be increased ~6-fold by the presence of homologous sequences previously shown to permit base pair "breathing." We conclude that human Mus81-Eme1 is a flap/fork endonuclease that is likely to play a role in the processing of stalled replication fork intermediates.

One of the greatest challenges that the cell has to face is to maintain genome integrity. A high level of fidelity during DNA replication is essential to achieve the accurate transmission of the genome from one generation to the next. To completely and correctly replicate the genome, replication needs to restart each time it encounters a block to replication fork progression. Such blocks could occur at nucleotide lesions (e.g. cyclobutane pyrimidine dimers), DNA backbone gaps, regions of secondary DNA structure (e.g. triplet repeats), or protein-DNA complexes (e.g. RNA polymerases, DNA topoisomerases) (1). In recent years, it has become increasingly evident that there is a connection between DNA replication and recombination that enables the replication fork to overcome such problems and facilitate faithful replication of the genome. Stalled replication forks can be repaired through a non-recombinogenic pathway (fork regression) or through a recombinogenic pathway in which the stalled replication intermediates are processed by endonucleolytic cleavage to generate a double-strand break that can be repaired by homologous recombination (1–9).

RecQ family helicases have been implicated in the non-recombinogenic pathway of replication fork restart. This family of proteins includes Sgs1 in *Saccharomyces cerevisiae*, Rqh1 in *Schizosaccharomyces pombe*, and Bloom’s, Werner’s, and Rothmund-Thomson’s helicases in humans (4). That these proteins can suppress recombination is indicated by the hyper-recombination phenotype observed in sgs1 and rqh1 mutants and cell lines derived from individuals with Bloom’s syndrome (5–8).

The observation of a synthetic lethality between Sgs1/Rqh1 helicase and the Mus81 endonuclease in yeast (9, 10) has raised the possibility that Mus81 is active in the recombinogenic pathway of replication restart. Consistent with this proposal, mus81 mutants in *S. cerevisiae* are sensitive to agents that stall replication forks (e.g. methyl methane sulfonate or UV) but not to agents such as x-rays that cause double-strand breaks (11).

Mus81 is a component of an endonuclease that exhibits sequence similarity to *S. cerevisiae* Rad1 (XPF in humans), which forms a stable heterodimer with Rad10 (ERCC1 in humans) (9, 11). Rad1/10 and ERCC1/XPF are involved in nucleotide excision repair where they promote incision close to a DNA lesion, as the DNA is opened by helicase activities (12–15).

In *S. pombe*, Mus81 requires a partner protein, Emel, for activity (16). Cells defective in *MUS81* or *EME1* have been shown to exhibit meiotic defects consistent with problems with the processing of recombination intermediates; this phenotype can be at least partially rescued by expression of the Rusa Holliday junction resolvase (16). These studies raised the possibility that Mus81-Eme1 possessed Holliday junction resolvas activity, a proposal that was supported by in vitro studies with Mus81-Eme1 protein. However, the ability of Mus81-Eme1 to cleave Holliday junctions is considerably lower than that observed with other DNA substrates such as 3′-flaps and replication fork structures (16–18).

In *S. cerevisiae*, Mus81 interacts with Mms4, a distant homolog of Emel (19). Budding yeast defective in *MUS81* or *MMS4* exhibit a mild meiotic defect (20), although recombinant Mus81-Mms4 protein shows the same specificity for forks and flaps as the *S. pombe* Mus81-Eme1 complex (18, 19).

In humans, immunoprecipitates of Mus81 complex derived from HeLa cells were shown to cleave Holliday junctions (21), raising the possibility that Mus81 endonuclease was a component of a Holliday junction-branch migration complex described previously (22). However, when HeLa cell-free extracts were extensively fractionated it was found that the Mus81 endonuclease separated from the Holliday junction resolvase (now designated Resolvase A) and that the two junction endonucleases were distinct entities (23). Moreover, whereas Mus81 exhibited a broad range of specificity, Resolvase A was found to be specific for Holliday junctions and exhibited a cleavage mechanism that was very similar to that observed with the *Escherichia coli* Holliday junction resolvase RuvC.

