Regulation of fragile sites expression in budding yeast by MEC1, RRM3 and hydroxyurea

Nadia Hashash, Anthony L. Johnson and Rita S. Cha*

Stem Cell Biology and Developmental Genetics, National Institute for Medical Research, MRC, The Ridgeway, London NW7 1AA, UK

*Author for correspondence (rcha@nimr.mrc.ac.uk)

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Summary
Fragile sites are specific loci within the genome that exhibit increased tendencies for chromosome breakage. They are conserved among mammals and are also found in lower eukaryotes including yeast and fly. Many conditions, including mutations and exogenous factors, contribute to fragile site expression, but the nature of interaction among them remains elusive. Here, we investigated this by examining the combined effects of rrm3Δ, mec1 and hydroxyurea (HU), three conditions that induce fragile sites, on expression of the replication slow zone (RSZ), a type of fragile site in budding yeast. Contrary to the expectation that each factor would contribute to fragile site expression in an independent manner, we show that rrm3Δ and high concentrations of HU suppressed RSZ expression in mec1-4ts cells. Further analyses revealed that rrm3Δ suppression occurs via promotion of Sm11 degradation, whereas HU suppresses RSZ via a premature commitment to inviability. Taken together, these observations demonstrate that: (1) the yeast genome contains different types of fragile site with regard to regulation of their expression, and (2) each fragile-site-inducing condition does not act independently, but can elicit a cellular response that can paradoxically prevent the expression of a specific type(s) of fragile sites.

Key words: Fragile sites, Chromosome breakage, MEC1, RRM3, SML1, HU, dNTPs, Checkpoint

Introduction
Accidental double strand breaks (DSBs) arise during unperturbed proliferation. Such breakage is observed in all organisms examined to date ranging from bacteria to humans, suggesting that the break formation is an undesired byproduct of an evolutionarily conserved process(es). Notably, these DSBs do not arise randomly throughout the genome but at specific locations, often referred to as fragile sites. Examples of such breakage-prone regions of a genome include the bacterial ter (Hill, 1996), the budding yeast replication slow zones (RSZs) (Cha and Kleckner, 2002), and the mammalian common and rare fragile sites (Durkin and Glover, 2007; Sutherland and Hecht, 1985).

Many conditions promote fragile site expression. For instance, inactivation of budding yeast Rrm3, an evolutionarily conserved DNA helicase, leads to chromosome breakage at ~1400 discrete fork pause sites in the genome, including tRNA genes, the telomeres, and centromeres (Azvolinsky et al., 2009; Torres et al., 2004a). Fragility at these loci stems from the presence of specific non-nucleosomal DNA–protein complexes, which directly impede fork progression (Torres et al., 2004a). Thermal inactivation of Mec1, the budding yeast ATR homolog, leads to chromosome breakages that are enriched at relatively large (~10 kb) loci referred to as replication slow zones (RSZs) (Cha and Kleckner, 2002). The RSZ is a genetic determinant that occurs alternately with active replication origins along the entire length of a chromosome, except in the centromeric region. Although the mechanism underlying RSZ expression is unknown, its suppression by sm11Δ, a mutation that leads to a twofold increase in the dNTP levels (Zhao et al., 1998), suggest that the breakage might be regulated by changes in dNTP levels (Cha and Kleckner, 2002). Chromosome breakage has also been observed in checkpoint alleles of MEC1 or its downstream target RAD53 following exposure to an acute dose (200 mM) of HU (Admire et al., 2006; Raveendranathan et al., 2006). When Rad53 function is compromised, breakage is observed preferentially around a subset of early firing origins, termed compromised early origins (CEOs) (Raveendranathan et al., 2006).

Notably, the preferred sites of chromosome breakage in rrm3Δ, mec1-4ts or mec1/rad53 + 200 mM HU do not overlap much, suggesting that different conditions lead to expression of a specific type(s) of fragile sites. The situation in mammals is similar, and the fragile sites are often classified according to their inducing conditions, for example, as folate-, aphicolin- or BrdU-sensitive fragile sites (Durkin and Glover, 2007; Sutherland and Hecht, 1985). Currently, the basis for this differential regulation of fragile site expression remains poorly understood.

The aim of this study was to examine the nature of interaction among different conditions that induce fragile sites. To this end, we assessed the effect of combining rrm3Δ, mec1 and HU on expression of RSZs. Unexpectedly, we found that both rrm3Δ and high concentrations of HU suppressed RSZ expression. The implication of these observations regarding the nature of eukaryotic fragile sites and regulation of their expression is discussed.

