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Covalent protein–DNA complexes at the 5′ strand termini of meiosis-specific double-strand breaks in yeast

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ABSTRACT **During meiosis in *Saccharomyces cerevisiae*, the first chemical step in homologous recombination is the occurrence of site-specific DNA double-strand breaks (DSBs). In wild-type cells, these breaks undergo resection of their 5′ strand termini to yield molecules with 3′ single-stranded tails. We have further characterized the breaks that accumulate in *rad50S* mutant stains defective in DSB resection. We find that these DSBs are tightly associated with protein via what appears to be a covalent linkage. When genomic DNA is prepared from meiotic *rad50S* cultures without protease treatment steps, the restriction fragments diagnostic of DSBs selectively partition to the organic–aqueous interphase in phenol extractions and band at lower than normal density in CsCl density gradients. Selective partitioning and decreased buoyant density are abolished if the DNA is treated with proteinase K prior to analysis. Similar results are obtained with *sae2-1* mutant strains, which have phenotypes identical to *rad50S* mutants. The protein is bound specifically to the 5′ strand termini of DSBs and is present at both 5′ ends in at least a fraction of breaks. The stability of the complex to various protein denaturants and the strand specificity of the attachment are most consistent with a covalent linkage to DSB termini. We propose that the DSB-associated protein is the catalytic subunit of the meiotic recombination initiation nuclease and that it cleaves DNA via a covalent protein–DNA intermediate.**

During meiosis, reciprocal recombination between homologous chromosomes is essential for establishing a mechanical connection between homologs that ensures their disjunction at the first meiotic division (1). In *Saccharomyces cerevisiae*, most, if not all, of this meiotic recombination initiates via the formation and processing of DNA double-strand breaks (DSBs) (ref. 2 and references therein).

The positions and frequencies of DSBs are influenced in part by the underlying chromatin structure, since there is a close correspondence between DSB sites and nuclease hypersensitive regions in mitotic chromatin (2, 3). In addition, these nuclease hypersensitive sites undergo meiosis-specific alterations in chromatin structure prior to the appearance of DSBs (3). Meiotic DSB formation requires three genes that are also involved in mitotic DSB repair (*RAD50, MRE11*, and *XRS2*), as well as at least six meiosis-specific genes (*SPO11, MEI4, REC102, REC104, REC114*, and *MER2*) (ref. 4 and references therein). A null mutation in any of these genes abolishes both DSB formation and meiotic recombination.

In wild-type cells, DSBs are subject to rapid resection of their 5′ strand termini (5), yielding molecules with 3′ single-stranded tails of ≈600 nt (6–8). These tails are presumably used to form strand-exchange products (refs. 9–11; A. Schwacha and N.K., unpublished results) that are ultimately converted into mature recombination products. Previous work in this laboratory has identified mutations that block the 5′ resection step. Strains homozygous for certain nonnull mutations in the *RAD50* gene (*rad50S*) accumulate unresected DSBs during meiosis (5, 6); null mutations in the recently identified *SAE2* gene confer an identical phenotype (A. McKee and N.K., unpublished results). Both *rad50S* and *sae2* mutations confer a severe defect in meiotic recombination even though they leave the total level of DSBs unperturbed.

The molecular mechanisms of DSB formation and resection and the roles of the individual gene products in these processes are not yet understood. As a step toward addressing these issues, we have analyzed the unresected DSBs that accumulate in *rad50S* and *sae2* mutants. We show that DSBs from these mutant strains are associated with protein at their 5′ strand termini via what appears to be a covalent protein–DNA linkage. These observations have important implications for the biochemical mechanism of DSB formation and for the control of exonucleolytic resection.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA and RNA polymerases were from New England Biolabs. Zymolyase 100T was from ICN. Unless otherwise noted, all other reagents were from Sigma.

