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Saccharomyces cerevisiae Mus81-Mms4 is a catalytic, DNA structure-selective endonuclease

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ABSTRACT
The DNA structure-selective endonuclease Mus81-Mms4/Eme1 is a context-specific recombination factor that supports DNA replication, but is not essential for DSB repair in Saccharomyces cerevisiae. We overexpressed Mus81-Mms4 in S. cerevisiae, purified the heterodimer to apparent homogeneity, and performed a classical enzymological characterization. Kinetic analysis (k_{cat}, K_M) demonstrated that Mus81-Mms4 is catalytically active and identified three substrate classes in vitro. Class I substrates reflect low K_M (3–7 nM) and high k_{cat} (~1 min^{-1}) and include the nicked Holliday junction, 3'-flapped and replication fork-like structures. Class II substrates share low K_M (1–6 nM) but low k_{cat} (~0.3 min^{-1}) relative to Class I substrates and include the D-loop and partial Holliday junction. The splayed Y junction defines a Class III substrate having high K_M (~30 nM) and low k_{cat} (0.26 min^{-1}). Holliday junctions assembled from oligonucleotides with or without a branch migratable core were negligibly cut in vitro. We found that Mus81 and Mms4 are phosphorylated constitutively and in the presence of the genotoxin MMS. The endogenous complex purified in either modification state is negligibly active on Holliday junctions. Hence, Holliday junction incision activity in vitro cannot be attributed to the Mus81-Mms4 heterodimer in isolation.

INTRODUCTION
Mus81-Mms4/Eme1 is a eukaryotic endonuclease that supports DNA replication fork recovery and functions in at least one subpathway of meiotic recombination (1–7). Support of DNA replication and the generation of pathological genetic changes by inappropriate applications of recombination during fork recovery may have important consequences to genetic stability (8). As an endonuclease that incises DNA joint molecules associated with replication fork recovery and recombination, Mus81-Mms4/Eme1 is likely a central factor in eukaryotic replication fork support (9–12). Its substrate(s) in vivo, however, are still uncertain. Biochemical studies in vitro demonstrate that the enzyme can cleave a number of DNA joint structures, but a quantitative enzymological analysis determining kinetic parameters for the native enzyme has been lacking.

DNA molecules exchange homologous sequence information during recombinational repair and damage tolerance mechanisms, and undertake structural interactions that require phosphodiester bond hydrolysis for separation. In the traditional double-strand break repair model, the interacting DNA molecules must be separated either by reversal of strand exchange (D-loop disruption), by dissolution (BLM-TOPOIIIα) or by endonucleolytic incision of the structure(s) joining the duplex molecules (4,13).

Prevailing models for DNA double-strand break repair have postulated that a single, symmetric intermediate DNA joint molecule, the Holliday junction, is the target for endonucleolytic separation of the joined duplex molecules (14–17). Support for the existence of this structure comes from physical analyses in bacteria and yeast and from the biochemical description of several endonucleases that deliver paired, symmetric incisions across a four-way branch point (18). Endonucleases that incise Holliday junctions have been characterized from bacteria (RuvC and RusA), archaea (Hjc and Hje) and bacteriophages (T4 endonuclease VII and T7 endonuclease I), but candidate nuclear enzymes have been absent from eukaryotic sources (4). To date, no sequence ortholog of any of the characterized resolvases has been recognized in eukaryotes, apart from the mitochondrial Cce1 that appears to be of bacterial origin (2–4,14).

In association with its interaction partner Mms4 in budding yeast and Emel in fission yeast and humans, Mus81 is a facultative associate of the RAD52 epistasis group and has been proposed to be a eukaryotic candidate for Holliday junction processing in vivo (3,4,19–24). Biochemical support for this idea is uneven.
Heterodimer preparations from partially purified, eukaryotic sources differ from those highly purified, recombinant sources in their ability to incise synthetic Holliday junctions \(2-4, 25\). Mus81-Eme1 partially purified from *Schizosaccharomyces pombe* by a tandem affinity purification protocol has shown a capacity to resolve model Holliday junctions to linear duplex products \(24\). *Schizosaccharomyces pombe* Mus81-Eme1 recovered in recombinant form from *Escherichia coli*, however, showed nearly no activity on Holliday junctions in *vitro* \(26, 27\). Recombinant *Saccharomyces cerevisiae* Mus81-Mms4 expressed and purified from *E. coli* was similarly inactive on the same Holliday junction substrates \(28\). In the case of human Mus81-Eme1, endogenous complex immunoprecipitated or fractionated from HeLa cells \(23, 29\) and recombinant complex immunoprecipitated from a eukaryotic expression source (insect SF9 cells) \(30\) has shown an incision capacity on model Holliday junctions, but no Holliday junction incision has been observed for recombinant human complex expressed in *E. coli* \(31, 32\).

What explains the difference between eukaryotic sources of Mus81-Mms4/Eme1 and recombinant sources in Holliday junction incision activity? One possibility has been the presence of post-translational modifications available in eukaryotic expression systems, but not in *E. coli*. Another concerns the association of an unidentified junction-targeting factor in partially pure eukaryotic enzyme preparations \(3\). Still, another possibility relates to the presence of factors in the partially purified preparations that alter junction presentation to Mus81-Mms4/Eme1 by nickase or helicase activity. Most recently, an explanation for the difference between recombinant and endogenous heterodimer abilities to cleave Holliday junctions was proposed by isolation of the first recombinant *S. pombe* and *S. cerevisiae* Mus81-Eme1/Mms4 fractions that exhibited Holliday junction cleavage in *vitro* \(20\). Gaskell *et al.* suggest that purification procedures that selected for the endonuclease complex in an oligomeric state greater than the single heterodimer conferred Holliday junction incision ability, in a manner responsive to metal ion concentration.

Because most recombinant preparations incise Holliday junctions poorly, a number of alternative potential physiological targets of Mus81-Mms4/Eme1 have been proposed, including a 3′-flapped structure, variants of a replication fork and the strand exchange intermediate, the displacement loop (D-loop). Whether partially purified from a eukaryotic source or purified from a recombinant source, all preparations of Mus81-Mms4/Eme1 are active on these substrates and it is only the Holliday junction activity that remains unclearly attributable to Mus81 \(20, 21, 23-29, 31, 32\). However, the lack of a quantitative comparison \(K_M, k_{cat}\) does not allow one to distinguish whether substrate recognition or catalysis drives the distinction.