In the work described here, we report the identification of a human homolog of Emel and show that it forms a stable complex with human Mus81 protein. A second, considerably

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† Present address: Institute de Biochimie, Universite de Lausanne, Switzerland.

§ To whom correspondence should be addressed. Tel.: 44-20-7269-3868; Fax: 44-20-7269-3811; E-mail: stephen.west@cancer.org.uk.

From Cancer Research UK, London Research Institute, Clare Hall Laboratories, South Mimms, Hertfordshire EN6 3LD, United Kingdom

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smaller, potential partner (Eme2) was also identified by sequence similarity. We show that purified recombinant Mus81-Eme1 complex is an endonuclease and that the protein is specific for flap and replication fork structures. Mus81-Eme1 has a weak ability to cleave Holliday structures but does so only when the junction contains a migratable crossover that is known to cause base pair breaking. For these reasons, we suggest that the activities of human Mus81-Eme1 are likely to be targeted to a primary role in replication fork processing and repair.

EXPERIMENTAL PROCEDURES

Gene Cloning—The human EME1 (accession number BC016470) and EME2 (accession number XM_113869) genes were identified by PSI-BLAST homology searches using the S. pombe EME1 gene as bait (16). EME1 was PCR-amplified from the IMAGE clone 28599896 and inserted into the Gateway vector pDEST17 (Invitrogen). The resultant plasmid pDEST17-ntsEME1 was used for overexpression of Eme1 with a 6-histidine tag at its N terminus. Similarly, MUS81 was PCR-amplified from IMAGE clone 4135990 into pDEST15 (Invitrogen) and pDEST17 to produce fusion proteins with N-terminal GST- and His-tags, respectively. The resultant plasmids are designated pDEST15-ntsMUS81 and pDEST17-ntsMUS81.

To co-express ccrMsus81 and nseEme1, we constructed the bicistronic plasmid vector pGex-ccrMUS81/ntsEME1. To do this, we amplified MUS81 and cloned it into the EcoRI and XhoI sites of pGex-bicis-His (a gift from D. R. McDonald), followed by the insertion of EME1 into the NdI and HindIII sites.

To co-express Mus81 and nseEme1, we inserted an adaptor (5’-CATGGATTACCATGAGGTGTTACACCGTC-3’) into the NcoI and XhoI sites of pET21d. An EcoRI-HindIII fragment from pGex-ccrMUS81/ntsEME1 encoding MUS81/ntsEME1 was then inserted into modified pET21d to produce pET21d-MUS81/ntsEME1. The inserts present in pDEST15-ntsMUS81, pDEST17-ntsMUS81, pDEST15-ntsEME1, pGex-ccrMUS81/ntsEME1 were all verified by DNA sequencing.

Protein Purification—pDEST15-ntsMUS81, pDEST17-ntsMUS81, pDEST15-ntsEME1, pGex-ccrMUS81/ntsEME1 were resuspended in 100 ml of phosphate buffer containing 0.5 M NaCl and 1 mM dithiothreitol and lysed using a French press. Following centrifugation, the supernatant was loaded on a 5-mL phosphocellulose column and eluted using 100 ml of 0.5–1.5 M NaCl gradient in the same buffer. Peak fractions were supplemented with 1.5 ml of Talon beads and incubated for 2 h at 4 °C. The beads were then washed with 10 ml of buffer containing 50 mM imidazole and eluted with 4 × 1.5 ml of buffer containing 0.5 M imidazole. Proteins were identified by SDS-PAGE, pooled and dialyzed for 2 h against 4 liters of storage buffer, and frozen in 25-μl aliquots at −80 °C. The final yield was 260 μg of ccrMus81 and 105 μg of ccrMus81-ntsEme1.