Results and Discussion
rrm3Δ is a mild suppressor of mec1-4ts
To examine the potential interaction between rrm3Δ and mec1 in RSZ expression, we assessed the status ChrIII in mec1-4ts rrm3Δ using the pulsed-field gel electrophoresis (PFGE) followed by Southern hybridization method (Fig. 1A). As shown before (Cha and Kleckner, 2002), chromosome breakage enriched at RSZs is observed in mec1-4ts cells (Fig. 1C,D). By contrast, the breakage was notably reduced in the mec1-4ts rrm3Δ sample, suggesting that inactivation of RRM3 suppressed the breakage. Ectopic expression of RRM3 restored the chromosome breakage (Fig. 1D), confirming that the suppression was due to the loss of RRM3. We found that rrm3Δ also improved viability of mec1-4ts at 30°C and...
34°C (Fig. 1E). The extent of suppression conferred by \( rrm3 \), however, was not as robust as that conferred by \( sml1 \) or \( RNR1 \) overexpression (Desany et al., 1998; Zhao et al., 1998). Taken together, we conclude that \( rrm3 \) is a weak suppressor of \( mec1 \), which prevents DSB formation at RSZs and improves viability at restrictive temperatures. \( rrm3 \) leads to Sml1 degradation and improves S-phase progression in \( mec1 \). Elimination of Rrm3 leads to chromosomal damage that activates the intra-S-phase checkpoint (Ivessa et al., 2003). The checkpoint activation in turn leads to the removal of Sml1, a direct inhibitor of Rnr1 (Chabes et al., 2003; Zhao et al., 2001), and higher levels of dNTPs in \( rrm3 \) (Cheng et al., 2009; Taylor et al., 2005). Given that elimination of Sml1 is an efficient means of suppressing \( mec1 \) lethality (Zhao et al., 1998), the \( rrm3 \) suppression might be mediated by \( MEC1 \)-independent intra-S-phase checkpoint activation and the subsequent removal of Sml1. We addressed this possibility by examining the effects of \( rrm3 \) on the status of Sml1. Deletion of Rrm3 led to a notable reduction in Sml1 levels in both \( MEC1 \) and \( mec1 \) backgrounds. For example, whereas the reduction in Sml1 levels was transient in the wild type (WT), where it was observed mostly during early S-phase (Fig. 2A,B), the level remained low in \( MEC1 \) \( rrm3 \) culture even after the completion of bulk genome duplication. The \( rrm3 \) \( mec1 \) double mutant also exhibited a modest decrease in Sml1 levels at the G1–S transition compared with the \( mec1 \) control, and a notable improvement in bulk genome duplication (Fig. 2A). In the absence of Mec1 (e.g. \( mec1 \) at restrictive temperature), a related kinase Tel1 carries out many of the functions of Mec1 (Carballo and Cha, 2007). Thus, if \( rrm3 \) suppression was via \( MEC1 \)-independent checkpoint activation, the suppression is likely to require \( TEL1 \). Indeed, we found that the \( rrm3 \) suppression of RSZ expression was \( TEL1 \) dependent (Fig. 2C). The current findings imply that chromosome breakage at RSZs and that at the \( rrm3 \)-sensitive sites are differentially regulated. Specifically, although the \( rrm3 \)-dependent downregulation of
Sml1 (above) and the subsequent increase in dNTP levels (Cheng et al., 2009; Taylor et al., 2005) can suppress the RSZ expression, these effects apparently exert no impact on the estimated ~1400 rrm3\(\Delta\)-sensitive fragile sites (Torres et al., 2004a). The observation that rrm3\(\Delta\) does not exhibit sensitivity to HU (Torres et al., 2004b), is also consistent with the notion that the nature of defect(s) conferred by rrm3\(\Delta\) is unrelated to a reduction in dNTP levels. We propose that RSZs and the rrm3\(\Delta\)-sensitive sites represent two different types of yeast fragile site whose expression is regulated, respectively, by a reduction in dNTP levels (this study) or the presence of non-nucleosomal proteins that physically impede fork progression (Ivessa et al., 2003).

