Mus81 cleavage of Holliday junctions: a failsafe for processing meiotic recombination intermediates?

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The Holliday junction (HJ) is a central intermediate of homologous recombination. Its cleavage is critical for the formation of crossover recombinants during meiosis, which in turn helps to establish chiasmata and promote genetic diversity. Enzymes that cleave HJs, called HJ resolvases, have been identified in all domains of life except eukaryotic nuclei. Controversially, the Mus81-Eme1 endonuclease has been proposed to be an example of a eukaryotic nuclear resolvase. However, hitherto little or no HJ cleavage has been detected in recombinant preparations of Mus81-Eme1. Here, we report the purification of active forms of recombinant *Schizosaccharomyces pombe* Mus81-Eme1 and *Saccharomyces cerevisiae* Mus81-Mms4, which display robust HJ cleavage *in vitro*, which, in the case of Mus81-Eme1, is as good as the archetypal HJ resolvase RuvC in single turnover kinetic analysis. We also present genetic evidence that suggests that this activity might be utilised as a back-up to Mus81-Eme1's main activity of cleaving nicked HJs during meiosis in *S. pombe*. The EMBO Journal (2007) 26, 1891–1901. doi:10.1038/sj.emboj.7601645; Published online 15 March 2007
Subject Categories: genome stability & dynamics
Keywords: Holliday junction; meiosis; Mus81; recombination

Introduction

Homologous recombination (HR) is important for repair, replication and segregation of chromosomes. Its mechanism is perhaps best defined during meiosis, where programmed DNA double-strand breaks (DSBs) specifically trigger HR so that crossing over between homologous chromosomes can occur (Roeder, 1997; Smith and Nicolas, 1998; Krogh and Symington, 2004). This promotes genetic diversity, and is critical for the establishment of chiasmata that direct faithful chromosome segregation during the first meiotic division.

Meiotic recombination has been studied most extensively in the budding yeast *Saccharomyces cerevisiae*. Here, DSBs are repaired by at least three distinct pathways of HR, each of which depends on Rad51 and/or Dmcl catalysing the invasion of a homologous duplex by a resected end of the

DSB to form a structure called a displacement (D)-loop (Hollingsworth and Brill, 2004; Whitby, 2005; Neale and Keeney, 2006). The invading strand primes new DNA synthesis, which copies genetic information that might have been deleted at the DSB. In most instances, it is believed that the invading strand is then unwound, and anneals to its complementary strand at the other end of the break to achieve repair. This mechanism is called synthesis-dependent strand annealing (SDSA), and generates noncrossover (NCO) recombinants (Paques and Haber, 1999; Allers and Lichten, 2001).

In the second pathway, the D-loop is not unwound and can itself anneal to the complementary strand at the other end of the break (second end capture). Further DNA synthesis and strand ligations then result in the formation of a structure called the double Holliday junction (dHJ) (Szostak *et al*, 1983; Schwacha and Kleckner, 1995). This is resolved by cleavage of a pair of strands at each HJ by a junction-specific endonuclease. The relative orientation of cleavage at each junction determines whether crossover (CO) or NCO recombinants are generated (Szostak *et al*, 1983). In principle, the symmetry of the dHJ means that resolution should occur without bias for CO or NCO, and *in vitro* this is true for junctions cleaved by the phage T7 endonuclease I resolvase (Plank and Hsieh, 2006). However, resolution of meiotic dHJs in *S. cerevisiae* generates solely COs (Allers and Lichten, 2001; Hunter and Kleckner, 2001; Borner *et al*, 2004). This CO pathway depends on the so-called ZMM (Zip1, Zip2, Zip3, Zip4, Msh4, Msh5 and Mer3) proteins, which may be important for activating and directing an as yet unidentified HJ resolvase (Hollingsworth and Brill, 2004; Whitby, 2005).

The third pathway depends on the Mus81 endonuclease and is thought to generate only CO recombinants (Osman *et al*, 2003; Smith *et al*, 2003; Hollingsworth and Brill, 2004; Whitby, 2005). The importance of this pathway varies among eukaryotes, for example in *S. cerevisiae*, and possibly mammals, it accounts for only a minor proportion of COs, whereas in the fission yeast *Schizosaccharomyces pombe* most, if not all, COs depend on Mus81 (Hollingsworth and Brill, 2004; Guillon *et al*, 2005).

The mechanism by which Mus81 generates COs is controversial. It was initially heralded as the first example of a nuclear-acting HJ resolvase in eukaryotes (Boddy *et al*, 2001; Chen *et al*, 2001). This was based mainly on the detection of an HJ cleavage activity that copurified with Mus81 from *S. pombe* (Boddy *et al*, 2001; Gaillard *et al*, 2003). However, recombinant yeast and human Mus81 produced in bacteria exhibit very little HJ cleavage activity, while delivering robust cleavage of a variety of other branched DNA structures, including D-loops, nicked HJs, forks and 3' flaps (Kaliraman *et al*, 2001; Doe *et al*, 2002; Ciccia *et al*, 2003; Gaillard *et al*, 2003; Osman *et al*, 2003; Whitby *et al*, 2003; Fricke *et al*, 2005). On the basis of this substrate specificity it was proposed that Mus81 cleaves the D-loop and nicked HJ that precede the formation of the dHJ (Osman *et al*, 2003). However, more recent evidence suggests that in *S. pombe*

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Received: 23 October 2006; accepted: 19 February 2007; published online: 15 March 2007

Figure 1 Model for Mus81-Eme1 generating crossovers and non-crossovers during meiosis by cleaving a mixture of nicked and intact HJs.

meiotic DSB repair may generate only one nicked HJ for Mus81 to cleave (Cromie *et al*, 2006). Irrespective of the number of junctions, their inherent asymmetry provides a guide for Mus81 cleavage and ensures that only COs are formed during DSB repair (Figure 1). An appealing feature of this model is that it provides a simple explanation for the strong CO bias in *S. pombe*, where typically between 60 and 80% of gene conversion events are associated with a CO (Young *et al*, 2002; Osman *et al*, 2003). However, this mechanism allows little room for error because D-loops and nicked HJs will mature into intact HJs if Mus81 fails to act promptly. It would therefore be prudent for the cell to have a backup strategy for processing intact HJs (Figure 1).