One approach to identify whether eukaryotic-expressed Mus81-Mms4/Eme1 is sufficient for Holliday junction processing in *vitro* is to isolate the heterodimer to apparent homogeneity from its eukaryotic expression source. This should distinguish whether eukaryotic expression alone is sufficient to confer Holliday junction resolution in *vitro*, or whether a state of partial purity is necessarily correlated with Holliday junction incision. Furthermore, a strategy to more clearly define Mus81-Mms4 substrate selectivity in *vitro* entails the design of kinetic assay conditions that supply a direct quantitative comparison among joint molecule substrates. Classically, enzymological assays demand stringency in substrate processing by presenting a limiting quantity of enzyme with an excess of substrate \(33\). Effective substrate processing is therefore demonstrated as turnover by a catalytic enzyme preparation.

We performed a kinetic analysis of Mus81-Mms4 cleavage, and present a quantitative comparison of its substrate selectivity. Mus81-Mms4 purified from *S. cerevisiae* exhibits low \(K_M\) and correspondingly slow turnover on a number of substrates. The incisable joint molecules can be grouped into three classes by differences in \(K_M\) and \(k_{cat}\); differences among these substrates reveal the need for duplex DNA flanking the branch point for low \(K_M\). Furthermore, we find that Holliday junction incision is not an enzymatic property of endogenous *S. cerevisiae* Mus81 endonuclease in isolation, and not a property likely to be conferred by post-translational modification of the heterodimer in response to genotoxic challenge.

**MATERIAL AND METHODS**

*Cloning of MUS81 and MMS4 into GAL1/10 divergent promoter, 2μ-based overexpression vector*

The binary expression vector is based on pJN58, a 2μ shuttle vector bearing the bidirectional yeast promoter GAL1/10 \(34\). pWDH619 (pJN58 with His10 insertion) was generated by removing the NotI fragment of pWDH423, blunted and cloned into the XbaI site (blunted) of pJN58. MUS81 was PCR-amplified using Elongase® enzyme (Invitrogen™) from plasmid pWDH484 \(p_{GAL1-MUS81}\), a kind gift of S. Brill) using primers oIJWDH320 and oJWDH321 that introduce FLAG and TEV protease recognition sequences, engineered as a cassette flanked by MuI restriction sites. MMS4 was PCR-amplified from WDHY668 yeast genomic DNA using primers oJWDH314 and oJWDH315 that introduce cloning flanking XhoI and SphI restriction sites. The MUS81 fragment was cloned into the MuI site of pWDH619 (in frame with a His10 sequence) to generate pWDH592, and the MMS4 fragment was cloned into the XhoI/SphI backbone of pWDH597 (in frame with GST and a PreScission™ protease recognition sequence) to generate pWDH620. The EcoNI/SphI fragment of pWDH592 was cloned into the EcoNI/SphI backbone of pWDH620 to generate the double-expression vector, pWDH595. Construction was independently verified by PsiI/BamHI and EcoRI/EcoNI double digestions. The GST-MMS4/His10-FLAG-mus81-dd overexpression vector (pWDH596) was generated by mutagenic PCR of pWDH592 using primers oJWDH374 and oJWDH375, to generate pWDH593. The EcoNI/SphI fragment of pWDH595 was cloned into the corresponding sites of pWDH593 to generate the double-expression vector.
Mutation of MUS81 at the XPF-family conserved D414A/D415A sites introduced a diagnostic NheI restriction site, the second such site for the plasmid. Sequencing of all clones confirmed their nucleotide sequences as published in the Saccharomyces Genome Database (SGD) or the engineered MUS81 active site mutations. Primer sequences are available upon request.

**Confirmation of fusion protein function by complementation of genotoxin sensitivity of a mus81-A mms4-A double mutant strain**

Complementation analysis was performed by drop dilution assays on solid YPD or YPG agarose with genotoxin where appropriate. WDHY1636 (W303 MATa ade2-1 ura3-1 his3-11,15 trpl-1 leu2-3,112 can1-100 RAD5) is the wild-type strain and WDHY2129 is the isogenic mms4::KANMX mus81::KANMX double mutant strain. mus81 mms4 cells were transformed with the overexpression vector pWHD619 lacking the MUS81 and MMS4 open reading frames, with pWHD595 expressing N-terminal fusion alleles of MUS81 and MMS4, or with pWDH596 expressing fusion alleles of mus81-dld and MMS4. Cells were spotted at 5-fold dilutions starting at 4 x 10^6 cells; plates were incubated at 30°C and photographed daily up to 3 days.

**Purification of GST-Mms4/His10-FLAG-Mus81 by sequential affinity chromatography**

A diploid protease-deficient expression strain, WDHY668 (MATa ura3-52 trp1 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL/MATa ura3-52 trp1 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 GAL), was transformed with pWHD595 or pWHD596 using a standard lithium acetate transformation protocol and expression was induced for 8 h at 25°C as described in Solinger et al. (35). All purification procedures were performed at 4°C. A total of 120 g of S. cerevisiae cells were thawed in PBS (phosphate-buffered saline: 137 mM NaCl/2.7 mM KCl/10 mM Na2HPO4/1.8 mM KH2PO4) adjusted to 500 mM NaCl, pH 7.5. Cells were mechanically disrupted in a bead chamber with 350 g small glass beads, for 30 s intervals separated by 2 min on ice. Lysate was cleared of cellular debris by centrifugation at 45,000 rpm for 45 min in a Beckman Optima™ LE-80K preparative ultracentrifuge. Solid ammonium sulfate (ICN Biomedicals, Inc.) was slowly added to 25% with constant stir. Precipitate was removed by centrifugation in a Beckman R2-MC centrifuge, 30 min at 15,000 rpm. The supernatant was then loaded onto an approximately 4 ml-bed volume glutathione-sepharose™ 4B resin (Amersham Biosciences) at 0.3 ml/min. flow rate. After washing the resin with PBS/500 mM NaCl, pH 7.5, GST-tagged material was eluted by competition with 20 mM l-glutathione (Sigma) in PBS/500 mM NaCl, pH 7.5. Fractions were analyzed for protein content by 10% SDS-PAGE and Coomassie Brilliant Blue staining. To remove free glutathione, pooled fractions were dialyzed against 11 PBS/500 mM NaCl with constant stirring for 1–2 h, then dialyzed against an additional 11 volume for 1 h. A 2 ml-bed volume Ni2+-NTA resin (Qiagen) was equilibrated with 10 mM imidazole (Fisher Scientific) in PBS/500 mM NaCl, pH 7.5. The dialyzed material was adjusted to 10 mM imidazole and loaded at 0.3 ml/min flow rate. The resin was washed with increasing imidazole stringency to 50 mM imidazole in PBS/500 mM NaCl, pH 7.5. Bound complex was eluted by competition with 250 mM imidazole, pH 7.0. Pooled fractions were dialyzed against two 11-volumes of storage buffer: 20 mM Tris–HCl, pH 7.5/500 mM NaCl/10% glycerol/1 mM EDTA/0.2 mM PMSF/0.1 mM DTT, 1–2 h. His10-FLAG-Mus81/GST-Mms4 heterodimer was concentrated by centrifugation at 1500 g in a Millipore Ultrafree-15 centrifugal filter device pre-rinsed with 10 ml storage buffer, 10 min at 1500 g. Concentrated protein was stored in 10 μl aliquots and flash-frozen in liquid N2 for long-term storage at −80°C. Protein concentration was determined by direct A280 reading using the calculated heterodimer extinction coefficient εM at 280 nm = 147.840 cm⁻¹ M⁻¹. Predicted Mr for GST-Mms4 is 106.4 kDa, and for His10-FLAG-Mus81 is 73.6 kDa.