To produce the Mus81-ntsEme1 heterodimer, 4 liters of cells carrying pET21d-MUS81/ntsEME1 were resuspended in 100 ml of phosphate buffer containing 0.5 M NaCl and lysed using a French press. After centrifugation, the supernatant was loaded on a 10-mL phosphocellulose column and eluted using 100 ml of 0.5–1.5 M NaCl gradient in the same buffer. Peak fractions were supplemented with 1.5 ml of Talon beads and incubated for 2 h at 4 °C. The beads were then washed with 10 ml of buffer containing 50 mM imidazole and eluted with 4 × 1.5 ml of buffer containing 0.5 M imidazole. Proteins were identified by SDS-PAGE, pooled and dialyzed for 2 h against 4 liters of storage buffer, and frozen in 25-μl aliquots at −80 °C. The final yield was 215 μg.

Coprecipitation Assays—A cell-free extract was prepared from an IPTG-induced culture (1 liter) of bacteria carrying pGex-ccrMUS81/ntsEME1. The extract (50 ml) in phosphate buffer containing 0.5 M NaCl was incubated with 0.5 ml of beads (either GST-Septoharose or Talon) for 2 h at 4 °C and eluted with 4 × 0.5 ml of the same buffer supplemented with 20 mM glutathione or 0.5 M imidazole, respectively.

DNA Substrates—All substrates were prepared by annealing appropriate combinations of oligonucleotides and purified by gel electrophoresis. Junction X26 (23), which was 32P-labeled in oligo 1, was composed of four oligonucleotides as follows:

**Oligo 1:** 5’-CCGCTACATCGTACCAATGGATTGTACACGGCATTTGCCCACCTGCAGGTTCACCC-3’
**Oligo 2:** 5’-TGGTTGAAACCTTGGATGTCGAGGAATGTTCTCAAGCAATCTACCGTATAGCAGTTCCC-3’
**Oligo 3:** 5’-GACCTGACGTATGCATAGATTTGAGTTGACGAGCATCCTTGTTGCCCATCAGCTGTTCTACCC-3’
**Oligo 4:** 5’-TGGTTGAAACCTTGGATGTCGAGGAATGTTCTCAAGCAATCTACCGTATAGCAGTTCCC-3’

Similarly, junction X0 (24) was prepared by annealing following four oligonucleotides:

**Oligo 1:** 5’-GACCTGACGTATGCATAGATTTGAGTTGACGAGCATCCTTGTTGCCCATCAGCTGTTCTACCC-3’
**Oligo 2:** 5’-GACCTGACGTATGCATAGATTTGAGTTGACGAGCATCCTTGTTGCCCATCAGCTGTTCTACCC-3’
**Oligo 3:** 5’-GACCTGACGTATGCATAGATTTGAGTTGACGAGCATCCTTGTTGCCCATCAGCTGTTCTACCC-3’
**Oligo 4:** 5’-GACCTGACGTATGCATAGATTTGAGTTGACGAGCATCCTTGTTGCCCATCAGCTGTTCTACCC-3’

The splayed arm substrate was derived from X0 and generated by annealing oligo 1 with oligo 4. The 3’-flap structure contained oligo 1 and oligo 4 3’term (5’-CATGGACGTCGTCTAGAGCTGTCGACCGTCTACCC-3’). The replication fork substrate contained oligo 1, oligo 4, oligo 3’term, and oligo 2’term (5’-TGGTTGAAACCTTGGATGTCGAGGAATGTTCTCAAGCAATCTACCGTATAGCAGTTCCC-3’). To facilitate comparisons, the splayed arm substrate, the 3’-flap structure, the replication fork, and junction X0 were all labeled in oligonucleotide which is common to each.

**Cleavage Assays—**Reactions (20 μl) contained 23P-labeled substrate (~1 ng) in 60 mM sodium phosphate, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. Following incubation for 30 min at 37 °C, the cleavage reactions were stopped and samples were deproteinized by a 15-min incubation in the presence of 2 mg/ml proteinase K and 0.4% SDS. Labeled DNA products were analyzed by electrophoresis through 10% neutral polyacrylamide gels followed by autoradiography.