Here, we show that although Mus81 has a binding and cleavage preference for nicked HJs, it nevertheless has a robust cleavage activity on intact HJs, which performs as well as the archetypal HJ resolvase RuvC in single turnover kinetic analysis. We also present genetic evidence that suggests that this activity might be utilised *in vivo* to generate a mix of CO and NCO recombinants.

Results and discussion

Purifying an active form of recombinant Mus81-Eme1 that cleaves both nicked and intact HJs

Mus81 is related to the XPF family of structure-specific endonucleases, which contain a conserved ERKX₃D active-

site motif and a pair of helix-hairpin-helix (HhH) motifs that are important for protein-protein and protein-DNA interactions (Boddy *et al*, 2000; Interthal and Heyer, 2000). Like other eukaryotic members of this family, Mus81 functions as a heterodimer, with a related but non-catalytic partner protein. In *S. pombe* and human, the partner of Mus81 is called Eme1 (Boddy *et al*, 2001; Ciccio *et al*, 2003), whereas in *S. cerevisiae* it is called Mms4 (Mullen *et al*, 2001). Functioning as a heterodimer Mus81-Eme1 or Mus81-Mms4 would have the requisite single active site for D-loop and nicked HJ cleavage, however, intact HJ resolution would require two active sites.

In a fresh attempt to evaluate recombinant *S. pombe* Mus81-Eme1 for HJ cleavage, we coexpressed Mus81 with a hexahistidine-tagged form of Eme1 in *Escherichia coli* cells, and purified them by fractionation on Ni-NTA, gel filtration and SP-sepharose columns (Figure 2A). SDS-PAGE analysis of the gel filtration fractions revealed that Eme1 elutes together with Mus81 in a wide range of fractions (data not shown). To determine gel filtration fractions that contain active Mus81-Eme1, we assessed them for cleavage of nicked (X0n) and intact (X0) versions of a synthetic HJ. X0(n) is referred to as a static HJ because its CO point is fixed by sequence heterology between its four junction arms. Plenty of X0n cleavage was detected in most of the fractions containing both Mus81 and Eme1 (Figure 2B). In contrast, only a small amount of X0 cleavage was detected, which peaked in fractions 27 and 28 (Figure 2B). A comparison with the elution profile of known molecular weight proteins showed that the peak of X0 cleavage corresponds to between 316 and 372 kDa, which is approximately twice the predicted molecular weight of the Mus81-Eme1 heterodimer (= 161.5 kDa including the His tag) (Figure 2C). Fractions 26–29 were pooled and further purified on an SP-sepharose column. The peak of Mus81-Eme1 eluted from this column between 0.4 and 0.5 M NaCl, and these fractions were pooled and used for all subsequent analyses of cleavage activity (Figure 2D, lane b). We also confirmed that when this material was reappplied to the gel filtration column, it eluted again as a peak in fractions 26–29 (data not shown). These data suggest that recombinant Mus81-Eme1 can be purified as a complex consisting of two Mus81 and two Eme1 subunits, which is capable of cleaving both nicked and intact HJs. To confirm that Mus81 is responsible for the X0 cleavage, we purified a catalytically inactive form of Mus81-Eme1, in which the two aspartates in Mus81's VERKXXDD active site motif are mutated to alanine (Figure 2D, lane c). This mutant form of Mus81 is called Mus81^{DD} (Boddy *et al*, 2001) and exhibited no X0 cleavage activity (Supplementary Figure S1 and Supplementary data), confirming that X0 cleavage is intrinsic to Mus81-Eme1.

To see whether Mus81 from other species behaves similarly, we purified *S. cerevisiae* Mus81-Mms4 using exactly the same protocol as for *S. pombe* Mus81-Eme1 (Supplementary Figure S2A). Mus81-Mms4 eluted in exactly the same fractions as Mus81-Eme1 from both gel filtration and SP-sepharose columns. As with Mus81-Eme1, fractions 26–29 from the gel filtration column contained the peak of X0 cleavage activity, which correspond to molecular weights that are equivalent to two heterodimers of Mus81-Mms4 (Supplementary Figure S2B and C). Furthermore, after fractionation on the SP-sepharose column, purified Mus81-Mms4 elutes again in fractions 26–29 when re-run on the gel

Figure 2 Purification of Mus81-Eme1 and analytical ultracentrifugation of Mus81-Mms4. **(A)** Summary of the purification scheme. **(B)** X0n and X0 cleavage activity in Mus81-Eme1 gel filtration fractions. Reactions (20 μ l) contained 4 μ l of the indicated gel filtration fraction, 1.1 nM of labelled junction DNA and 10 mM $MgCl_2$, and were analysed by native PAGE. **(C)** An estimation of the molecular weight of Mus81-Eme1 based on its gel filtration profile relative to known standards. The standards are thyroglobulin (~670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa) and equine myoglobin (17 kDa). V_C is the column volume, V_O is the void volume and V_R is the elution volume. **(D)** SDS-PAGE analysis of purified Mus81-Eme1 and Mus81^{DD}-Eme1. The gel is stained with Coomassie blue. Note that the identity of His-Eme1 and Mus81 was confirmed by N-terminal amino-acid sequencing. **(E)** $g(s)$ profile calculated from 20 sample distribution data sets using Sedfit (Schuck and Rossmanith, 2000). The data are shown as open circular symbols with a six-peak Gaussian fit plotted over as a black line. The individual peaks found by that fit are shown as broken red lines and have values $1.5 \pm 0.1S$, $2.1 \pm 0.2S$, $4.2 \pm 0.03S$, $7.3 \pm 0.04S$, $9.2 \pm 0.1S$ and $14.2 \pm 0.04S$. **(F)** Plots of apparent molecular weight against the absorbance of the sample at the midpoint of the 6000 r.p.m. profile for each dilution used. Black symbols and line at 6000 r.p.m.; red at 8000 r.p.m.; green at 10500 r.p.m.; blue at 15000 r.p.m. and cyan at 18000 r.p.m.