Yeast Strains. The strains used in this study are isogenic heterothallic derivatives from a single spore clone of the rapidly sporulating strain SK1 (12). NKY2059 = **a**/α, *ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his4XLEU2/his4BLEU2, rad50KI-81::URA3/rad50KI-81::URA3*. NKY2629 = **a**/α, *HO/HO, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, ste7-1/ste7-1, trp1::hisG/TRP1, ade2::LK/ADE2, his4XLEU2 (Bam)-URA3/his4BLEU2, sae2-1/sae2-1*. NKY2642 = **a**/α, *ho::LYS2/ho::LYS2, lys2/lys2, ura3/ura3, leu2::hisG/leu2::hisG, his4XLEU2/his4BLEU2, arg4-Nsp/ARG4*. Synchronous meiotic cultures were prepared by pregrowth in YPA [1% yeast extract/2% (wt/vol) Bacto Peptone/1% potassium acetate] followed by transfer to sporulation medium (SPM = 0.3% potassium acetate/0.02% raffinose) as described (5, 13).

Genomic DNA Preparations. Genomic DNA was prepared without protease treatment and without purification steps expected to cause loss of stable protein–DNA complexes. For small-scale preparations, 10 ml of meiotic culture (5×10^8 cells) was harvested and the DNA was isolated as described (14) except that proteinase K treatment was omitted. For large-scale preparations, 400 ml of meiotic culture was harvested 3–6 hr after transfer to SPM. Spheroplasts were prepared and lysed as described (14), and then the DNA was sheared by two passes through a 21-gauge needle and purified by CsCl density gradient centrifugation as described (15). CsCl-banded DNA was dialyzed at 4°C against three changes of 10 mM Tris·HCl, pH 8.0/1 mM EDTA/50 mM NaCl.

Analysis of Meiotic DSBs. Where indicated, DNA was deproteinized with proteinase K (200 μg/ml) in 0.5% SDS/100 mM Tris·HCl, pH 8.0/10 mM EDTA at 58°C for at least 1 hr. For analysis of meiotic DSBs, DNA was electrophoresed on

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Abbreviation: DSB, DNA double-strand break.

0.8% agarose gels in 1× TAE buffer (16) and blotted by capillary transfer to Hybond N+ membranes (Amersham). The *THR4* locus was probed with the 0.87-kb *Pst* I fragment spanning the 5′ portion of the *THR4* coding region (17). *HIS4LEU2* probes 155 and 291 were as described (18); probe 207 is the 0.6-kb *Hin*dIII–*Eco*RI fragment between *HIS4* and *STE50*. Strand-specific RNA probes were generated from plasmids pNKY290 (yielding a probe complementary to the 5′ strand) or pNKY291 (yielding a probe complementary to the 3′ strand) (8). Double-strand DNA probes and strand-specific RNA probes were radiolabeled as described (8). Blots were probed by using standard methods (19) and were visualized by autoradiography and quantitated with a Fuji phosphor imager.

Phenol Extraction and Recovery of DSB Fragments. Restriction enzyme-digested genomic DNA (20 μg in 0.5 ml) was extracted with 0.5 ml of phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol). The aqueous phase was recovered, avoiding the material at the interphase, and the DNA was precipitated with ethanol and resuspended in TE. The organic phase was back-extracted twice with TE and then mixed with 0.5 ml of water/0.1 ml of 3 M sodium acetate/100 μg of tRNA/2 ml of ethanol. The precipitate was collected by centrifugation, digested with proteinase K as described above, then reprecipitated with ethanol, and examined by electrophoresis and Southern blot analysis. (Protein-associated DSBs are not detected under the standard gel and blotting conditions unless they are deproteinized prior to electrophoresis.)

CsCl Density Gradient Centrifugation. Genomic DNA (40 μg) was digested with *Kpn* I in 0.5 ml. The samples were then digested with proteinase K (200 μg/ml) or left untreated. Samples were brought to 10 ml in 20 mM Tris·HCl, pH 8.0/5 mM EDTA/0.5% sarkosyl, plus CsCl at a final refractive index (η_D) of 1.3992 (corresponding to a density of 1.699 g/ml). Samples were centrifuged 90 hr in a Beckman type 70.1 Ti rotor at 40,000 rpm, 20°C, and then fractions (≈0.2 ml) were collected from the bottoms of the tubes. Densities were estimated from the measured η_D by the formula [density (g/ml) = 10.8601 × η_D − 13.4974] (20). DNA was precipitated by addition of 0.6 ml of double-distilled H_2O and 0.4 ml of ethanol, with 20 μg of tRNA as carrier. Pellets were resuspended in 20 μl of 10 mM Tris·HCl, pH 8.0/2 mM EDTA/0.5% SDS, digested 3 hr at 58°C with proteinase K (2 mg/ml), and then analyzed on Southern blots.