**RESULTS**

**Identification of Human Homologs of S. pombe Eme1—**To identify a human homolog of Eme1, a PSI-BLAST search of the NCBI data base was performed using S. pombe Eme1 as bait. Similar attempts to identify human homologs of S. cerevisiae Mms4 were unsuccessful. However, among the sequences similar to S. pombe Eme1, we observed S. cerevisiae Mms4 and a Neospora crassa Eme1 homolog (accession number AL536173). Additionally, two human proteins were identified, which we here designate Eme1 (accession number BC016470) and Eme2 (accession number XM_113869). Both are weak homologs, because human Eme1 exhibits just 17% identity and 26% similarity to S. pombe Eme1 and Eme2 shows 15% identity and 29% similarity (Fig. 1).

Human Eme1 is a 583-amino acid protein with a molecular
mass of 65 kDa. The chromosomal localization of the EME1 gene is 17q21.3. In the NCBI data base there is a second version of the gene (accession number AK055926), which encodes a protein that is 13 amino acids shorter (residues 372 to 384 are missing). It is possible that these are two tissue-specific splicing variants, because the full-length Eme1 was identified from cDNA from choriocarcinoma cells, whereas the shorter variant was from a neuroblastoma. The EME2 gene, which localizes to 16p13.3, encodes a 245-amino acid protein with a molecular mass of 26 kDa. Eme2 shows 44% identity with Eme1 and aligns mainly with the C-terminal region of Eme1 (Fig. 1).

When human Eme1 and Eme2 were aligned with S. pombe Eme1, the C-terminal regions were the most highly conserved (Fig. 1). To determine whether the human Eme1 and Eme2 C-terminal sequences were conserved in other proteins, a PSI-BLAST search was initiated using the last 160 amino acids of Eme1 and Eme2. Remarkably, a close match was identified in the human Mus81 protein. The alignment of full-length Eme1 and Eme2 with Mus81 is shown in Fig. 2.

Previously, it was shown that S. pombe Mus81 and Eme1 interact via their C-terminal regions (16). The Mus81 C-terminal is also related to the C-terminal regions of the S. cerevisiae RAD1 and human XPF proteins, and it has been shown that these regions are necessary for interactions between RAD1 and RAD10 (25) and between XPF and ERCC1 (26). From our sequence alignments, it appears likely that human Mus81 also interacts with its partner proteins via C-terminal contacts.

Interaction of Human Eme1 with Mus81—Because the human Eme1 protein (65 kDa) is similar in size to S. pombe Eme1 and S. cerevisiae Mms4, we tested it for specific interactions with human Mus81. For this purpose, a bicistronic vector for bacterial coexpression of GSTMus81 and HISEme1 was constructed. Following expression of the two proteins in E. coli, GST-Sepharose beads were used in pull-down assays carried out with cell-free extracts containing GSTMus81 and HISEme1. We observed that the GST beads pulled out both GSTMus81 and HISEme1 (Fig. 3, lane c), as determined by SDS-PAGE in comparison with purified GSTMus81-HISEme1 complex (lane g). Similarly, incubation of histidine-binding Talon beads with the extract led to the co-precipitation of both GSTMus81 and HISEme1 (lane d). As expected, extracts prepared from E. coli expressing only one of the two proteins (either GSTMus81 or HISEme1) showed only single bands in the respective pull-
Furthermore, interactions between Mus81 and Eme1 were verified in Talon pull-downs from extracts prepared from *E. coli* carrying a bicistronic vector containing untagged Mus81 and HISeme1 (data not shown). We conclude that the gene identified here as human *EME1* encodes the mammalian homolog of *S. pombe* Eme1 and that human Eme1 makes a stable complex with Mus81 protein.

When similar studies were carried out with Eme2, we observed only a weak affinity for Mus81 (data not shown). Moreover, preliminary attempts to purify the Mus81-Eme2 complex were unsuccessful because the two proteins failed to form a stable complex. For these reasons, our subsequent efforts were focused on the Mus81-Eme1 complex.