filtration column (Supplementary Figure S2D). Using this purification protocol we were able to generate much greater quantities of Mus81-Mms4 than were possible for Mus81-Eme1. We attribute this difference to the greater solubility of

the Mus81-Mms4 protein when expressed in *E. coli*. The greater quantity and concentration of Mus81-Mms4 enabled us to perform sedimentation velocity and sedimentation equilibrium experiments to determine more accurately

the molecular weight of Mus81-Mms4 (see below). It also enabled us to analyse a more concentrated sample of protein by SDS-PAGE (Supplementary Figure S2E). This confirmed the purity of the sample and showed that when Mus81-Mms4 is not boiled before SDS-PAGE analysis, a small amount of material runs at a molecular weight in excess of 250 kDa (Supplementary Figure S2E, compare lanes b and c). This is consistent with a tetrameric form of Mus81-Mms4 (~300 kDa).

The data above contrast with that obtained with our earlier preparations of Mus81-Eme1 and Mus81-Mms4, which did not show a peak of XO or XOn cleavage in gel filtration column fractions 26–29 (Doe *et al*, 2002; Whitby *et al*, 2003). We suspect that the reason for this is that our new preparations are made from far more concentrated protein samples, which may encourage the putative Mus81-Eme1/Mms4 dimer of heterodimers to hold together better during the purification.

Analysis of Mus81-Mms4 by analytical ultracentrifugation

To gain a better idea of the assemblage of Mus81-Mms4 (and by extrapolation Mus81-Eme1), we performed a number of sedimentation velocity experiments to observe the species present in solution. In the Supplementary data (Supplementary Figure S3) we show the profile of sedimenting species (g(s)) observed in three experiments at 40k r.p.m. and two at 50k r.p.m. The results of the different experiments are consistent with one another, and are exemplified by the profile shown in Figure 2E, which derives from the largest data set to display several species and therefore represents the best-attested description of the sample. Here, there are at least six different species, with *s* values of 1.5S, 2.1S, 4.2S, 7.3S, 9.2S and 14.2S. The smallest protein species in the sample has a molecular weight of ~72 kDa, which would be about 6.1S, if spherical. This indicates that the monomeric forms of the proteins are hydrodynamically inefficient and probably elongated, or otherwise extended such as unfolded. A protein of ~150 kDa, such as a dimer, would be 9.9S, and we therefore conclude that the species observed at 4.2S, 7.3S and 9.2S are various forms of hetero- and homodimers, with varying degrees of elongation. The peak at 14.2S is most likely a tetrameric assembly; a sphere of 300 kDa would be 15.8S. We also performed sedimentation equilibrium experiments (Figure 2F), which show that the sample is very polydisperse, consistent with the velocity profile. As the speed of the centrifuge is raised the apparent molecular weight falls; at the highest speeds investigated, the apparent weight is in the range of 75 kDa, whereas it is around 150 kDa at the lowest speed. Furthermore, at intermediate speeds of 8000 and 10500 r.p.m. there is a strong inverse relationship between sample concentration and apparent weight; this indicates a high degree of non-ideality in the sample (interactions and crowding effects). These data are entirely in agreement with the velocity data and indicate that species of weight greater than a heterodimer (~150 kDa) are present in the sample, as well as heterodimeric (~150 kDa) and monomeric (~72 and ~81 kDa) species. The polydisperse nature of Mus81-Mms4 in solution contrasts with its discrete elution profile from gel filtration after purification on an SP-sepharose column (Supplementary Figure S2D). It is possible that under gel filtration conditions the putative tetrameric

assemblage of Mus81-Mms4 is stabilised, whereas this stability is lost in the analytical ultracentrifuge. Nevertheless, the above data are consistent with the notion that Mus81-Mms4 can form a tetrameric assemblage in solution, and therefore could bind as such to a HJ to perform dual strand incisions.

HJ cleavage is optimal at low Mg^{2+} concentration

Quite a broad range of Mg^{2+} concentrations (1–10 mM) supports 3' flap cleavage by Mus81-Mms4 (Fricke *et al*, 2005). This is also true for the cleavage of XOn by Mus81-Eme1 (Figure 3A and B). The cleavage reactions in Figure 2B and Supplementary Figure S2B contain 10 mM $MgCl_2$, which although optimal for XOn cleavage might not be optimal for XO cleavage. To check this, we assessed XO cleavage by Mus81-Eme1 in a range of $MgCl_2$ concentrations (Figure 3A and B). Compared with XOn cleavage, the range of $MgCl_2$ concentrations that support XO cleavage is more restricted, with an optimum of ~2.5 mM (Figure 3B). In particular, XO cleavage is severely inhibited at 10 mM $MgCl_2$ and completely blocked at 20 mM (Figure 3A and B). In contrast, XOn cleavage is close to optimal at these $MgCl_2$ concentrations (Figure 3A and B). In the case of Mus81-Mms4, the difference in optimal $MgCl_2$ concentration for cleaving XO and XOn is even more striking, with the cleavage of XO and XOn being optimal at 2.5 and 20–40 mM, respectively (Supplementary Figure S4). The latter concentrations are inhibitory for XO cleavage.

