Meiosis-Specific DNA Double-Strand Breaks Are Catalyzed by Spo11, a Member of a Widely Conserved Protein Family

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Summary

Meiotic recombination in S. cerevisiae is initiated by double-strand breaks (DSBs). In certain mutants, breaks accumulate with a covalently attached protein, suggesting that cleavage is catalyzed by the DSB-associated protein via a topoisomerase-like transesterase mechanism. We have purified these protein–DNA complexes and identified the protein as Spo11, one of several proteins required for DSB formation. These findings strongly implicate Spo11 as the catalytic subunit of the meiotic DNA cleavage activity. This is the first identification of a biochemical function for any of the gene products involved in DSB formation. Spo11 defines a protein family with other members in fission yeast, nematodes, and archaebacteria. The S. pombe homolog, rec12p, is also known to be required for meiotic recombination. Thus, these findings provide direct evidence that the mechanism of meiotic recombination initiation is evolutionarily conserved.

Introduction

In most sexually reproducing organisms, recombination plays a crucial role. It provides a potent source of genetic variation but also plays a mechanical role during meiosis, the cell division pathway that gives rise to haploid gametes during the sexual cycle. Specifically, crossover recombination between homologous chromosomes forms an essential physical connection between them that allows them to orient properly on the spindle and thus to segregate accurately to opposite poles at the first division. Correspondingly, the process of recombination occupies much of meiotic prophase and is under the control of a large number of meiosis-specific functions, which serve to coordinate temporally and spatially the stages of recombination with changes of higher-order chromosome structure and with other cellular events (see Orr-Weaver, 1995; Roeder, 1995; Kleckner, 1996 for recent reviews).

In Saccharomyces cerevisiae, meiotic recombination results from the specific induction of a pathway for the formation and subsequent processing of DNA double-strand breaks (DSBs) (Game et al., 1989; Sun et al., 1989; Cao et al., 1990). DSB formation requires the products of at least nine genes. Of these, six are known, or presumed, to be meiosis-specific: *SPO11* (Cao et al., 1990); *MEI4* (Menees et al., 1992; S. K., unpublished data); *MER2* (Rockmill et al., 1995), and *REC102*, *REC104*, and

REC114 (Bullard et al., 1996). The remaining three also function in the repair of DSBs in nonmeiotic cells: *RAD50* (Alani et al., 1990; Sugawara and Haber, 1992; Schiestl et al., 1994), *MRE11* (Ajimura et al., 1992; Johzuka and Ogawa, 1995), and *XRS2* (Ivanov et al., 1992, 1994). A null mutation in any of these genes blocks DSB formation and meiotic recombination, resulting in chromosome nondisjunction at the first meiotic division. At least some of these gene products associate with one another (e.g., Johzuka and Ogawa, 1995), presumably in some type of "DSB complex," but the biochemical roles of these proteins are unknown. Three other meiosis-specific proteins that are required for full levels of DSBs (Red1p, Hop1p, and Mek1p/Mre4p) (Mao-Draayer et al., 1996; Xu et al., 1997) appear to be either components or modulators of chromosome or chromatin structure (Hollingsworth et al., 1990; Roeder, 1995; Smith and Roeder, 1997).

Intriguingly, mutations in some, and possibly all, of the nine genes that are required for DSB formation also confer defects in other events of meiotic prophase, such as DSB-independent chromosome pairing (Weiner and Kleckner, 1994), axial element formation (Giroux et al., 1989; Alani et al., 1990), synapsis of homologous chromosomes (Giroux et al., 1989; Alani et al., 1990; Menees et al., 1992; Loidl et al., 1994; Rockmill et al., 1995), and the timing of the first meiotic division (Klapholz et al., 1985; Giroux et al., 1993). These observations highlight the interplay between the events of recombination initiation and other cellular processes of meiotic prophase.