RESULTS

The *HIS4LEU2* meiotic recombination hot spot specifies two prominent DSB sites (6): site I at the *HIS4*-distal boundary of the *LEU2* insert and the weaker site II at or near a tRNA gene upstream of the *LEU2* coding region (Fig. 1). Meiotic DSBs can be detected here and elsewhere as diagnostic fragments in Southern blots of genomic DNA from meiotic cultures (e.g., ref. 6). The protocols used to prepare DNA for such analysis usually include a protease treatment step. Previous studies demonstrated that recovery of the unresected DSBs from *rad50S* mutant strains was greatly reduced when this step was omitted (D. Bishop and N.K., unpublished observations; ref. 21), suggesting that the unresected DSBs in this mutant might be tightly associated with protein.

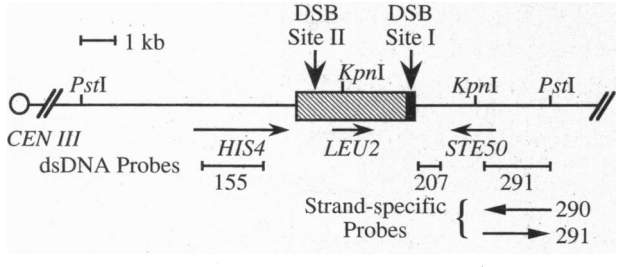

FIG. 1. Restriction map of the *HIS4LEU2* locus. A 2.8-kb segment from the *LEU2* region (hatched box) was inserted into the *HIS4* region of chromosome III (6). The solid box represents a 77-bp fragment of bacterial DNA that appeared during construction (18, 21). Horizontal arrows indicate the location and direction of transcription of the *HIS4*, *LEU2*, and *STE50* coding regions.

To investigate this possibility more carefully, we prepared genomic DNA from meiotic *rad50S* cultures without a protease treatment step and then compared DSB fragments with the parental restriction fragments by two procedures that differentiate protein–DNA complexes from naked DNA. (*i*) Stable protein–DNA complexes should selectively partition to the organic–aqueous interphase in phenol extractions. (*ii*) Such complexes should band at lower than normal density in CsCl density gradients. Moreover, DSB fragments should behave identically to parental DNA in both procedures if samples are treated with proteinase K prior to analysis.

Unresected DSBs in *rad50S* Mutants Are Stably Associated with Protein. If *rad50S* genomic DNA is treated with proteinase K and then extracted with phenol/chloroform, DNA fragments diagnostic of unresected DSBs at *HIS4LEU2* are observed at high levels and with the expected timing (Fig. 2*A Right*). The yield of such fragments is significantly reduced, however, if samples are not deproteinized prior to extraction