The different $MgCl_2$ concentrations for optimal nicked and intact HJ cleavage suggest that Mus81-Eme1/Mms4 might have two modes of action. Magnesium concentration can affect the oligomerisation of proteins (e.g. Deprez *et al*, 2000), so perhaps a low magnesium concentration favours the putative dimer of heterodimers form of Mus81-Eme1, whereas high magnesium concentrations favour its heterodimeric form. Alternatively, magnesium concentration may influence Mus81 cleavage by affecting junction folding. Divalent metal ions promote the pairwise stacking of junction arms, and some HJ binding proteins are unable to bind to this stacked form (e.g. Whitby and Lloyd, 1998). Perhaps Mus81-Eme1/Mms4 only binds to the open conformation of a HJ. Nicked HJs adopt a stacked conformation different from intact HJs (Pohler *et al*, 1994), and therefore may be bound more readily by Mus81-Eme1/Mms4 at higher magnesium concentrations.

Static and mobile X-junctions are cleaved with equal ability by Mus81-Eme1

Although previous preparations of recombinant Mus81-Eme1 exhibited no cleavage of XO, low levels of cleavage of mobile HJs, such as X12 and X26, were detected (Doe *et al*, 2002; Ciccio *et al*, 2003; Gaillard *et al*, 2003). Mobile HJs contain a core of homologous sequences (12 bp in the case of X12 and 26 bp in the case of X26), in which the CO point can branch migrate. Unlike static HJs, mobile junctions are prone to transient thermal denaturation or basepair breathing, and therefore it has been suggested that Mus81-Eme1's ability to cleave X12 and X26 is due its recognition of the transient 'bubble' structures that would be generated at the junction centre (Ciccio *et al*, 2003). If the same is true for our recombinant preparation of Mus81-Eme1, then we should see much better cleavage of X12 than XO. However, a titration of Mus81-Eme1 with a fixed amount of XO and X12 reveals

Figure 3 Characterizing the cleavage of X0 by Mus81-Eme1. (A) The effect of $MgCl_2$ concentration on X0 and X0n cleavage by Mus81-Eme1. Reactions (20 μ l) contained 1.1 nM of labelled junction, together with the indicated amounts of Mus81-Eme1 and $MgCl_2$. (B) Histogram showing the mean data from three experiments represented in (A). Error bars represent the standard deviations. (C) Comparison of the cleavage of X0, X0n and X12 by Mus81-Eme1. Reactions (20 μ l) contained 1.1 nM junction DNA and the specified amounts of protein. (D) Comparison of the rate of cleavage of X0 and X0n by Mus81-Eme1. Reactions (80 μ l) contained 1.3 nM labelled junction and 0.8 nM of protein. A 9 μ l volume of samples was withdrawn at the stated intervals for native PAGE analysis. The data are the mean of three experiments, and error bars represent the standard deviations. (E) Single turnover kinetic analysis of X0 cleavage by Mus81-Eme1. Reactions (40 μ l) contained 13.4 nM Mus81-Eme1 and 0.9 nM X0. A gel mobility shift assay confirmed that all of the X0 was bound by Mus81-Eme1 under these conditions (data not shown). The data are the mean of three experiments, and error bars represent the standard deviations.

little difference in the amount of cleavage between these junctions (Figure 3C, compare lanes a–e with k–o). Similar data were obtained when we compared X0 with X26 (data not shown). These data confirm that Mus81-Eme1 is recognising and cleaving intact four-way junctions rather than some transient feature that mimics a preferred substrate.

Additional factors are not required to explain the HJ cleavage activity of endogenous Mus81

Endogenous Mus81-Eme1 that is partially purified from *S. pombe* cells exhibits robust X0 cleavage activity (Gaillard *et al*, 2003). Until now this was in marked contrast to preparations of recombinant Mus81-Eme1 and led to the suggestion that endogenous Mus81-Eme1 copurified with some kind of specific or nonspecific activating factor (Gaillard *et al*, 2003). In order to gauge how our new recombinant Mus81-Eme1 measures-up to its endogenous form, we compared its rate of X0n and X0 cleavage under conditions that are similar to those used previously for endogenous Mus81-Eme1 (Gaillard *et al*, 2003). With an amount of Mus81-Eme1 that gave approximately the same rate of X0n cleavage as obtained with 3 μ l of endogenous enzyme, we observed a slightly faster rate of cleavage of X0 with the recombinant enzyme than had been recorded for the endogenous enzyme (Figure 3D). These data show that both endogenous and recombinant Mus81-Eme1 perform similarly *in vitro*, and therefore there is no need to invoke the idea of an activating factor in endogenous Mus81-Eme1 preparations.

The rate of HJ cleavage by Mus81-Eme1 and RuvC are comparable under single turnover conditions

RuvC from *E. coli* is one of the best known and characterised HJ resolvases. It cleaves HJs with a single turnover rate of 0.25 min^{-1} at 37°C (Fogg *et al*, 1999). In order to see how well Mus81-Eme1 compares to RuvC for HJ cleavage, we measured its single turnover rate for cleavage of X0. To do this, X0 was preincubated with a molar excess of Mus81-Eme1 at 30°C for 5 min. The reaction was then started by the addition of 2.5 mM $MgCl_2$ and samples were withdrawn at timed intervals for assessment of the amount of junction cleavage by native PAGE analysis. The mean data from three experiments are plotted in Figure 3E, from which a single turnover rate of 0.26 min^{-1} can be calculated. This is almost identical to the value obtained for RuvC, and shows that in terms of the chemical steps of catalysis, Mus81-Eme1 is just as capable as RuvC of cleaving HJs *in vitro*.