The chemical alterations in the DNA phosphodiester backbone that accompany recombination initiation have been characterized extensively. The primary cleavage species consists of a pair of nicks closely spaced on opposite strands, with the 5′ termini on either side of the DSB covalently bound to protein (Keeney and Kleckner, 1995; Xu and Kleckner, 1995; de Massy et al., 1995; Liu et al., 1995; Xu and Petes, 1996). The simplest explanation for this structure is that the DSB-associated protein is the catalytic subunit of the meiotic DNA cleaving activity and that it cleaves DNA by a mechanism in which a nucleophilic protein side chain attacks the DNA backbone, generating a phosphodiester linkage between the 5′ strand terminus and the protein. According to this interpretation, meiotic DSB formation does not proceed by simple hydrolysis of the phosphodiester DNA backbone, but instead consists of a transesterification reaction analogous to those catalyzed by many families of enzymes, including DNA topoisomerases (reviewed in Wang, 1985).

In wild-type cells, DSBs are subject to rapid exonucleolytic resection of their 5′ strand termini to yield molecules with 3′ single-stranded tails of approximately 600 nucleotides (Alani et al., 1990; Cao et al., 1990; Sun et al., 1991; Bishop et al., 1992). Several mutations block this resection step. These include certain nonnull alleles of the *RAD50* gene (*rad50S* mutations; Alani et al., 1990) and null mutations in the *SAE2/COM1* gene (Keeney and Kleckner, 1995; McKee and Kleckner, 1997; Prinz et al., 1997). Strains homozygous for any of these mutations

accumulate high levels of the protein–DNA complex and are therefore profoundly defective for meiotic recombination even though they form essentially normal levels of the initiating lesion (Alani et al., 1990; McKee and Kleckner, 1997; Prinz et al., 1997).

We show here that the protein bound to DSB 5′ strand termini in *rad50S* cells is the product of the *SPO11* gene. *SPO11* was one of the first meiotic recombination genes identified (Esposito and Esposito, 1969; Klapholz et al., 1985) and is essential for DSB formation (see above). This finding strongly implicates Spo11 as the catalytic subunit of the meiotic DSB transesterase, thus providing the first identification of a biochemical function for any of the gene products required for DSB formation.

We further report that Spo11 defines a widely conserved family of proteins. The Schizosaccharomyces pombe homolog, rec12 protein, is also required for meiotic recombination (Lin and Smith, 1994). The structural and functional similarity between proteins from these two widely diverged fungal species strongly suggests that the mechanism of meiotic recombination initiation is evolutionarily conserved.

Results

Experimental Approach
We sought to identify the protein on the 5′ strand termini of meiotic DSBs by a direct biochemical approach. Covalent protein–DNA complexes were purified by a two-phase procedure from mutant cells that accumulate high levels of these intermediates. In the first phase, complexes were purified away from bulk proteins; in the second phase, complexes were purified away from bulk DNA. Sequence analysis of the DNA-associated proteins present after this procedure identified a single strong candidate polypeptide. Control experiments confirmed that this candidate protein is absent when protein–DNA complexes are purified from cells lacking the DSB intermediate. Finally, the identity of this polypeptide as the 5′ terminal protein was demonstrated directly: immunoprecipitation of this protein specifically coprecipitates DSB fragments but not bulk genomic DNA.

Development of a Two-Phase Procedure for Purification of Meiotic Protein–DNA Complexes
The purification procedure is outlined in Figure 1. Meiotic *rad50S* cells were harvested and spheroplasted and their nuclei separated from soluble components by hypotonic lysis of cells in the presence of protease inhibitors. The nuclear isolation step substantially reduces contamination by proteases, which are predominantly extranuclear. Preliminary experiments suggested that standard techniques, in which DNA is extracted from whole meiotic cells, result in extensive proteolysis of the DSB-associated protein, in keeping with the known induction of proteases in yeast cells entering meiosis (Zubenko and Jones, 1981).

Genomic DNA was then extracted in guanidine-HCl plus ionic detergent at 65°C and then purified away from bulk protein on a CsCl gradient. These steps are sufficiently harsh that only very tightly bound proteins, primarily covalently attached ones, would remain associated with the DNA. Protein–DNA complexes were then
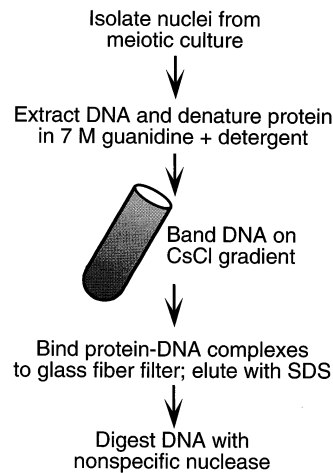