**Isolation of heterodimer from MMS- and HU-stressed cells**

Heterodimer was purified essentially as described for the wild-type and mutant heterodimer, except with the following modifications. At 6 h during protein overexpression, MMS (Sigma-Aldrich) was added to 0.1% or HU (US Biologicals) was added to 100 mM and cells were grown 2 h before harvest. During extraction and throughput purification, 0.1 mM NaVO₃ (Acros Organics) was added to buffers until dialysis prior to the Ni²⁺-NTA resin [see Bashkirov et al. (36)].

**Preparation of DNA oligonucleotide joint molecules for kinetic analysis**

Oligonucleotides (Qiagen Operon) were diluted to ~100 pmol/μl. Non-radiolabeled oligo structures were prepared to define joint molecule concentration in nuclease assays; radiolabeled oligo structures were prepared in parallel to ‘spike’ reactions for nuclease activity detection. Radiolabeling was performed using T4 PNK (New England BioLabs) and ³²P-γ-ATP (PerkinElmer). Oligonucleotides were annealed in an MJ Research PTC-200 Peltier thermal cycler under a staged temperature decline program: 95°C/3 min, 65°C/10 min, 37°C/10 min, 25°C/10 min, 0°C/10 min. For non-radiolabeled structures, 600 pmol 50-mer and 1200 pmol 25-mers were annealed in oligo annealing buffer (150 mM NaCl/15 mM Na3C6H5O7/i n6 0.1 M HEPES, pH 7.4) in 60 μl volumes; for radiolabeled structures, one oligonucleotide was first radiolabeled and then 20 pmol radiolabeled oligo and 100 pmol non-radiolabeled 50-mers, 200 pmol non-radiolabeled 25-mers were annealed. The reactions were adjusted to 5% glycerol using DNA loading dye with bromophenol blue and the full volume was loaded onto a 1.5 mm-thick 10 × 10 cm or 10 × 20 cm 10% native PAGE-TBE gel and electrophoresed at 150 V for 65 min. Following electrophoresis, DNA was identified in the PAGE-TBE gel by UV shadowing on Baker-flex® cellulose PEI-F thin layer chromatography paper (J.T. Baker). Bands corresponding to the fully annealed DNA structures were excised and stored at 4°C.
For radiolabeled structures, the PAGE-TBE gel was exposed to Kodak Scientific Imaging film for 15 min. Fully annealed joint structure bands were excised using the processed film as a template on a light table. Completely annealed structures were verified by their slow electrophoretic mobility relative to structures annealed with all possible partial oligonucleotide compositions. Heat-denaturation of substrates verified their component strands. DNA joint structures were electrophoresed from the polyacrylamide slice in a Millipore Microelutor in TBE buffer at 150 V for 2 h at room temperature or at 4°C. Samples were concentrated in the Millipore Centricron device by centrifugation in a Beckman Avanti J2-21 centrifuge at 4000 rpm for 1 h to 1 h 45 min at 4°C. Samples were collected by inverting the Centricon unit with centrifugation at 300 g, 2 min, and dialyzed against two volumes of 200 ml TE, pH 7.5 in a Tube-o-dialyzer Medi tube, MWCO 15 000 (GenoTech, Inc.) for 1 h at 4°C. Substrate concentration was determined by spectrophotometry at \( \lambda = 260 \text{ nm} \) using a NanoDrop® ND-1000 spectrophotometer for non-radiolabeled olo structures, or by scintillation count to determine c.p.m./\( \mu l \) for radiolabeled olo structures. When further concentration was needed, DNA samples were transferred to a Microcon YM-10 centrifugal filter device (MWCO 10 000; Millipore/Amiccon) and concentrated in a microcentrifuge at 12 000 rpm for 30 min, 4°C. The sequences of oligonucleotides are those reported by Kaliraman et al. and Osman et al. (28,37), other than the oligonucleotides used in preparation of the D-loop structure (DL). These sequences are: [DL-0/olWDH684] 5’-CGTTGACGGCTGCAGCCGATTTCAACTCAGCTCGT GCCTTGCTAGGACATCTTTGTGGCCACCTGCAAGT TCACCCATCCG-3’; [DL-1/olWDH685] 5’-GGCGATG GGTTGAACCTGAGGTGCTGGCGCCTGTCATCGT AGGTAGTAGTGAGTGAATTTGTAGGCTGCGCAGTC GAAC-3’; [DL-2/olWDH686] 5’-GATGTCGAAAGGCA AGATGTTCTATAGAATGCTTACAGGCAAAGGCA CGCAG-3’; [DL-3/olWDH687] 5’-TATAGAACACTTT GCTTTACAGTCT-3’.