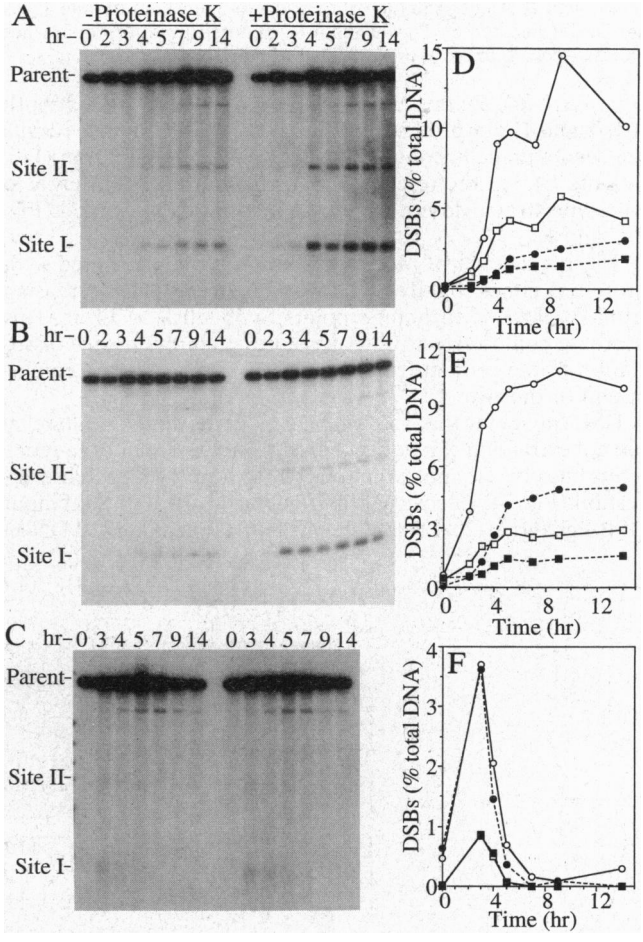

FIG. 2. Time course of meiotic DSBs in *rad50S* (*A* and *D*), *sae2-1* (*B* and *E*), and wild-type (*C* and *F*) strains. (*A–C*) Genomic DNA was isolated under nonproteolyzing conditions from meiotic cultures at the indicated times after transfer to sporulation medium. DNA was digested with *Pst* I and extracted with phenol/chloroform with (*Right*) or without (*Left*) prior proteinase K treatment. (*D–F*) Quantitation of DSBs in *A–C*. ○ and ●, Site I with or without proteinase K treatment, respectively; □ and ■, site II with or without proteinase K treatment, respectively.

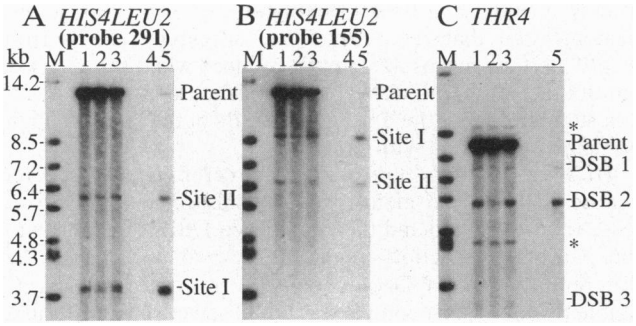

FIG. 3. Partitioning of DSB fragments to the organic–aqueous interphase. Genomic DNA prepared from a meiotic *rad50S* culture under nonproteolyzing conditions was digested with *Pst* I (*A* and *B*) or *Eco*RI (*C*) and extracted with phenol. The aqueous and organic phases were separated and the DNA from each was recovered by ethanol precipitation, deproteinized, and examined by Southern blot analysis with the indicated probes. To provide signals with similar intensities, all of the material from each organic phase but only 10% of the material from each aqueous phase was loaded on the gel. Lanes: M, λ *Bst*EII molecular size markers; 1, aqueous phase of a sample treated with proteinase K prior to extraction; 2, aqueous phase without prior proteinase K treatment; 3, sample treated with proteinase K with no phenol extraction; 4, organic phase of sample treated with proteinase K prior to extraction; 5, organic phase treated with proteinase K subsequent (but not prior) to extraction. An equivalent of lane 4 was not performed for *C*. The bands in *C* denoted by asterisks are not meiosis-specific and are presumably due to cross-hybridization.

(Fig. 2*A Left*). By this criterion, 70–90% of the DSBs at both sites I and II are protein-associated (Fig. 2*D*). Similar results are seen with cells homozygous for the *sae2-1* mutation (Fig. 2 *B* and *E*), but the resected breaks that appear transiently in wild-type strains do not appear to be protein-associated (Fig. 2 *C* and *F*).

The percentage of *rad50S* and *sae2* DSBs associated with protein declines slightly at later points in meiosis, decreasing from 70–90% at early time points to 50–70% by 14 hr. This decrease could reflect proteolysis of the complex, slow dissociation of the protein from the DNA *in vivo*, or some combination of the two.

DSB fragments that are removed from the aqueous phase by phenol extraction can be recovered from the organic–aqueous interphase by ethanol precipitation followed by protease digestion (Fig. 3). Recovery is inefficient: 15–20% of total input DSB signal (i.e., 20–25% of the protein-associated DSB DNA) is recovered by this procedure. However, this partitioning is specific for DSB fragments since only 0.06–0.14% of the input parental restriction fragment was recovered from the interphase.