HU induction of RSZ expression

If a RSZ is a dNTP-sensitive fragile site, exposure to HU might promote its expression. The effects of HU might also depend on the dose as well as the basal level dNTPs in the cell. To test this idea, we assessed the effects of HU in three mec1 situations that differed with respect to the basal level dNTP concentrations: (1) mec1-4ts at 30°C, with less than WT levels of dNTPs as a result of the thermal inactivation of Mec1, (2) mec1-4ts at 23°C, with near-WT levels of dNTPs, and (3) mec1\(\Delta\) sml1\(\Delta\) with greater than WT levels of dNTPs because of sml1\(\Delta\). As expected, in the absence of HU, only the mec1-4ts at 30°C acquired chromosome breakage at RSZs (Fig. 3Ai,Bi; Fig. 4Ai). mec1-4ts cells incubated at 23°C or the mec1\(\Delta\) sml1\(\Delta\) culture, in the absence of HU, efficiently completed bulk genome duplication without any signs of chromosome breakage or loss of viability (Fig. 3Bi–iii, Fig. 4A). Addition of HU in the latter two led to RSZ expression, demonstrating that HU-mediated reduction in dNTPs can promote fragile site expression irrespective of the status of Mec1’s essential function or SML1. Note that the amount of HU required for RSZ expression in mec1\(\Delta\) sml1\(\Delta\) cells was four times higher (10 mM) than that in mec1-4ts at 23°C (2.5 mM), reflecting the need for additional HU to overcome the effects of sml1\(\Delta\). We conclude that RSZ is a dNTP sensitive fragile site whose expression is triggered when the dNTP levels in the cell fall below certain threshold level(s). dNTP levels, in turn, would be determined by Mec1 and Sml1–HU, which regulate RNR activity in a positive and negative manner, respectively (Fig. 4C).

Suppression of RSZ expression by high concentrations of HU

Both mec1-4ts and mec1\(\Delta\) sml1\(\Delta\) cells exhibit a dose-dependent sensitivity to HU (Fig. 3Aii,Bii, Fig. 4Aii). Unexpectedly, however, a notable reduction in RSZ breakage is observed in the presence of highest concentrations of HU (Fig. 3Aii,Bii, Fig. 4A). We reasoned that this reduction could be due to the effects of HU on either or both of the following two requirements for RSZ expression: (1) replication fork stalling at the loci (Cha and Kleckner, 2002), and (2) the onset of a Top2-, condensin- or Cdc14-mediated mitotic chromosome event (N.H., unpublished results). Given that MEC1 was cloned based on its role in preventing G2–M progression in the presence of HU (Weinert et al., 1994), the suppression is
unlikely to be due to cell cycle arrest before onset of the required mitotic chromosomal event. Indeed, we did not observe any notable effects of HU on cell cycle progression as assessed by DAPI staining and budding morphology (Fig. 3Aiv,Biv).

mec1/rad53 mutants exposed to high concentrations of HU become committed to inviability shortly after cell initiate genome duplication (Desany et al., 1998; Cha and Kleckner, 2002). Commitment to inviability, defined as the inability of the cell to resume cell division following the removal of a sensitizing condition(s), such as HU or high temperature, is an indication that an irrecoverable damage to the cell has occurred. Evidence indicates that the commitment in mec1/rad53 cells exposed to high levels of HU is mediated by irreversible fork collapse, majority of which occurs within 3–7 kb away from an origin during early S-phase (Santocanale and Diffley, 1998; Shirahige et al., 1998; Sogo et al., 2002). Commitment to inviability in mec1ts cells at high temperature, however, is mediated by chromosome breakage at RSZs during G2–M transition (Cha and Kleckner, 2002). These considerations raise the possibility that the suppression of RSZ expression by high concentrations of HU might be due to collapse of replication forks before they reach RSZs, prematurely committing cells to inviability.

To test the above notion, we assessed the effects of HU on the kinetics of commitment to inviability in mec1-4ts cells incubated at 30°C. As expected, HU inhibited bulk genome duplication in a dose-dependent manner with a notable delay at 10 mM and an even earlier block at 50 mM (Fig. 3Aiii). The analysis reveals that chromosome breakage at RSZs is observed in the cultures (0, 0.5 mM, and 2.5 mM HU) where commitment to inviability occurred comparatively late. In these cultures, the approximate time at which 50% of the culture becomes commitment to inviability (T50) occurred at 150 minutes, almost 100 minutes after the genome duplication reached mid-late S-phase (Fig. 3Aiv,vii), by which time, replication forks have reached RSZs (Cha and Kleckner, 2002). In contrast, the T50 in the mec1-4ts cultures without RSZ expression (10 mM and 50 mM HU) occurred notably earlier, at 40 and 20 minutes following the release, respectively (Fig. 3Aiv). At these times, the cells in the corresponding cultures were either just exiting (10 mM) or still in (50 mM) the small budded stage, with the genome duplication stuck at its early stage (Fig. 3Aiii). Note that the reduced break signals in the 10 mM or 50 mM HU cultures, when dNTP concentrations fall below the viable threshold (asterisks), mec1 or rad53 cells lose viability. If the extent of dNTP depletion is modest (the area marked by the grey box), RSZ expression is observed (grey circles). When the dNTP concentration falls below this level, the cells become prematurely committed to death before RSZ expression (black circles).