Nuclease assays

In salt and pH activity assays, 100 nM 3’-flapped substrate was used (defined by non-radiolabeled substrate, but spiked with radiolabeled 3’-FL as a reporter), and heterodimer was at 5 nM. The radiolabeled spike was confirmed to contribute negligible concentration by DNA DipStickSM analysis (Invitrogen) and NanoDrop® ND-1000 spectrophotometric readings at \( \lambda = 260 \text{ nm} \) after decay to background. Heterodimer was diluted in standard enzyme diluent (SED: 10 mM Tris–HCl, pH 7.5, 0.5 mg/ml BSA) to 50 nM. Buffers were used at 25 mM reaction concentration, with 10 mM MgCl2/1 mM DTT/0.1 \( \mu \text{g/ml BSA} \). MES, PIPES, MOPS, HEPES, TAPS (all from Sigma) and Tris–HCl (US Biologicals) were titrated as 8 × reaction stocks to the appropriate pH at 30°C. Reactions were pre-incubated in a 30°C water bath for 10 min, and were initiated by addition of 1 \( \mu l \) 50 nM heterodimer with gentle mixing. Ionic strength at each pH value was titrated as defined by [NaCl], at 0, 50, 100, 150, 200, 300 and 500 mM. For metal ion characterization, buffer composition was 25 mM HEPES, pH 7.5/100 mM NaCl/1 mM DTT/0.1 \( \mu \text{g/ml BSA} \) (New England BioLabs) with the appropriate concentration of the metal acetate salt (magnesium acetate from Fisher; manganese and calcium acetates from Sigma-Aldrich). Aliquots of 0.5 \( \mu l \) were removed from each [substrate] reaction at 3, 6, 10, 15, 20, 30, 45 and 60 min and quenched immediately into pre- aliquoted 0.5 \( \mu l \) volumes of 0.5 x nuclease stop buffer (1 x is 200 mM EDTA, 2.5% SDS, 10 mg/ml Proteinase K). Nine \( \mu l \) of DNA loading dye (5% glycerol/bromophenol blue) were added to each time point and samples were electrophoresed by 10 × 20 cm native 10% TBE-PAGE at 100 V for 65 min. Gels were equilibrated in 5% glycerol or 3% glycerol/20% methanol for 30 min and vacuum-dried to Whatman® paper at 65°C, then exposed overnight to a phosphorimager screen. For DL analyses, time point volumes were boiled in formamide/bromophenol blue for 2 min at 95°C before electrophoresis on a 7 mA urea/10% TBE-PAGE denaturing gel at 150 V for 40 min. These gels were equilibrated as described and vacuum-dried to DE81 anion exchanger chromatography paper (Whatman®). The comparison of wild-type heterodimer with the catalytic mutant control was performed at optimized assay conditions, 25 mM HEPES, pH 7.5/3 mM Mg(OAc)2/100 mM NaCl/0.1 mM DTT/0.1 \( \mu \text{g/ml BSA} \).

Kinetic analysis

Substrate concentrations used for initial velocity determinations were chosen to flank a predicted \( K_m \) on the order of 1–10 nM. At least three independent trials were performed for each substrate at 0.5, 1, 2.5, 5, 10, 20, 50 and 100 nM substrate and enzyme fixed at 5 nM, with all substrate concentrations processed in parallel. Nuclease reactions were performed as described for assay optimization, with fixed time points quenched at 0, 3, 6, 10, 15, 20, 30, 45 and 60 min reaction time. Reaction progress was quantified by Storm Phosphorimagery and ImageQuant software. Initial velocities were extrapolated from non-linear regression curves defined by Graphpad Prism at 30 s reaction time. Michaelis–Menten analysis was performed using GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, California USA, www. graphpad.com. Regions of the assay time that followed linear initial velocity were verified and typically lasted between 3 and 5 min or more for each substrate concentration in every reaction. Rates are expressed as nanomolar joint molecule substrate incised/minute, and where calculated for \( k_{cat} \) rates are expressed as number of joint molecule substrates incised per heterodimer molecule per minute.

RESULTS

Purification of GST-Mms4/His10-FLAG-Mus81 heterodimer

Galactose-induced overexpression of the fusion-tagged heterodimer followed by sequential affinity selection for
the N-terminal GST and His10 tags (Figure 1A) resulted in the isolation of GST-Mms4/His10-FLAG-Mus81 heterodimer that is homogeneous by Coomassie staining. Passage of ammonium sulfate-fractionated extract over glutathione-sepharose yielded heterodimer with a slight excess of GST-tagged subunit, as expected if some GST-Mms4 was not associated with Mus81. Passage of the glutathione-sepharose eluants over an immobilized metal chelate resin (Ni²⁺-NT-agarose) selected for the intact heterodimer with subunits in a 1:1 stoichiometry as determined by Coomassie staining (Figure 1B). The predicted catalytic mutant complex (His10-FLAG-Mus81 D414A, D415A; abbreviated Mus81-dd) behaved like the wild-type heterodimer during its purification.

**Complementation of mus81-Δ mms4-Δ double mutant genotoxin sensitivity by fusion protein expression**

*Saccharomyces cerevisiae* cells deficient in Mus81-Mms4 are sensitive to alkylation, topoisomerase I inhibition, deoxyribonucleotide depletion, and other perturbations that disturb replication forks by reducing processivity, or by generating template lesions that prohibit replisome progression or provoke its disassembly. We verified that the addition of fusion tags designed for affinity purification of the heterodimer does not interfere with heterodimer function by establishing that the tagged heterodimer (pWDH595) expressed at low level fully complements the genotoxin sensitivity of the double mutant strain (Figure 1C). The heterodimer expression construct is under galactose-inducible control for protein overexpression, but on glucose in the absence of galactose, there is only a low level of transcription from the *GAL1/10* promoter. The expression vector bearing instead the *mus81* D414A, D415A mutant allele (pWDH596), predicted to encode a catalytically inactive nuclease, does not complement the double mutant strain for sensitivity to 0.015% MMS. This mutant also does not complement sensitivity to the ribonucleotide reductase inhibitor hydroxyurea or the topoisomerase I cleavage complex inhibitor camptothecin (not shown). The fusion complex...
is therefore active in vivo and competent to perform the DNA joint molecule incision role(s) demanded of native Mus81-Mms4.

**Mus81-Mms4 is catalytically active**

Although evolutionary selection for Mus81-Mms4 has taken place under circumstances expected to generate DNA structures that typically occur with low numerical incidence but regular frequency in cycling cells, a priori it is unclear whether the heterodimer should function stoichiometrically or catalytically with respect to substrate turnover. This has been demonstrated for few structure-selective endonucleases; the 5'-flap endonuclease Fen1 functions catalytically in vitro (38), but work with XPF-ERCC1 and the canonical Holliday junction resolvase RuvC has been typically performed under conditions that do not show catalysis or do not allow substrate turnover (39–43). We therefore assayed whether purified Mus81-Mms4 shows catalytic turnover of DNA joint molecule substrates in vitro. We chose to optimize assay conditions on a 3'-flapped structure because this substrate has consistently shown strong incision by both recombinant and partially purified eukaryotic heterodimer preparations reported to date. We verified that DNA structure incision was dependent on Mus81 catalytic activity; limiting endonuclease (5 nM) was sufficient for turnover of excess 3'-flapped substrate (100 nM) within 30 min at 30°C. No 3'-flapped substrate incision was observed during incubation with an equivalent concentration of the predicted catalytically inactive complex (Figure 2A). The active heterodimer in the presence of 20-fold substrate excess exhibits a time course of 3'-flapped incision that can approach completion within an hour at 30°C (Figure 2B and C). No contaminating helicase, exonuclease or non-specific nicking activities or thermal denaturation of substrate during the assay time course is observed, indicating that the heterodimer preparation and assay design is free of activities that are likely to alter substrate properties from the anticipated annealed structures during incubation. Specifically, there is no loss of label due to phosphatase or 5'-3' exonuclease, no fragmentation due to contaminating endo- or 3'-5' exonucleases, and no evidence for helicase or thermal denaturation of substrate during the assay time course (Figures 2 and 3).