Identical results are seen regardless of which side of the *HIS4LEU2* region is probed (Fig. 3 *A* and *B*). Since >50% of the DNA from each side is protein-associated, at least a fraction of the DSBs must have protein bound on both sides of the break.

DSBs are also associated with protein at another, naturally occurring hot spot for meiotic recombination, the *THR4* locus (17) (Fig. 3*C*). Therefore, these protein–DNA complexes are not a special property of the *HIS4LEU2* locus but instead are a general feature of unresected DSBs in *rad50S* and *sae2-1* mutants.

Equilibrium Density Gradient Analysis. If protein is associated with a relatively small DNA fragment such that the protein contributes a significant fraction of the mass of the complex, the buoyant density of the complex is expected to be less than that of free DNA (22–24). Therefore, *rad50S* genomic DNA was digested with *Kpn* I and subjected to equilibrium density gradient centrifugation (Fig. 4). This restriction digest yields a *HIS4LEU2* parental DNA fragment of 3.5 kb and a DSB fragment of 1.8 kb, resulting from cleavage at site I (Fig. 1). Sarkosyl was included in the CsCl gradients because it greatly improved the recovery of the protein–DNA complexes. The same qualitative results are obtained in the absence of detergent, however (data not shown).

Under the conditions used, the majority of the parental DNA bands on the gradient at an average buoyant density of ≈1.66 g/ml (fractions 29–32) (Fig. 4*A*). This density is significantly lower than the 1.699 g/ml expected from the G+C content of 39.4% (25). This aberrant banding pattern appears to be due to binding of sarkosyl to the DNA, since all of the parental DNA fragment bands at the expected density if detergent is omitted from the gradient (data not shown). The minor band at ≈1.69 g/ml (fractions 22–24) presumably represents DNA not associated with sarkosyl. A second minor band at the top of the gradient (fractions 45 and 46) is sarkosyl-dependent and is most likely due to DNA fragments trapped in sarkosyl micelles.

If the DNA is treated with proteinase K prior to banding on CsCl, the DSB fragment bands at the same position as the parental fragment (Fig. 4*A*). If the DNA is not pretreated with proteinase K, however, the DSB signal trails into the lower-density portion of the gradient (Fig. 4*B*). There is also a

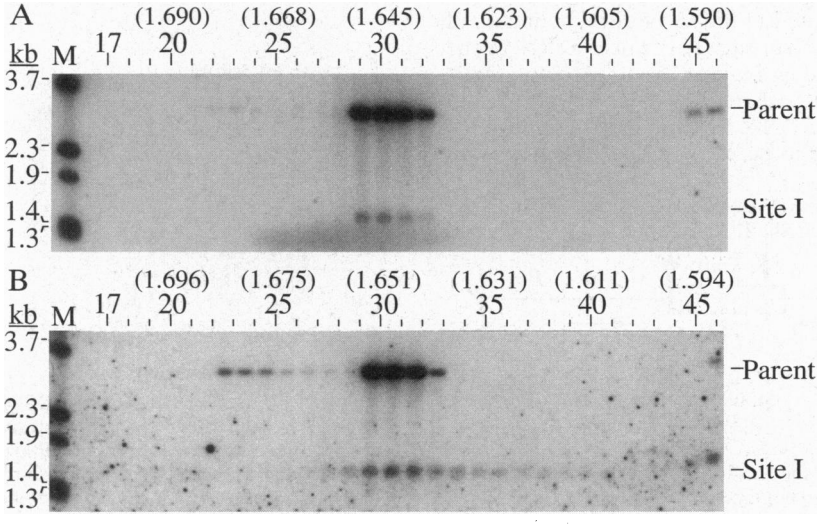

FIG. 4. Equilibrium density gradient centrifugation. Genomic DNA from a *rad50S* strain was digested with *Kpn* I and analyzed on CsCl gradients with (*A*) or without (*B*) prior treatment with proteinase K. Fractions were examined by Southern blot analysis and hybridization with probe 207. Densities (g/ml) of selected fractions are indicated in parentheses. The tops of the gradients are to the right.