Single turnover kinetic analysis does not measure substrate association and product dissociation. These steps may be rate limiting for Mus81-Eme1. Indeed, although we have been able to measure multiple turnover for X0n cleavage by Mus81-Eme1, we have been unable to do so for X0 cleavage (data not shown). After X0 cleavage, Mus81-Eme1 may remain bound to the reaction products. Alternatively, the putative dimer of heterodimers form of Mus81-Eme1 might be disrupted following catalysis, and then be unable to reform *in vitro*. If true, then multiple turnover for nicked HJ cleavage might be explained by Mus81-Eme1 functioning as a single heterodimer.

Figure 4 Comparing the binding of X0 and X0n by Mus81-Mms4. (A) Binding of X0 and X0n with increasing concentrations of Mus81-Mms4 in the presence of EDTA. (B, C) The effect of linear double-stranded competitor DNA on X0 and X0n binding by Mus81-Mms4 in the presence of EDTA (B) and 200 μM CaCl₂ (C). In both (B) and (C), the right-hand panels show mean data from three experiments represented in the left-hand panels. Error bars represent the standard deviations.

What underlies Mus81's preference for nicked HJs versus intact HJs?

So far we have established that Mus81 can cleave intact HJs *in vitro*; however, it is also clear that it cleaves nicked HJs more efficiently (e.g. Figure 3C and D). This difference could be explained by a nick and counter-nick mechanism for cleaving intact junctions (Gaillard *et al*, 2003). Here, the first incision is made with much slower kinetics than the second incision. Cleavage of the nicked HJ would be more efficient because it would proceed with the kinetics of the second incision. A number of HJ resolvases, including RuvC, operate by a nick and counter-nick mechanism, where the first incision is slower than the second (Lilley and White, 2001), and like Mus81, a difference in cleavage efficiency between nicked and intact HJs can be observed (e.g. in Figure 5, RuvC converts the nicked version of junction M1 into products better than the intact version).

Mus81's preference for nicked HJs may also reflect a difference in DNA binding affinities. To investigate this, we

Figure 5 The effect of arm length on X junction cleavage. (A) Native gels showing the cleavage of long-armed M1 and M1n by Mus81-Eme1 and RuvC. Reactions (20 μl) contained 1 nM junction DNA and 6.7 nM Mus81-Eme1 or 10 nM RuvC as indicated. RuvC cleavage reactions contained 10 mM MgCl₂. The bottom panel is a schematic representation of the long-armed M1(n) showing its bi-mobile core and the length of its arms. The asterisk indicates the radiolabel. (B) Same as in (A) but using short-armed M1(n). Note that the M1n short-armed junction runs as a smear because it is relatively unstable and tends to fall apart during PAGE.

employed gel mobility shift assays. In preliminary experiments, weak gel shifts were detected with Mus81-Eme1 incubated with either X0 or X0n (data not shown). However, more robust DNA binding was obtained with Mus81-Mms4, and therefore we focused on this enzyme for our analysis of junction binding. Both X0 and X0n were shifted to a low-mobility complex (complex 2) by a 33- to 50-fold excess of the putative Mus81-Mms4 tetramer (Figure 4A, lanes e, f, k and l). Addition of an antibody directed to the His tag on Mms4 resulted in a super-shift of this complex, confirming that it is likely to contain Mus81-Mms4 (Supplementary Figure S5A). At lower concentrations of protein, a faster migrating complex (complex 1) is visible with X0n (Figure 4A, lanes i and j). Intriguingly, the *E. coli* HJ-binding protein RuvA, which binds as two tetramers to both X0 and X0n, has virtually the same mobility as complex 1 (Figure 4A, compare lanes b and h with i and j). Two tetramers of RuvA have a molecular weight of ~176 kDa, so it is tempting to speculate that complex 1 represents the binding of a single heterodimer of Mus81-Mms4 to X0n, whereas complex 2 represents the binding of two heterodimers. However, gel electrophoretic mobility of protein-junction complexes is not only dependent on size, but also on shape and charge, and therefore further experiments are needed to ascertain the stoichiometry of Mus81-Mms4 binding to X0 and X0n.

Having established that we can detect binding of Mus81-Mms4 to both X0 and X0n, we next compared Mus81-Mms4's

specificity for these junctions by measuring complex formation in the presence of increasing amounts of a nonspecific unlabelled competitor DNA. Mindful of our observation that MgCl_2 concentration dramatically affects the relative cleavage efficiencies of X0 and X0n (Figure 3 and Supplementary Figure S3), we performed this experiment both in the absence (Figure 4B) and presence (Figure 4C) of divalent metal ions. To prevent junction cleavage, we substituted MgCl_2 with CaCl_2 . In the absence of CaCl_2 , increasing amounts of competitor DNA resulted in similar reductions in overall X0 and X0n DNA binding, with ~80% of X0 and ~65% of X0n DNA binding being lost with a 55-fold excess (100 ng) of competitor DNA over junction DNA (Figure 4B). However, X0 complex 2 is more resistant to competitor DNA than X0n complex 2, which is seemingly lost in favour of complex 1 (Figure 4B, compare lanes d–g with k–n). At present we are uncertain of the significance of this. In the presence of sufficient CaCl_2 to promote junction folding, there is a more dramatic difference, with X0n binding becoming more resistant to competitor DNA and X0 binding becoming less resistant (Figure 4C). These data indicate that in the presence of divalent metal ions, Mus81's binding affinity for nicked HJs increases, whereas it decreases for intact HJs. This behaviour is not observed for other HJ binding proteins such as RuvA and RuvC, which show the same binding affinities for X0 and X0n in 200 μM CaCl_2 (Supplementary Figure S5B and C). It also provides an explanation for why X0 cleavage is favoured at low concentrations of MgCl_2 , whereas X0n cleavage is optimal at relatively high concentrations (Figure 3A and B and Supplementary Figure S4). As discussed above, divalent metal ions affect junction folding and possibly Mus81-Mms4 conformation. We suspect that one or both of these factors underlies its effect on Mus81's affinity for nicked and intact HJs.