**Activities of the Mus81-HISeme1 Complex** — The Mus81-HISeme1 complex was purified from *E. coli* carrying the bicistronic vector pET21d-MUS81/HISME1. Following induction with IPTG, a high level of expression of Mus81 and HISeme1 was observed (Fig. 4, lane c), but most of the overexpressed protein was found to be insoluble and precipitated during high-speed centrifugation. However, the remaining Mus81-HISeme1 present in the cleared supernatant (lane d) was purified by phosphocellulose chromatography (lane e), followed by Talon binding (lane f). The identity of Mus81 and Eme1 was confirmed by mass spectrometry.

To determine the substrate specificities of the Mus81-HISeme1 complex, a series of branched DNA substrates was generated by annealing partially complementary oligonucleotides. These included splayed arm, 3' flaps, replication fork, and Holliday junction structures (Fig. 5), each of which was 5' 32P-end labeled in the strand common to all substrates. Using equal amounts of protein, we observed that the 3' flap (lane j) and the replication fork (lane o) substrates were cut by Mus81-HISeme1. In contrast, very little cleavage was observed with untagged Mus81 and HISeme1 (data not shown). We conclude that the gene identified here as human *EME1* encodes the mammalian homolog of *S. pombe* Eme1 and that human Eme1 makes a stable complex with Mus81 protein.

**Fig. 2.** Sequence alignment between human Eme1 (HsEme1), human Mus81 (HsMus81), and human Eme2 (HsEme2). Identical and similar residues are indicated in dark and light blue, respectively. Sequence alignments were carried out using ClustalW.

**Fig. 3.** Specific interaction of human Mus81 with Eme1. Cell-free extracts were prepared from *E. coli* carrying the bicistronic vector pGex-csrMUS81/hsEME1 (lane b), and pull-down assays were carried out using GST-Sepharose (lane c) or Talon (lane d) beads. Marker proteins were purified csrMus81 (lane e), HISeme1 (lane f), and csrMus81-hsEme1 (lane g). Pull-down complexes and purified protein markers were separated by 10% SDS-PAGE, and the gel was stained with Coomassie Blue.

downs (lanes e and f). Furthermore, interactions between Mus81 and Eme1 were verified in Talon pull-downs from extracts prepared from *E. coli* carrying a bicistronic vector containing untagged Mus81 and HISeme1 (data not shown). We conclude that the gene identified here as human *EME1* encodes the mammalian homolog of *S. pombe* Eme1 and that human Eme1 makes a stable complex with Mus81 protein.

When similar studies were carried out with Eme2, we observed only a weak affinity for Mus81 (data not shown). Moreover, preliminary attempts to purify the Mus81-Eme2 complex were unsuccessful because the two proteins failed to form a stable complex. For these reasons, our subsequent efforts were focused on the Mus81-Eme1 complex.

**Activities of the Mus81-Eme1 Complex** — The Mus81-Eme1 complex was purified from *E. coli* carrying the bicistronic vector pET21d-MUS81/hisEME1. Following induction with IPTG, a high level of expression of Mus81 and hisEme1 was observed (Fig. 4, lane c), but most of the overexpressed protein was found to be insoluble and precipitated during high-speed centrifugation. However, the remaining Mus81-hisEme1 present in the cleared supernatant (lane d) was purified by phosphocellulose chromatography (lane e), followed by Talon binding (lane f). The identity of Mus81 and Eme1 was confirmed by mass spectrometry.

To determine the substrate specificities of the Mus81-hisEme1 complex, a series of branched DNA substrates was generated by annealing partially complementary oligonucleotides. These included splayed arm, 3'-flap, replication fork, and Holliday junction structures (Fig. 5), each of which was 5' 32P-end labeled in the strand common to all substrates. Using equal amounts of protein, we observed that the 3'-flap (lane j) and the replication fork (lane o) substrates were cut by Mus81-hisEme1. In contrast, very little cleavage was observed with
the splayed arm (lane e) or Holliday junction (lane t) substrates. Previously, we showed that a partially purified fraction from HeLa cells containing Mus81 could cut 3′-flap and replication fork structures (23). The purified Mus81-HISEme1 complex described here exhibited the same substrate specificity (compare lanes g with j, l with o, and q with t). Moreover, we found that the recombinant protein produced similar cleavage patterns as the HeLa fraction with the fork structure as determined by denaturing gel electrophoresis (data not shown). When the levels of cleavage of flap and fork structures by Mus81-HISEme1 were quantified by phosphorimaging, we observed that the flap and fork substrates were cut with a similar efficiency and that the specific activity of the nuclease with these substrates was 75× greater than that observed with the synthetic Holliday junction (Fig. 6).