Fig. 4. HU induction of RSZ expression in various checkpoint mutants. (A) α-factor-arrested mec1Δ sml1Δ cells were released into YPD medium containing varying concentrations of HU at 30°C. Samples were collected at the indicated times and subjected to different analyses. (i) Status of ChrIII. (ii) Direct comparison between the distribution of HU induced DSBs in mec1Δ sml1Δ cells and that induced by thermal inactivation of Mec1. Roman numerals correspond to the six RSZs in ChrIII (Fig. 1B). (iii) The number of CFUs. Dotted lines denote the time at which 50% of the culture becomes commitment to inviability (T50). (iv) FACS profiles. (B) Effects of HU on the indicated checkpoint mutants. (C) A model for dNTP regulation of RSZ expression. The extent of dNTP depletion regulates RSZ expression in mec1 or rad53K277A mutants. The overall dNTP levels in each strain are determined by the status of MEC1, SML1 and the concentrations of HU. When dNTP concentrations fall below the viable threshold (asterisks), mec1 or rad53 cells lose viability. If the extent of dNTP depletion is modest (the area marked by the grey box), RSZ expression is observed (grey circles). When the dNTP concentration falls below this level, the cells become prematurely committed to death before RSZ expression (black circles).
cell to resume cell division if returned to permissive temperature before G2–M, whereas those stalled elsewhere undergo rapid collapse, remains unknown.

**RSZ expression in other checkpoint mutants**  
Is RSZ expression specific to Mec1 inactivation? To address this question, we assessed the effects of HU on various checkpoint mutants that were either sensitive (rad53 and mec1) or insensitive (rad9 or rad24) to HU (Fig. 4B). RSZ expression was observed only in the rad53 mutant demonstrating that checkpoint inactivation or HU sensitivity are not sufficient for RSZ expression; we propose that the failure to downregulate Sml1 in response to HU is a key requirement for RSZ expression. Note that RSZ expression in rad53 cells was observed in cultures exposed to 2.5 mM or 10 mM HU, but not 50 mM (Fig. 4B). The latter is consistent with the notion that the extent of dNTP depletion should be modest (Fig. 4C; above). We conclude that RSZ expression is not mecl-dependent per se, but Mec1 inactivation happens to provide all the necessary conditions — i.e. modest reduction in dNTP levels, the inability to upregulate dNTP synthesis via Sml1 downregulation, and the loss of both intra-S and G2–M checkpoint regulation.

We began the current study to address the nature of interactions among different fragile-site-inducing factors, predicting that rrm3Δ or HU would increase RSZ expression in mecl-4ts by elevating fork stalling. The unexpected results presented here reveal that different fragile-site-inducing factors do not act independently, but rather interact in a complex manner that can suppress expression of a specific type of fragile sites. The current study also reveals that the yeast genome contains both dNTP-sensitive (e.g. RSZs) and dNTP-insensitive (e.g. centromeres, rDNA genes, and telomeres) fragile sites. Given the similarities between the nature of mammalian and yeast fragile sites, the current findings provide highly relevant insights into the nature of mammalian fragile sites and regulation of their expression.

**Materials and Methods**  
**Yeast manipulation and media**  
Standard yeast manipulation procedures and growth media were used. All strains were of the SK1 background; relevant genotypes of the strains used in the current study are listed in supplementary material Table S1. To obtain a synchronous study are listed in supplementary material Table S1. To obtain a synchronous

**Spot test**  
Indicated strains were grown to log phase at 23°C in YPD. Each culture was adjusted to OD600 of 0.4 and tenfold serial dilution was spotted onto YPD agar. The agar plates were then incubated at the indicated temperature for 3 days.

**Pulse-field gel electrophoresis (PFGE)**  
Agarose plugs containing chromosome-sized DNA were prepared as previously described (Cha and Kleckner, 2002). Electrophoresis was performed using 1/3 plug per lane in a Bio-RAD CHEF Mapper using the following parameters: 6 V/cm, 5–30 seconds, 14°C, 1% agarose, 0.5× TBE and a 24 hour running time.

**Western blots**  
Whole-cell extracts were prepared from cell suspensions in 20% trichloroacetic acid by agitation with glass beads. Precipitated proteins were solubilized in SDS-PAGE sample buffer. Proteins were separated by 15% PAGE and analyzed by western blot using anti-Myc and anti-tubulin antibodies. Antibodies for western blotting were mouse monoclonal anti-Myc 9E10 (1:1000), rat monoclonal anti-tubulin YL1/2 (1:5000) and goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000; Sigma), and donkey anti-rat IgG conjugated to horseradish peroxidase (1:10,000; Sigma).

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**Supplementary material available online at**  
http://jcs.biologists.org/cgi/content/full/124/2/181/DC1

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