We sampled fixed time points during the course of the nuclease assay to gauge product accumulation over time. The progress curves at 5 nM and 10 nM heterodimer show a window of time during which the initial reaction rate remains linear (up to ~5 min assay time, or 10–20% substrate depletion). The reaction velocity linearity over this extensive window of substrate depletion can probably be explained by a low $K_M$ of Mus81-Mms4 for the 3'-flapped substrate; even as substrate concentration drops by turnover to product, the remaining substrate concentration continues to substantially exceed $K_M$ (Table 1). When twice the amount of enzyme is added to 100 nM 3'-flapped structure, the initial rate correspondingly responds by a factor of two (Figure 2C).

**Mus81-Mms4 is responsive to metal ion character and concentration**

Having established that S. cerevisiae fusion-tagged Mus81-Mms4 is catalytically active and shows DNA joint molecule substrate turnover under conditions of limiting enzyme relative to substrate concentration, we defined optimal in vitro assay conditions to profile the enzyme’s response to metal ion character and concentration, buffer and pH, and ionic strength as defined by NaCl concentration. We titrated Mg(OAc)$_2$, Mn(OAc)$_2$, and Ca(OAc)$_2$ to define optima by the criterion of initial reaction velocity. Complete 60 min progress curves were collected as in Figure 2C to define the region of initial reaction velocity linearity, with 5 nM heterodimer in the presence of 100 nM 3'-flapped substrate. Like most DNA-acting enzymes, Mus81-Mms4 uses Mg$^{2+}$ optimally for phosphodiester bond hydrolysis in DNA substrates, with an empirical optimum at approximately 3 mM (Figure 2D). The heterodimer can also use Mn$^{2+}$ as cofactor, but over a more narrow concentration range with a maximum at less than or equal to 1 mM. Ca$^{2+}$ is completely ineffective as cofactor, and the enzyme shows absolutely no incision on the 3'-flapped structure at any Ca$^{2+}$ concentration assayed up to 20 mM.

**Mus81-Mms4 is sensitive to buffer, pH and NaCl effects**

Although a number of buffers are appropriate and widely applied to biological assays in vitro, some are known to interact non-specifically with certain macromolecules. Although physiological conditions are frequently cited as pH 7.5 with total ionic strength approaching 150 mM, some enzymes are especially active at pH regions outside this standard range. We therefore assayed Mus81-Mms4 initial reaction velocity on the 3'-flapped substrate as a function of pH and ionic strength defined by NaCl concentration (Figure 2E). We chose buffers with pKa values that represented a pH span from 6 to 9, and titrated NaCl at 0, 50, 100, 150, 200, 300 and 500 mM at every pH value represented across the buffers MES, PIPES, HEPES, Tris–HCl and TAPS. For the common biological buffers HEPES and Tris–HCl, we assayed initial reaction velocity at several pH values defined at 30°C. First, we observed buffer-specific effects primarily at low pH. PIPES, pH 6.5 is an especially poor buffer for heterodimer activity. Reaction velocity is even poorer than in MES, pH 6.0, suggesting that low pH is not solely responsible for reduced activity. Nuclease activity improves at pH above 7.0, regardless of the buffer identity (Tris–HCl, HEPES or TAPS). Heterodimer nuclease activity is most sensitive to increasing NaCl concentration at low pH, but becomes more resistant to increasing NaCl concentration at higher pH. Mus81-Mms4 shows improved activity at NaCl concentrations approximating physiological ionic strength, but becomes salt-sensitive at concentrations above 200 mM NaCl. Mus81-Mms4 nuclease activity is more resistant to increasing NaCl concentration at high pH than at low pH. Mus81-Mms4 shows improved activity at NaCl concentrations approximating physiological ionic strength, but becomes salt-sensitive at concentrations above 200 mM NaCl. Mus81-Mms4 nuclease activity is more resistant to increasing NaCl concentration at high pH than at low pH. Mus81-Mms4 shows improved activity at NaCl concentrations approximating physiological ionic strength, but becomes salt-sensitive at concentrations above 200 mM NaCl. Mus81-Mms4 nuclease activity is more resistant to increasing NaCl concentration at high pH than at low pH. Mus81-Mms4 shows improved activity at NaCl concentrations approximating physiological ionic strength, but becomes salt-sensitive at concentrations above 200 mM NaCl. Mus81-Mms4 nuclease activity is more resistant to increasing NaCl concentration at high pH than at low pH. Mus81-Mms4 shows improved activity at NaCl concentrations approximating physiological ionic strength, but becomes salt-sensitive at concentrations above 200 mM NaCl. Mus81-Mms4 nuclease activity is more resistant to increasing NaCl concentration at high pH than at low pH.
Mus81-Mms4 is active on any tested DNA joint molecule that meets minimal criteria of a branch point coincident with a strand discontinuity.

Having defined nuclease assay conditions that promote optimal reaction velocity on the 3' flapped substrate, we examined the heterodimer’s substrate selection on a number of DNA joint molecules in vitro. A broad spectrum of DNA joint molecules is readily incised by limiting enzyme (5–10 nM), including the 3'-flapped (3'-FL) and replication fork-like (RF-like) structures, nicked four-way junctions (nXO12), three-way junctions with 3' or 5'-emanating single-stranded DNA (pXO12-3' and pXO12-5'), and the displacement loop structure (DL). All these structures share properties previously defined as relevant to Mus81-Mms4/Eme1 recognition and processing, primarily the presence of duplex DNA flanking...
a phosphodiester backbone discontinuity (nick), which defines a branch point from which emanates duplex or single-stranded DNA. In the case of single-stranded DNA, the polarity must be 5'→3' for optimal cleavage adjacent to the branch point, although this is less important in substrates with three duplex arms (pXO12 structures). Increasing the concentration of enzyme relative to substrate (20–100 nM enzyme) broadens the class of substrates that can be readily incised by *S. cerevisiae* Mus81-Mms4, including the 5'-flapped (5'-FL) and splayed arm (Y) joints. The apparent selectability of a joint molecule substrate can therefore be modulated by enzyme: substrate stoichiometry (Figure 3A and B). These observations underscore the