The effect of junction size on Mus81-Eme1 cleavage

While comparing Mus81 with RuvC for HJ cleavage we discovered that junction arm length is critical for intact HJ cleavage by Mus81-Eme1. For example, when we compare cleavage of a bi-mobile HJ, called M1, with 24–25 bp arms, with a version of M1 that has 15 bp arms, only the longer-armed junction is cleaved (Figure 5, compare lanes b and h, and Supplementary Figure S6). In contrast, both junctions are cleaved equally well if they contain a nick at the CO point (lanes e and k). Similar data were obtained with Mus81-Mms4 (data not shown). Shortening the arms of M1 does not fundamentally alter its characteristics as an HJ, because both short- and long-armed forms of M1 are cleaved equally well by RuvC (note that the core sequence of M1 provides a sub optimal cleavage site for RuvC; Sha *et al*, 2000) (Figure 5, lanes c, f, i and l). Therefore, these data suggest that Mus81-Eme1 requires a larger binding interface for cleaving intact HJs than for cleaving nicked HJs. So far we have not observed an obvious difference in the binding of the short- and long-armed forms of M1 by Mus81-Mms4 (data not shown). Nevertheless, we suspect that intact HJ cleavage requires a larger footprint of protein–DNA contacts than is needed for nicked HJ cleavage.

Mus81-Eme1 is directed by a nick whereas RuvC is not

In *S. pombe*, interhomologue recombination during meiosis mostly generates COs (Young *et al*, 2002). This CO bias has

been attributed to Mus81-Eme1's ability to cleave nicked HJs (Osman *et al*, 2003). However, if the junction branch migrates a small distance away from the nick, will it be recognised as an intact HJ by Mus81-Eme1, and therefore be cleaved without a CO bias? To answer this question, we compared the Mus81-Eme1 cleavage sites within intact and nicked forms of X26, which contain a 26 bp homologous core, in which the junction point can branch migrate. In the intact form of X26, cleavage sites are found throughout most of the homologous core, albeit certain sites are cleaved more than others (Figure 6A and B, lane c, and data not shown). The preferred sites tend to be towards the 5' end of each strand within the homologous core, which is probably due to the fact that Mus81-Eme1 cleaves 5' of a junction point (Figure 6C and D, and data not shown). Site preferences may also be due to limited sequence dependence for cleavage, or the fact that the junction point is likely to reside at certain sites more than others. In the nicked form of X26 (X26n), the majority of cleavages are redirected away from strands 1, 2 and 4, to be concentrated in a region of 13 nucleotides within strand 3, which are symmetrically related to sites that are 5' of the nick in strand 1 (Figure 6A and B, lane f, and data not shown). Similar data were obtained for *S. cerevisiae* Mus81-Mms4 (Supplementary Figure S7). Quantification of the amount of cleavage in strand 2 of X26 and X26n illustrates the magnitude of the redirection of strand cleavage by the nick (Figure 6E). The redirection of Mus81-Eme1 cleavage is in marked contrast with RuvC, which cleaves at the same sites within X26 and X26n (Figure 6A and B, lanes b and e). These data highlight an important distinction between Mus81-Eme1 and HJ resolvases like RuvC, which is that the orientation of HJ cleavage by Mus81-Eme1 is directed by the existence of a pre-existing nick in the vicinity of the CO point, whereas HJ resolvases, like RuvC, only cleave junctions that are located at specific nucleotide sequences (Shah *et al*, 1994; Lilley and White, 2001). In other words, RuvC will only cleave opposite an existing nick if the appropriate sequence is present. Sequence specificity is thought to endow a resolvase with structure selectivity for cleaving only HJs (Lilley and White, 2001). This is because HJs, unlike many other branched and distorted DNAs, can relocate their junction point to a cleavable sequence by undergoing branch migration. A sequence dependence for cleavage is not a constraint that is imposed on Mus81-Eme1. In this regard, Mus81-Eme1 is more akin to the bacteriophage resolvases T4 endonuclease VII and T7 endonuclease I, which have only a limited sequence requirement for cleavage (Picksley *et al*, 1990). Consequently, these enzymes are able to cleave a wider range of junctions.

Evidence that Mus81-Eme1 might cleave intact HJs *in vivo*

Having established that Mus81-Eme1 can cleave intact HJs *in vitro*, the question arises whether it does the same *in vivo*? We are trying to develop ways of directly answering this question by distinguishing nicked and intact HJs during a meiotic time course in *S. pombe*. This is technically demanding and might take a long time to achieve. In the meantime we can offer a less direct experiment to address this question. This depends on the ability of the *E. coli* RusA HJ resolvase to substitute for Mus81-Eme1 during meiotic recombination in *S. pombe* (Boddy *et al*, 2001; Osman *et al*, 2003; Smith *et al*, 2003). RusA, like RuvC, is highly selective for cleaving HJs, and its

Figure 6 The effect of a strand nick on cleavage site selection by Mus81-Eme1 and RuvC. (A, B) Denaturing gels showing cleavages in strands 2 and 3 of X26 and X26n made by Mus81-Eme1 and RuvC. Reactions (40 μ l) contained 1 nM junction DNA and either 50 nM RuvC or 6.7 nM Mus81-Eme1. RuvC cleavage reactions contained 10 mM $MgCl_2$. Selected cleavage sites are numbered. (C, D) Schematic of X26(n) showing the positions of the selected Mus81-Eme1 cleavage sites shown in (A) and (B). The asterisk indicates the site of the nick in X26n. (E) Histogram of the amount of cleavage by Mus81-Eme1 at each of the 18 selected sites in strand 2 of X26 and X26n shown in (B). The data are the mean of three experiments. Error bars are omitted for clarity.