Whereas purified Mus81-HISEme1 exhibited flap and fork endonuclease activity, neither HISEme1 (Fig. 5, lanes c, h, m, and r) nor HISMus81 (lanes d, i, n, and s) alone exhibited nuclease activity. We conclude that human Mus81-Eme1, like the yeast homologs, is functional as a heterodimer. However, at the present time, we have been unable to reconstitute the nuclease activity by mixing separately purified recombinant HISEme1 and HISMus81 subunits (data not shown).

The synthetic Holliday junction (X0) used in the above experiments contains an immobile crossover. We therefore compared the cleavage efficiency of Mus81-HISEme1 complex on X0 with a second Holliday junction containing a 26-bp homologous core (X26) through which the junction is freely able to branch migrate. We observed that X26 served as a better substrate for Mus81-HISEme1 and was cut with an efficiency that was 6-fold greater than X0 (Fig. 7, compare lanes b and c with g and h). Similar results were obtained with the partially purified Mus81 fraction from HeLa cell-free extracts (lanes d and i). In contrast to recombinant Mus81-HISEme1 or the HeLa Mus81 fraction, fractionated extracts from HeLa cells enriched for the human Holliday junction resolvase A (23) cleaved X0 and X26 equally and efficiently (lanes e and j).

Taken together, these results show that the endonuclease
activity of Mus81-Eme1 complex is specifically targeted to flap/fork structures and that the efficiency of cleavage of Holliday junctions can be enhanced by inclusion of homologous sequences that are known to result in breathing of the synthetic junction substrate (27).

**DISCUSSION**

In this paper, we have reported the cloning of the human *EME1* gene and show that Eme1 protein interacts with Mus81. Human *EME1* was identified in a data base search using *S. pombe* EME1, although a similar approach using *S. cerevisiae* MMS4 was unsuccessful. Although it has been shown that recombinant Mus81-Eme1 and Mus81-Mms4 complexes cleave the same substrates with similar efficiencies (18), Eme1 and Mms4 exhibit little (16%) sequence homology. A second potential Mus81 partner was also identified and named Eme2. This protein is 44% identical to Eme1 but, being less than half the length of Eme1, corresponds only to the Eme1 C terminus. Both Eme1 and Eme2 share sequence homology with the C-terminal portion of Mus81, and it is likely that this region is important for dimerization, as shown in *S. pombe* (16). Although our preliminary efforts to purify Mus81-Eme2 complex were unsuccessful, the possibility remains that human Mus81 may have more than one partner and raises questions relating to their functions in DNA metabolism. In future studies, it will be interesting to define whether Eme2 is a true interacting partner, to determine whether Mus81-Eme1 and Mus81-Eme2 exhibit related or different substrate specificities, and to determine whether there may be tissue-specific expression or induction following DNA damage or replicative stress.

**Activity of Mus81-Eme1 Complex**—Recombinant human Mus81-Eme1 complex, purified after overexpression in *E. coli*, was found to exhibit nuclease activities very similar to recombinant *S. pombe* Mus81-Eme1 and *S. cerevisiae* Mus81-Mms4 (17–19). Human Mus81-Eme1 preferentially cleaved flap and replication fork structures while exhibiting relatively little (~75-fold reduced) activity with Holliday junctions. However, we did find that junction X26, which contains a 26-bp region of homology through which the crossover can move, was cut with a greater efficiency than the static junction X0. Previously, using chemical probes that could detect the formation of transient regions of single-stranded DNA, we observed that mobile junctions exhibit a transient single-stranded character suggestive of base pair breathing (27). We therefore suggest that the ability of Mus81-Eme1 to cut four-way junctions is likely to be because of the recognition of transient flap structures that are formed as the junction undergoes spontaneous thermal denaturation. Consistent with this proposal, it was recently shown (23) that cleavage of Holliday junctions by Mus81 fractions prepared from HeLa extracts occurred at the 5’-side of the substrate and that the nicks were introduced without symmetry leading to the formation of non-ligatable products. These results contrasted with data obtained in similar experiments with Resolvase A prepared from the same HeLa extracts. We conclude that Holliday junction cleavage by Mus81-Eme1 is a secondary effect of its flap activity, rather than the sign of a bona fide Holliday junction resolvase.