**Figure 3.** Mus81-Mms4 from *S. cerevisiae* is active on a number of DNA joint molecule substrates, but not on intact Holliday junctions *in vitro*. (A) Heterodimer titration on a number of synthetic DNA joint molecules. Reactions were performed with joint molecule concentration defined by non-radiolabeled substrate at 50 nM and spiked with radiolabeled substrate to report on turnover of the substrate population during 30 min incubation at 30°C. Relative to substrate concentration, Mus81-Mms4 was added to limiting concentration at 5 nM, 10 nM and 20 nM heterodimer, at stoichiometric concentration at 50 nM heterodimer, or at excess concentration at 100 nM heterodimer. (B) Quantitation of A.
Mus81-Mms4 in isolation is not active on four-way Holliday junctions

Having shown that Mus81-Mms4 readily incises a number of DNA joint molecules in vitro, and that even structures that are not considered likely targets by structural criteria can be incised when heterodimer approaches or exceeds substrate concentration, we focused on the Holliday junction substrates that have been the most controversial amongst the DNA joint molecules exhibiting certain minimal structural features (data not shown). We tested whether Mg\(^{2+}\) or Mn\(^{2+}\) concentration influences Mus81-Mms4 nuclease. This is true for several preparations of Mus81-Mms4 isolated in our laboratory, ranging from ~1 \(\mu\)M to 3 \(\mu\)M heterodimer. We tested whether Mg\(^{2+}\) or Mn\(^{2+}\) concentration influences Mus81-Mms4 capacity to incise intact Holliday junctions (Figure 4). Although the fixed-branch point structure XO12 shows incision up to 20 \(\mu\)M Mg\(^{2+}\), the percent product turnover is nearly negligible (<2\% product after 30 min incubation at 30\(^\circ\)C). The junction with the branch-migratable core is incised even more poorly, with a small peak in incision near 10 \(\mu\)M Mg\(^{2+}\) and Mn\(^{2+}\).

Purified endogenous Mus81-Mms4 is multiply phosphorylated in the absence and presence of genotoxic stress, but these modification states are not sufficient for Holliday junction incision competence in vitro

Because it had been speculated that eukaryotic post-translational modifications could account for the difference in Holliday junction incision activity observed between recombinant and eukaryotic preparations of Mus81-Mms4/Eme1, we examined the phosphorylation status of purified Mus81 and Mms4. Purified Mms4 can be collapsed to a species with faster relative electrophoretic mobility by treatment with phosphatase (Figure 5A). Mus81 does not show a detectable electrophoretic mobility shift under the polyacrylamide gel electrophoresis conditions described. The shift of Mms4 is unlikely due to phosphorylation of GST, as other GST-fusions (Rad54, Rad57) do not show electrophoretic mobility shifts under similar treatment (W.-D.H., data not shown). In addition, mass spectrometric analysis of the purified complex identified a number of constitutive and DNA damage-induced phosphorylated serine and threonine residues on each Mus81 and Mms4 subunit (unpublished data). Mus81 and Mms4 also show electrophoretic mobility shifts in vivo in response to genotoxins including the alkylating agent MMS, the chemical UV mimetic 4-nitroquinoline (4-NQO) and the ribonucleotide reductase inhibitor hydroxyurea (HU) (Figure 5B and C). The heterodimer purified in a modification state induced by genotoxic stress (Figure 5C) remains unable to incise Holliday junctions in vitro (Figure 5D), although the endonuclease is active (Figure 5D and E). It is unclear whether the reduced activity of the heterodimer isolated from MMS-challenged cells reflects a physiologically relevant modification of enzyme behavior; the heterodimer isolated from HU-challenged cells shows nuclease activity similar to the heterodimer isolated from cells in the absence of exogenous genotoxic stress (Figure 5D and E). The reduced specific activity of the preparation from MMS-challenged cells may be associated with chemical modification of the complex by alkylation. In sum, these observations indicate that eukaryotic post-translational modification profiles constitutive to the overexpressed heterodimer complex or modification profiles induced by global genotoxic stress do not confer Holliday junction incision capacity as an enzymatic property latent to the heterodimer. Global dephosphorylation of the complex by PP1 phosphatase treatment also does not alter substrate selection as tested for 3'-flapped and Holliday junction substrates (data not shown).

### Determination of \(K_M\) and \(k_{cat}\) on DNA joint molecules

DNA joint molecules exhibiting certain minimal structural features are bound and catalytically processed by Mus81-Mms4/Eme1 (Table 1). Some of these structural features...
have been defined, including foremost the presence of a hydroxyl or phosphate group at the 5' deoxyribose position adjacent to a backbone discontinuity at a branch point (44). The discontinuity must occur at the junction of at least three DNA arms, two of which must be duplex DNA for optimal cleavage. We find that nevertheless, the splayed Y structure can be incised, although its initial binding parameter \(K_M\) is highest of all tested substrates. This is consistent with the observation that duplex DNA 3' to a structural branch point marked by a strand discontinuity is important for optimal enzyme binding to substrate.

Figure 6 shows an example for a [substrate] versus velocity plot for the 3' flapped substrate. For the 3' flapped, RF-like, and pXO12 substrates, expression of the data points at concentrations up to 50 nM in an equation that describes a rectangular hyperbolic function using the \(K_M\) and \(V_{max}\) calculated by non-linear regression shows that the data points are appropriately modeled by a rectangular hyperbolic function. Enzyme action on these substrates is therefore accommodated by an Henri–Michaelis–Menten kinetic model. The initial velocity at increasing substrate concentration (100 nM) is reduced relative to the maximal velocity defined by the rectangular hyperbolic region of the [substrate] versus velocity plot, which indicates that at high concentration of these oligonucleotide joint molecules, the endonuclease exhibits substrate inhibition \textit{in vitro}. On the nicked four-way junction and D-loop structure, substrate inhibition occurs at 10-fold lower substrate concentrations (maximal velocity at 5 nM) relative to that observed for the 3'-flapped, RF-like and pXO12 substrates. The substrate inhibition observed for Mus81-Mms4 may reflect the presence of at least two semi-independent binding sites for at least two discrete substrate regions. These binding sites may be shared between a single heterodimeric Mus81-Mms4 unit, or they may be shared between a homodimeric complex of two Mus81-Mms4 heterodimeric subunits. Analysis and possible implications of substrate inhibition on the mechanism of Mus81-Mms4 are the subject of further analysis to be reported in a subsequent manuscript.