orientation of cleavage is not affected by the presence of a nick (Bolt and Lloyd, 2002; Osman *et al*, 2003). Consequently, RusA should resolve HJs that accumulate in the absence of Mus81-Eme1 to give an equal ratio of COs and NCOs. Therefore, if Mus81-Eme1 generates only COs, then its substitution by RusA should give half the number of wild-type COs. To test this, we measured the effect of RusA expression on the number of gene conversion events at *ade6* that are associated with a CO of flanking *ura4-aim2* and *his3-aim* markers by random spore analysis, both in a wild-type and a *mus81*⁻ mutant strain (Figure 7). RusA was expressed from the *nmt1* promoter in plasmid pREP1 (Doe *et al*, 2000), and therefore as controls we used strains containing the empty pREP1 vector (in the wild type), as well as a plasmid that expresses both Mus81 and Eme1 from independent *nmt1* promoters (in both the wild type and *mus81*⁻ mutant). In the wild type, containing pREP1, the mean frequency of *ade*⁺ convertants is 0.57%, and on average 66.3% of these *ade*⁺ convertants are associated with a CO of the flanking *ura4-aim2* and *his3-aim* markers (Figure 7B and Supplementary Table S1). Similar values were obtained with RusA expression and Mus81-Eme1 overexpression in the wild type and with Mus81-Eme1 overexpression in the *mus81*⁻ mutant (Figure 7B and Supplementary Table S1). In contrast, the expression of RusA in the *mus81*⁻ mutant, whilst delivering a wild-type frequency of *ade*⁺ convertants, results in a reduction in the percentage of convertants that are associated with a CO (Figure 7B and Supplementary Table S1). However, this reduction is not to half the wild type value (33.15%), but to a value (39.63%) that is significantly higher ($P = 5 \times 10^{-6}$).

Figure 7 Comparing Mus81-Eme1 and RuvC for CO formation during meiosis in *S. pombe*. (A) Schematic of the cross to assess meiotic recombination at the *ade6* locus. The filled circles indicate the relative positions of the M26 and L469 mutations. (B) Bar diagram showing the percentage of Ade⁺ recombinants associated with crossing over in the *ura4-aim2 - ade6 - his3-aim* interval for the indicated crosses. Error bars represent standard deviations about the mean values.

It is also significantly ($P = 5 \times 10^{-8}$) more than half the value for the *mus81*[−] mutant containing the Mus81-Eme1 expression plasmid (=31.56%). These data show that the strong CO bias in *S. pombe* is dependent on Mus81-Eme1 and cannot be achieved by any HJ resolvase. However, they are not in accord with the idea that Mus81-Eme1 only generates COs.

If we assume that RsaA only resolves HJs that accumulate in the absence of Mus81-Eme1, and in so doing generates an equal number of COs and NCOs, then the percentage of ade6⁺ convertants that come from a Mus81-dependent pathway is approximately 79%. This is significantly more than the number of COs observed in the wild type, and suggests that Mus81-Eme1 is responsible for the formation of more than a third of NCOs, the remaining ones presumably derive from SDSA. As the cleavage of D-loops and nicked HJs by Mus81-Eme1 would only generate COs, we propose that the NCOs come from the cleavage of intact HJs by Mus81-Eme1, which will occur without an orientation bias. Likewise, a percentage of COs will come from intact HJ cleavage. However, this argument is based on the assumption that RsaA is not itself affected by the protein environment *in vivo*, so more direct experiments are needed to substantiate this claim.

Conclusion

There has been much controversy concerning whether Mus81-Eme1 should be classed as an HJ resolvase (Haber and Heyer, 2001; Hollingsworth and Brill, 2004). Here, we show that it can perform the central catalytic steps of dual strand cleavage as efficiently as the HJ resolvase RuvC. However, the dual incisions made by classical HJ resolvases are symmetrical, and therefore yield two nicked duplex species, which are directly repairable by DNA ligase (Lilley and White, 2001). The dual incisions made by Mus81-Eme1 appear to be asymmetric, yielding linear products with short single-strand gaps or flaps that cannot be repaired directly by ligation (Boddy *et al*, 2001; Chen *et al*, 2001; Constantinou *et al*, 2002, and our unpublished data). This slightly messier junction resolution should not be problematic *in vivo*, where there are polymerases and flap endonucleases that could 'tidy-up' the products of a Mus81-Eme1 cleavage ready for ligation. Nevertheless, Mus81-Eme1 fails to satisfy one of the criteria of a classical resolvase, and therefore is perhaps best placed in a new sub-class of HJ resolvases.

Although Mus81-Eme1 can cleave intact HJs, it clearly prefers to bind and cleave nicked HJs. It is therefore reasonable to suspect that during meiosis, Mus81-Eme1 primarily acts on D-loops and/or nicked HJs, and thereby establishes a CO bias (Figure 1). However, when a nicked HJ is ligated before cleavage, Mus81-Eme1 could resort to its back-up activity of intact HJ cleavage. Several observations suggest that Mus81-Eme1's mode of actions for cleaving intact and nicked HJs are different. For example, intact HJ cleavage is inhibited at Mg²⁺ concentrations that are optimal for cleaving nicked HJs. Intact HJ cleavage also requires a larger binding interface, and is limited to a single turnover under our current *in vitro* conditions. We suspect that Mus81-Eme1 can cleave nicked HJs in its heterodimeric form, whereas the cleavage of intact HJs requires Mus81-Eme1 to assemble into a dimer of heterodimers. Modulating Mus81-Eme1's switch between these two forms could provide a means of controlling its action *in vivo*.