significant enrichment of DSB fragments in the band at the top of the gradient (fraction 46), relative to the same portion of the gradient containing the proteinase K-treated sample (Fig. 4*A*). This enrichment is likely due to increased affinity of detergent for the protein–DNA complex relative to free DNA. The DSB signal in Fig. 4*B* is spread through a broader portion of the gradient than observed for either the parental band or the deproteinized DSB fragment. This feature is seen whether detergent is included in the gradient or not (data no shown) and may reflect heterogeneity in the size of the protein due to proteolysis during isolation. Heterogeneity in the observed buoyant densities could also result if each protein–DNA complex contains one of two or more proteins of different sizes.

Protein Is Attached to the 5′ Terminal Strand. The finding that protein is associated only with the unresected breaks in certain mutants and not with the resected breaks that appear transiently in wild-type cells suggested to us the possibility that the protein might be attached specifically to the resected (5′) strand. To investigate this possibility, partitioning of each strand of the DSB DNA to the organic–aqueous interphase was examined after restriction digestion and denaturation, with 5′ and 3′ strands monitored individually with strand-specific probes (Fig. 1).

The 5′ terminal strand partitioned to the organic phase regardless of whether the DNA was double-stranded or denatured by boiling prior to extraction (Fig. 5 *Left*). In contrast, the 3′ strand only partitioned to the organic phase when duplex DNA was extracted (Fig. 5 *Right*). Thus, the protein is specifically bound to the strand that is degraded in wild-type cells.

The Protein–DNA Association Appears to Be Covalent. The protein–DNA complex withstands a number of treatments that effectively disrupt noncovalent protein–DNA interactions, such as incubation in SDS at 65°C (data not shown), incubation in 4.5 M guanidinium hydrochloride/0.5% sarkosyl at 65°C (as part of the DNA isolation protocol; ref. 14), equilibrium centrifugation in CsCl (Fig. 4), organic extraction (Fig. 3), and boiling (Fig. 5). These properties are most consistent with a covalent protein–DNA complex.

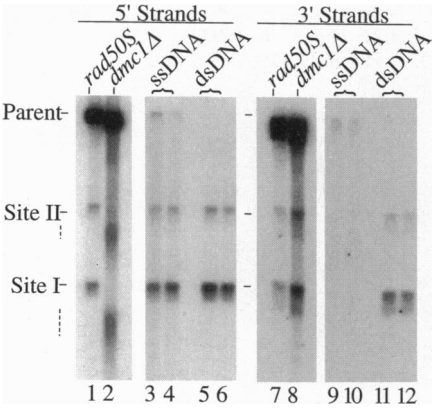

FIG. 5. Strand specificity of protein attachment to DSB DNA. *Pst* I-digested *rad50S* genomic DNA, denatured by boiling (ssDNA, lanes 3, 4, 9, and 10) or nondenatured (dsDNA, lanes 5, 6, 11, and 12), was extracted with phenol/chloroform. The material partitioning to the interphase was recovered by ethanol precipitation, deproteinized, and then analyzed on alkaline 0.8% agarose gels (16) by Southern blot analysis and hybridization with probes specific for either the 5′ strand (probe 290) (*Left*) or the 3′ strand (probe 291) (*Right*). *Pst* I-digested deproteinized genomic DNA from *rad50S* (lanes 1 and 7) or *dmc1*Δ (lanes 2 and 8) strains was electrophoresed in parallel as markers. The 5′ strands of DSBs observed in the *dmc1*Δ strain are hyperresected (8); the positions of the corresponding fragments are indicated by vertical dashed lines.

DISCUSSION

The *rad50S* and *sae2-1* mutations confer an intermediate block to meiotic recombination and cause an accumulation of unresected DSBs at meiotic recombination hot spots. We show here that these unresected DSBs are associated with protein via a stable linkage to the 5′ terminal strand on each side of the DSB. The stability of the complex strongly suggests a covalent protein–DNA linkage. Although we expect that the protein is attached at the 5′ terminus, the data do not rule out the possibility of linkage to an internal position on the 5′ strands. Our results agree with observations of two other groups who have obtained independent evidence for a *rad50S* protein–DNA complex in the course of nucleotide-resolution mapping of DSB cleavage sites (26, 27).