**DISCUSSION**

We describe the isolation and characterization of a catalytically active preparation of \textit{S. cerevisiae} Mus81-Mms4, purified to apparent homogeneity after overexpression in its native eukaryotic source. To our knowledge, we provide the first demonstration of catalytic turnover of DNA joint molecules by a native XPF-family endonuclease. We use this catalytic preparation to supply the first quantitative comparison of DNA joint molecule turnover by a native Mus81 preparation at apparent homogeneity, under conditions of limiting enzyme relative to substrate concentration. We first optimized assay conditions for buffer, pH, ionic strength and metal ion character. Because the anticipated \textit{in vivo} concentration of a single substrate in a yeast nucleus is low (on the order of 0.5–1 nM), there is no \textit{a priori} expectation as to whether the enzyme should function catalytically or whether stoichiometric behavior is sufficient (presumably, stoichiometric behavior would be explained by some manner of hysteresis, in which the enzyme adopts an inactive conformation after a catalytic cycle). We show that Mus81-Mms4 performs catalytic turnover on a number of DNA joint molecules that meet minimal structural criteria. We further show that catalysis is not directed to model oligonucleotide Holliday junctions by native Mus81-Mms4 in isolation.

Substrates that are catalytically processed by the enzyme share low \(K_M\) (on the order of 1–7 nM) and...
incubated for 30 min in each reaction. (E. coli complex is nucleolytically active (nXO12). The 50 nM substrate was incompetent to incise intact Holliday junctions (XO12), although the purified in a modification state induced by genotoxic stress remains D stress by 0.1% MMS treatment or by 100 mM HU. (C) Mms4 exhibits a phosphatase-sensitive electrophoretic mobility shift. (D) Complex purified from cells subjected to genotoxic stress by 0.1% MMS treatment or by 100 mM HU. (D) Heterodimer purified in a modification state induced by genotoxic stress remains incompetent to incise intact Holliday junctions (XO12), although the complex is nucleolytically active (nXO12). The 50 nM substrate was incubated for 30 min in each reaction. (E) Quantitation of data in D.

correspondingly slow turnover (estimated on the order of 1–4 min for 3′-flapped, replication fork-like, nicked four-way, partial three-way and splayed Y junctions, and up to 10.8 min for the D-loop). Three substrate classes can be distinguished by their kinetic parameters K_M and k_cat. Class I substrates (nickled Holliday junction, 3′-flapped junction, and replication fork-like junction) are characterized by low K_M (3–7 nM) and highest k_cat (~1 min^{-1}) among the substrates examined. Class II substrates (partial Holliday junction and D-loop) also demonstrate low K_M recognition by Mus81-Mms4 (1–6 nM), but lower k_cat (~0.3 min^{-1}) relative to the Class I substrates. The splayed Y junction represents a Class III substrate that is poorly cleaved relative to Class I and II substrates, with both a higher relative K_M (~30 nM) and low k_cat (0.26 min^{-1}). Comparing Class I and II substrates to the Class III Y substrate reveals that duplex DNA flanking the branch point is key to a low K_M parameter for Mus81-Mms4 substrates, although duplex DNA is not absolutely required 3′ to the branch point for turnover.

Despite the low K_M for Class I and II substrates, the average K_M is several-fold greater than the anticipated substrate concentration on which Mus81-Mms4 is expected to act in vivo. This may imply that additional factors are needed to target the enzyme to its joint molecule substrate. Mus81-Mms4 was first identified by a physical interaction with the Snf2-like ATPase Rad54, a DNA translocase that promotes Rad51-mediated DNA strand exchange, heteroduplex extension and Rad51 turnover from the DNA strand exchange product (6,45). This physical interaction may suggest a role for Rad54 in placement of Mus81-Mms4 at targeted substrates.

Our kinetic analysis of Mus81-Mms4 isolated from the cognate host demonstrates commonalities and interesting differences to a previous analysis of Mus81-Mms4 isolated from E. coli (25). While the overall substrate selectivity of both preparations appears similar, including exceedingly poor cleavage of intact HJs, the absolute kinetic parameters differ substantially for any given substrate. Moreover, the response to NaCl (improved activity up to 150 mM within pH 7–9 instead of inhibition), a more narrow magnesium optimum (3 mM) and discrimination between Mg^{2+} and Mn^{2+} as cofactors distinguish the native enzyme from the bacterial preparation. The reasons for these differences are unclear and may include the presence of different tags, post-translational modifications or differences in assay design and analysis.

Figure 6. [Substrate] versus velocity plots for a catalytic preparation of Mus81-Mms4, optimized for pH, ionic strength and metal ion cofactor. (A) 3′-FL structure up to 100 nM substrate; (B) Lineweaver–Burk transformation of data in A.
Mus81-Mms4 incises a broad class of DNA joint molecules in vitro, but not intact four-way junctions

Coordinated Holliday junction incision in vitro remains an activity that is difficult to attribute with certainty solely to Mus81-Mms4/Eme1. In cases where human HeLa cell Mus81 immunoprecipitates and partially purified S. pombe preparations show activity on Holliday junctions, the duplex products are gapped and flapped (23,24). These products differ from the nicked duplex products associated with symmetric incision by RuvC, RuvA, Hjc, Hje and bacteriophage junction-resolving enzymes T4 endonuclease VII and T7 endonuclease I. This suggests that the Holliday junction incision observed for some Mus81-Mms4/Eme1 preparations (i) may occur in a manner distinct from that of other classified four-way junction-specific enzymes, (ii) requires other factors to impose symmetric and coordinated cleavage or (iii) is an off-target outcome of independent incision events that occur at high enzyme: substrate ratios or in the presence of assay components that alter four-way junction presentation.

We show that the apparent selectivity of the enzyme toward substrates can be modulated by protein: substrate concentration ratios. 5'-flapped substrates have been poor substrates for nuclease incision and excluded as potential candidate in vivo targets. At protein: substrate concentration ratios greater than or equal to 1:1, however, even a 5'-flapped substrate can be incised by endogenous S. cerevisiae Mus81-Mms4 (Figure 2). This demonstrates that protein: substrate ratios are in fact critical determinants to apparent selectivity in vitro. The direct comparison of Mus81-Mms4 substrate selectivity under conditions of limiting enzyme is therefore needed to more completely describe Mus81 biochemistry.