**Possible In Vivo Mus81-Eme1 Functions**—The observation that recombinant Mus81-Eme1 complex exhibits the same nucleolytic activity on 3’-flap and replication fork structures as Mus81-containing fractions purified from HeLa cells provides further support for a role for Mus81-Eme1 in replication fork restart. The primary mechanism by which stalled forks are repaired is likely to involve fork regression such that the newly synthesized leading and lagging strands can anneal to form a fork that has a fourth arm, in a structure reminiscent of a chicken foot (28, 29). Fork regression can allow repair of the blocking lesion, such that the repaired fork can subsequently be restored by the actions of RecQ family helicases, such as Sgs1 and human Bloom’s and Werner’s helicases. These proteins have been shown to exhibit branch migration activity *in vitro* (30–32).

An alternative pathway for fork restoration may involve Mus81-Eme1-mediated nucleolytic cleavage of the stalled fork or the structure formed by fork regression. The resulting double-strand break would be expected to initiate recombinational repair reactions that would give rise to a new replication fork. Recently, electron microscopic studies of stalled replication forks in yeast showed that some regressed forks exhibited a single-stranded reverse arm structure (28). Because *S. cerevisiae* Mus81-Mms4 and *S. pombe* Mus81-Eme1 cleave synthetic replication fork structures with single-stranded regressed tails (18), it is to be expected that similar cleavage reactions would take place *in vivo*. Additionally, Mus81 complex may be able to cleave the chicken foot intermediate, albeit with reduced efficiency. Finally, the stalled fork itself would be expected to present an efficient substrate for Mus81 complex, because related structures are good *in vitro* substrates.

The possibility that Mus81 and RecQ family helicases provide alternative or complementary pathways for replication fork restart is supported by the finding that mus81 is synthetic lethal in combination with sgs1 or rgh1 in yeast (9, 10). Moreover, the observation that mus81 sgs1 lethality can be rescued by mutations in genes involved in homologous recombination (RAD51, RAD52, RAD54, RAD55, and RAD57) indicates a role for Mus81 that is dependent on the initiation of recombination (33). Considering the high specificity that Mus81 shows for 3’-flaps, it has been proposed that Mus81 could be involved in the cleavage of 3’-overhangs that protrude from duplex DNA after reinvision of the original strand in synthesis-dependent strand annealing recombination. This process could be initiated by single-stranded gaps that form at stalled replication forks (33).

The identification of the interaction between Mus81 and the forkhead-associated domain (FHA) of replication checkpoint kinase Rad53/Cds1/Chk2 both in yeast and in mammalian cells (9, 21, 24) points to a possible checkpoint regulation of Mus81 activity. Preliminary data suggest that Sgs1 also interacts with the FHA domain of *S. cerevisiae* Rad53 (35). Consistent with these results, Rad53 has been implicated in the stabilization of stalled replication forks in *S. cerevisiae* (36, 37). It is possible that Rad53, interacting with both Mus81 and Sgs1, could regulate the choice of pathway utilized for replication fork restart under particular stress conditions.

In summary, the biochemical data presented here support the notion that the primary cellular function of human Mus81-Eme1 is likely to be similar to that proposed to occur in yeast, in which aberrant replication fork intermediates serve as targets for the nuclease. Defects in this activity in mammalian cells are likely to result in genome instability and a predisposition to carcinogenesis.

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Alberto Ciccia, Angelos Constantinou and Stephen C. West

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