Figure 1. Scheme for Purifying from Meiotic Cells Proteins That Are Stably Associated with Genomic DNA

See text for details.

purified away from protein-free DNA by passing the CsCl gradient–purified material over a glass fiber filter, to which proteins adsorb specifically (Thomas et al., 1979). The adsorbed material was eluted from the filter and treated with a nonspecific nuclease to release proteins from the bulk of the linked DNA. The constellation of protein species was then analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). In pilot-scale purifications, this procedure yielded a relatively small number of DNA-associated proteins (Figure 2A).

To investigate whether this procedure identifies protein(s) likely to be DSB-associated, it was carried out in parallel on *rad50S* cells and on meiotic cells of a mutant in which DSBs do not occur. The polypeptide of interest should be present in the former but not the latter. Coincidentally, a *spo11* mutant was selected for this purpose. In this experiment, a limited number of bands that seemed to have the desired specificity could be discerned (Figure 2A). Polypeptides of approximately 27, 28, 34, and 45 kDa copurified with genomic DNA from *rad50S* cells but not with DNA from *spo11Δ* cells. In addition, proteins of 74 and 91 kDa copurified with genomic DNA from the *spo11Δ* strain (Figure 2A, open circles). However, these proteins are not *spo11Δ*-specific since they also were found in subsequent genomic DNA preparations from a *rad50S* strain (see below).

Spo11 Copurifies with *rad50S* Genomic DNA under Protein-Denaturing Conditions
For the next step in the analysis, stable protein–DNA complexes were purified from *rad50S* cells on a preparative scale. Candidate proteins were indentified by analysis of silver-stained SDS-PAGE protein profiles before and after nuclease digestion. A protein covalently bound to DNA is expected to run as a discrete species only if the DNA has been removed.

A number of bands (more than were observed in pilot studies) were present irrespective of nuclease digestion (Figure 2B). These bands most likely represented noncovalently attached proteins that copurified with genomic DNA in this preparation because the guanidine extraction was carried out at a lower temperature than in pilot
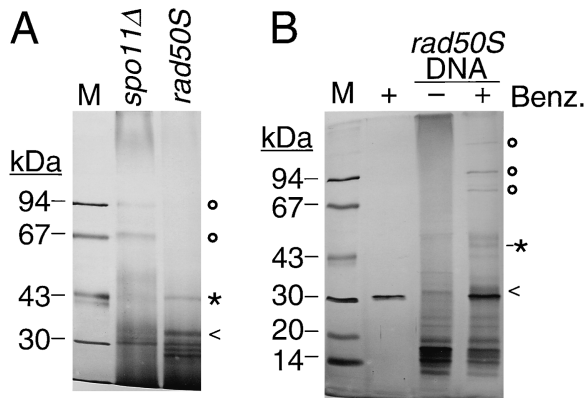
Figure 2. Purification of Meiotic DNA-Associated Proteins

(A) Pilot scale. Stable protein–DNA complexes were purified from *spo11*Δ (NKY2967)and *rad50S* (NKY2059) meiotic cultures; the DNA was digested with Benzonase; and then proteins were analyzed on a silver-stained SDS-polyacrylamide gel. The sizes of molecular mass markers (M) are indicated. The lane for each strain contains material from the equivalent of 600 ml of meiotic culture (∼3 × 10¹⁰ cells). The approximately 30 kDa band present in both the *spo11*Δ and the *rad50S* samples is Benzonase.

(B) Preparative scale. Genomic DNA-associated proteins were purified from 60 liters of meiotic *rad50S* culture (NKY2059). Aliquots (1% of total) were analyzed on silver-stained SDS-polyacrylamide gels before (−) or after (+) digestion with Benzonase (Benz). The sizes of molecular mass markers (M) are indicated. The second lane from the left contains Benzonase alone.