Despite the capacity of endogenous Mus81-Mms4 to incise a non-canonical substrate such as the 5'-flap at high protein concentration, S. cerevisiae Mus81-Mms4 is not active on synthetic Holliday junctions, even at excess protein: substrate ratios (2:1). Less than 2% substrate turnover can be demonstrated on 50 nM substrate with 2-fold excess enzyme; this is true for any Mg2+ or Mn2+ concentration tested from 1 to 20 mM. Hence the X12 and XO12 structures are inaccessible for K_M and k_cat determinations. These observations suggest that S. cerevisiae Mus81-Mms4 does not resolve Holliday junctions, and by extension to S. pombe and human Mus81-Eme1, may suggest that the heterodimer alone is not responsible for the model Holliday junction processing observed in partially purified eukaryotic preparations. Gaillard et al. (21) proposed that the inability of recombinant S. pombe and human Mus81-Eme1 preparations to cut model Holliday junctions could be explained by an inability of recombinant enzyme to make an initial nick in the junction core. In these studies, S. pombe Mus81-Eme1 has shown greater incision activity on X12 cruciform structures having 12 base-pair homologous cores that allow branch migration, as opposed to XO12 cruciform structures having a fixed branch point. X12 structures can undergo thermal breathing of the interior core with transient single-stranded DNA nature (46). These authors conclude that a coactivator in endogenous preparations may help Mus81-Eme1 open the Holliday junction core at the branch point, as a requirement for incision. Although our observations cannot exclude this possibility for S. cerevisiae Mus81-Mms4, our Mus81-Mms4 preparation shows least activity on X12 junctions, and minimal activity on XO12 junctions. This may be explained by an absence of the coactivator proposed by Gaillard et al., or it may alternatively be explained if the preferential incision of X12 substrates in partially purified endogenous preparations relates to a trace contaminating endonuclease that makes an initial nick at the transiently unpaired X12 core. Moreover, enzyme at high concentration excess as performed in these assays may have allowed incision of a partially unpaired (splayed) core, a behavior that is enhanced on the Y substrate in our assays with increasing heterodimer concentration.

Phosphorylation status of endogenous S. cerevisiae Mus81-Mms4 does not confer a latent Holliday junction incision activity to the endonuclease

Given that purified endogenous S. cerevisiae Mus81-Mms4 shows weak activity on model Holliday junctions, we explored alternative global phosphorylation profiles of the native complex. Mus81 complex overexpressed and purified from S. cerevisiae is multiply phosphorylated in a constitutive fashion on both subunits, but this apparently is not sufficient to confer a latent Holliday junction incision activity. Furthermore, enzyme overexpressed and purified from cells challenged by the genotoxin MMS exhibits a different phosphorylation profile, yet remains incompetent to incise Holliday junctions. The damage-modified complex, whether isolated from MMS- or HU-challenged cells, remains competent to incise other structures, however. Eukaryotic post-translational modifications in S. cerevisiae are therefore not sufficient to explain the Holliday junction incision behavior described for partially purified heterodimer preparations from S. pombe and human HeLa cells. If Holliday junction incision is a latent activity of S. cerevisiae Mus81-Mms4, it likely requires an additional factor that functions by a mechanism that remains to be described.

Most challenging to reconcile at this time is the difference in our preparation and the recombinant preparation recently reported by Gaskell et al. (20). These authors propose that recombinant preparations of S. pombe Mus81-Eme1 and S. cerevisiae Mus81-Mms4 can incise Holliday junctions when a purification protocol has selected for oligomeric states greater than the single heterodimer, and that quaternary structural differences account for disparities in Holliday junction incision competence observed to date. Their gel filtration protocol isolated fractions containing Mus81-Mms4/Eme1 with weak Holliday junction incision ability; in these peak fractions, however, strand dissociation of a fraction of the model junction population can also be detected and gel filtration fractions were not normalized for protein concentration. This report further suggests that magnesium ion concentration modulates the oligomeric state of
the enzyme, to explain the magnesium-response profile for Holliday junction incision observed in their recombinant preparations. Whereas nicked model Holliday junctions were incised over a broad range of magnesium concentrations (0.5–20 mM), intact Holliday junctions were incised 3- to 4-fold more effectively at magnesium concentrations below 5 mM. Modulation of substrate selectivity by metal ion concentration is without precedent, and a mechanism for oligomerization in response to metal ion concentration remains to be explained. Rather than reflecting different magnesium optima for cleavage of different substrates by Mus81-Mms4/Eme1, the enhanced Holliday junction incision at low Mg2+ concentration may alternatively be interpreted as modulation of a trace activity that responds to metal ion concentration and may alter the substrate presentation to Mus81-Mms4/Eme1. Our results indicate that magnesium or manganese titration does not alter S. cerevisiae Mus81-Mms4 activity on model Holliday junctions. Furthermore, our operative conditions for S. cerevisiae Mus81-Mms4 biochemical assays are not consistent with a requirement for isolation above threshold protein concentrations. Our nuclease assays require dilution of the enzyme 200- to 500-fold for meaningful rate determinations on excess substrate, suggesting that enzyme concentration is not a primary limiting factor for catalytic activity and therefore not likely to bear on active oligomeric state.

The DNA structure selectivity of Mus81-Mms4 is likely to be circumscribed by protein–protein interactions specific to the context in which the joint molecule is generated

Functional specialization of nucleases to targeted substrates has probably been permitted by virtue of an inherent plasticity in substrate selection. This plasticity explains Mus81-Mms4/Eme1 selectivity in vitro. Substrate specificity has been accomplished in vivo by specialization of paralogs in the context of pathways in which their DNA structural targets are generated. A component of the ‘specificity’ of enzymes in vivo stems from their placement relative to potential substrates, and proximity may therefore become central to understanding how the apparent substrate selectivity of Mus81-Mms4/Eme1 in vitro is proscribed to a target or subset of targets in a context-specific manner in vivo.

Consistent with this restriction of substrate specificity in vivo, genetic studies indicate that Mus81-Mms4 has no role in 3′-flap cleavage during single-stranded DNA annealing (SSA) in budding yeast, even though the 3′-flapped structure is a biochemical target in vitro (4,47). Instead, the evolutionarily related endonuclease Rad1-Rad10 is assigned to this pathway, and Mus81-Mms4 is not recruited to the 3′-flap intermediates inherent to this Rad52-promoted context and cannot substitute in the absence of Rad1-Rad10. These observations underscore the probable role of protein–protein interactions in the sanction of substrate specificity by evolutionarily evolved associations within pathways. Substrate assignment to Mus81-Mms4/Eme1 in vitro is most likely to be understood in context of the interacting proteins that generate the DNA substrates the endonuclease targets.

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