Several lines of evidence support the view that the unresected DSBs seen in *rad50S* (and *sae2*) mutants are true intermediates of meiotic recombination. They occur with the same timing, at the same sites, and with the same relative frequencies among different sites as do the transient resected DSBs observed in wild-type cells (ref. 6; Fig. 2). Also, formation of *rad50S* DSBs depends on the same early functions, such as *SPO11* (6), that are required for wild-type DSB formation.

If we accept that the unresected DSBs are true intermediates, it is reasonable to suppose that the covalent protein–DNA complexes are also intermediates. In support of this conclusion, these protein–DNA complexes are specific for DSBs made by the meiotic recombination initiation nuclease: site-specific DSBs made by HO endonuclease, in meiosis or mitosis, are subject to resection of their 5′ strands even in a *rad50S* mutant background, strongly suggesting that they are not covalently associated with the meiotic DSB protein *in vivo* (A. Malkova and J. E. Haber, personal communication).

The observations presented here are most easily explained if the DSB-associated protein is itself the catalytic subunit of the meiotic DSB nuclease, which cuts DNA via a covalent protein–DNA intermediate. Because protein is bound to both sides of the DSB (Fig. 3), the nuclease should consist of at least two subunits, each of which nicks one strand.

Covalent protein–DNA linkages are prominent intermediates in the DNA cleavage reactions catalyzed by several classes of enzymes, including type I and type II DNA topoisomerases (for review, see ref. 28); Tn*3* resolvase and related bacteriophage invertases (for review, see ref. 29); λ Int protein (30) and the related FLP recombinase of *S. cerevisiae* (31); and a family of proteins including virD2 protein of *Agrobacterium tumefaciens* (for review, see ref. 32) and the conjugative mobilization and rolling circle replication initiation proteins of various prokaryotic plasmids and phages (33, 34). It remains to be seen whether the DSB-associated protein is a member of one of these groups or is unrelated to previously characterized activities (see below).

A covalent protein–DNA intermediate in meiotic DSB formation is attractive for two reasons. (*i*) The bound protein may serve to protect the DSB from inappropriate degradation. Meiotic DSBs are formed with short 5′ overhangs (18, 26) or with blunt ends (27). For either terminal structure, 5′ resection would be the step that irreversibly commits the DSB to being repaired by homologous recombination, since a resected DSB can no longer be resealed by direct religation of the break. If the bound protein blocks degradation of the 5′ strand, release of the protein could serve as a critical control point for this step. (*ii*) For the DNA cleavage enzymes listed above, cleavage and resealing are isoenergetic. If the same holds for the meiotic DSB protein, then the covalent intermediate could itself reseal the DSB by direct religation. Since two subunits are required for cleavage (see above), protein–protein interactions between the subunits may serve to hold the ends of the DSB together to allow resealing.

An important question at this point is the identity of the DSB-associated protein. DNA topoisomerase II cleaves DNA via a covalent attachment to 5′ termini (28) and is known to interact with some nuclease-hypersensitive sites in mitotic chromatin *in vivo* (35, 36). However, this enzyme is not an attractive candidate for the meiotic nuclease because *top2* mutants initiate normal levels of meiotic recombination (37). Moreover, anti-topoisomerase II antiserum does not immunoprecipitate *rad50S* DSBs, and meiotic DSB sites do not correlate well with sites of drug-induced DNA cleavage by topoisomerase II *in vivo* (unpublished observations).

If the protein is the catalytic subunit of the meiotic nuclease, a null mutation in its structural gene might be expected to confer a DSB-defective phenotype. The six meiosis-specific genes required for DSB formation are thus obvious candidates. Interestingly, a portion of the MEI4 protein sequence shares similarity to a domain conserved among a family of plasmid mobilization and rolling circle replication initiation proteins (ref. 33; unpublished observations) that cleave DNA to form a covalent protein–DNA linkage to the 5′ terminal strand (see above). This sequence similarity raises the possibility that MEI4 protein may be the catalytic subunit of the meiotic nuclease and that this nuclease is evolutionarily related to a diverse family of recombination and replication initiation proteins.

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