Finally, although our focus has been on meiotic recombination, Mus81-Eme1 also functions in mitotic cells to promote the repair of DNA interstrand crosslinks and stalled and broken replication forks (Doe *et al*, 2002; Abraham *et al*, 2003; Bastin-Shanower *et al*, 2003; McPherson *et al*, 2004; Dendouga *et al*, 2005; Hiyama *et al*, 2006). HJ cleavage may be important for these processes too, for example, the repair of a broken replication fork probably involves the formation of a single D-loop, which can mature into a nicked and then intact HJ (Paques and Haber, 1999; McGlynn and Lloyd, 2002). Mus81-Eme1's versatility as a junction resolvase would ensure that it could process the junction formed during fork repair at any stage during its maturation.

Materials and methods

Proteins

The purification of recombinant *S. pombe* Mus81-Eme1, Mus81^{DD}-Eme1 and *S. cerevisiae* Mus81-Mms4 was adapted from an earlier protocol (Doe *et al*, 2002; Whitby *et al*, 2003) and is described in full in Supplementary data. RuvC was purified as described (Dunderdale *et al*, 1994), with some modifications detailed in Supplementary data. RuvA was purified as described (Lloyd and Sharples, 1993).

Protein concentrations were estimated using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Amounts of RuvC and RuvA are expressed in moles of monomer, whereas both Mus81-Eme1 and Mus81-Mms4 are expressed in moles of dimers of heterodimers.

Analytical ultracentrifugation

Sedimentation velocity and equilibrium measurements were performed using a Beckman Ultima XL-I analytical ultracentrifuge. Velocity experiments used both 40 000 and 50 000 r.p.m. rotor speeds; equilibrium experiments used 6000, 8000, 10 500, 15 000 and 18 000 r.p.m. Data were collected using absorbance optics (280 nm incident light) and analysed using Sedfit (Schuck and Rossmanith, 2000) for velocity data and UltraSpin (Altamirano *et al*, 2001) for the equilibrium data. Velocity analysis used the g(s) mode in which different species are apparent as distinct Gaussian distributions of apparent sedimentation coefficient. Equilibrium analysis used a single-species equation to calculate a whole-cell weight-average molecular weight, which then displays self-association or non-ideality in comparison of apparent values over a concentration range.

DNA substrates

The oligonucleotides used to make the DNA substrates are described in Supplementary data. The procedures for annealing and substrate preparation have been described previously (Parsons *et al*, 1990; Whitby and Dixon, 1998). In some cases (Figures 3A and B, 4, 6 and Supplementary Figures S5 and S7) the nicked X junction contains a 5' phosphate at the nick site. In all other cases the nick site is not phosphorylated. Phosphorylation of the nick site in X0n and X26n has little or no effect on the efficiency of junction binding/cleavage by Mus81-Eme1 or Mus81-Mms4 (our unpublished data). DNA substrates were radiolabelled at the 5'-end of one of their component oligonucleotides, as indicated using [γ -³²P]ATP and polynucleotide kinase. The concentration of DNA substrates was estimated by relating the specific activity of the labelled oligonucleotide to the activity of the purified substrate, and is expressed in molar concentrations of DNA substrate.

Nuclease assays

Cleavage reactions and their analysis by native (10% polyacrylamide) and denaturing (15% polyacrylamide) PAGE have been described previously (Whitby *et al*, 2003). The reaction buffer contained 25 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 100 µg/ml bovine serum albumin, 6% glycerol, and 2.5 mM MgCl₂, unless otherwise stated. Reactions were started by the addition of protein and were incubated at 30°C for 30 min, unless otherwise stated. Reactions were stopped by the addition of one-fifth volume of stop mix (2.5% SDS, 200 mM EDTA and 10 mg/ml proteinase K).

followed by incubation for a further 15 min at 30°C to deproteinize the mixture. For native PAGE, samples were mixed with loading dye before running on the gel. For denaturing gels, reactions were extracted with phenolchloroformisoamyl 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. Maxam-Gilbert sequence ladders of the appropriate radiolabelled oligo were used to map cleavage sites by denaturing PAGE. A 1.5 base allowance was made to compensate for the nucleoside eliminated in the sequencing reaction. Dried gels were analyzed with a Fuji FLA3000 PhosphorImager.

Gel mobility shift assays

Reaction mixtures (20 µl) contained 1.5 nM labelled junction DNA in binding buffer (25 mM Tris-HCl, pH 8.0, 1 mM DTT, 100 µg/ml BSA and 6% glycerol) plus 0.2 mM CaCl₂ or 0.2 mM EDTA together with double-stranded competitor DNA (poly [dI.dC]-poly [dI.dC], Amersham Pharmacia Biotech) as indicated. Reactions were started by the addition of protein (as indicated) and incubated on ice for 30 min before loading onto a 4% native polyacrylamide gel in low ionic strength buffer (6.7 mM Tris-HCl, pH 8.0, 3.3 mM sodium acetate and 2 mM EDTA). Gels for analysing the effect of Ca²⁺ on binding contained 200 µM CaCl₂ instead of EDTA in the buffer.

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Electrophoresis of gels was typically performed for 2 h at 160 V at room temperature, with continuous buffer recirculation, using gels and buffer that were precooled at 4°C. Dried gels were analyzed with a Fuji FLA3000 PhosphorImager.

Strains and plasmids

The strains and plasmids used in the recombination assay are described in Supplementary data.

Recombination assay

Recombination assays were essentially as described (Osman *et al*, 2003) and details are given in Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Julie Dixon for technical assistance. This work was supported by a Senior Research Fellowship (057586/Z/99/A) from the Wellcome Trust awarded to MCW. LG was funded by a Prize Studentship from the Wellcome Trust and RG by a University Research Fellowship from the Royal Society.

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