Carets (p34) and asterisks (p45) indicate bands that were subjected to micro–sequence analysis; the 45 kDa species is Spo11 (see Table 1 and text). Open circles indicate presumptive topoisomerase bands (see text).

studies (see Experimental Procedures). Among the bands that occurred specifically after nuclease digestion, however, two (Figure 2B, caret and asterisk, respectively) appeared to correspond to the 34 and 45 kDa polypeptides previously observed in the pilot study specifically in the *rad50S* sample but not in the *spo11*Δ sample. These bands were excised from a preparative SDS-PAGE gel, and tryptic peptides derived from them were sequenced by tandem mass spectrometry (Table 1).

The 45 kDa band is Spo11. The sequences of each of three peptides from this band exactly match sequences encoded by the *SPO11* gene (Table 1). Furthermore, the electrophoretic mobility of this protein agrees with the predicted mass of the *SPO11* gene product (45.4 kDa).

Since *SPO11* is required for DSB formation, this analysis identified Spo11 as a prime candidate for the DSB-associated protein.

The 34 kDa band contained a mixture of at least four polypeptides (Table 1). Two of the peptide sequences from this sample match ribosomal proteins S4 and L4. Given the abundance of ribosomal proteins in the cell and the avid binding of these proteins to nucleic acids, these proteins could be expected as contaminants unrelated to DSB formation. Two additional peptide sequences match either of two open reading frames (ORFs), YGR086c and YPL004c, whose predicted products are more than 70% identical to one another (data not shown). The fifth peptide sequence obtained from this sample is predicted in a region that is identical in the two ORFs. It remains to be determined whether there is any functional significance to the presence of these two proteins. Although these proteins are not the DSB-associated protein (see below), they still may play some other meiosis-specific (*rad50S*-specific) role. The high similarity of the two ORFs raises the possibility that they represent redundant functions, in which case they might not have been identified in genetic screens for meiotic mutants.

The 74 and 91 kDa proteins observed in the pilot-scale *spo11*Δ sample (Figure 2A, open circles) also appeared in the preparative-scale *rad50S* sample (Figure 2B, open circles). These proteins, along with a third with an apparent mass much greater than that of the 94 kDa marker, have sizes consistent with those of the known yeast DNA topoisomerases III (74 kDa), I (90 kDa), and II (164 kDa), respectively. The presence of these proteins would not be surprising since a small fraction of topoisomerase–DNA covalent complexes can usually be trapped by protein denaturants (Liu et al., 1983). Since these bands are not *rad50S*-dependent, they were not investigated further.
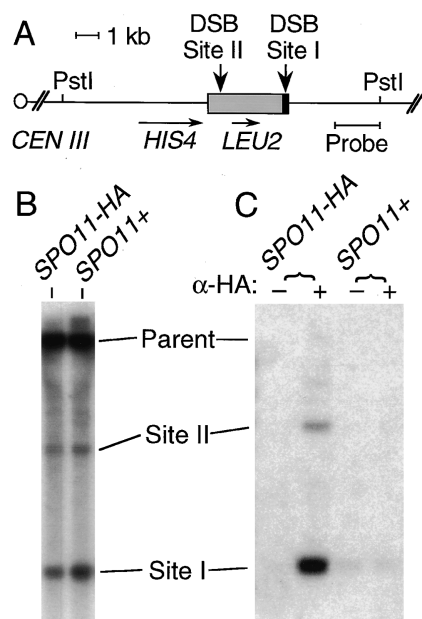
Spo11 Is Specifically Associated with DSB DNA

The preceding experiments show that Spo11 binds tightly to DNA from meiotic cells, even under conditions in which proteins are denatured. However, demonstration that Spo11 is the DSB-associated protein requires, in addition, that Spo11 be bound tightly to DSB DNA fragments specifically and not to bulk genomic DNA. We therefore tested whether immunoprecipitation of Spo11 from a *rad50S* genomic DNA preparation would specifically coprecipitate DSB fragments.

Table 1. Tryptic Peptide Sequences of Genomic DNA-Associated Proteins

Protein Band	Peptide Sequence	Sequence Match (residue nos.)
p45	[R] I* T Q* V L* A Q* N N E V H N K*—	Spo11 (320–334)
	[K] L* S T N T M L* I* T G K*—	Spo11 (247–258)
	[K] S I* Q L* L* S L* N Q* R—	Spo11 (334–344)
p34	[K] G V P Y V V T H D G R—	Ribosomal protein S4 (134–145)
	[K] N F G I* G Q* A V Q* P K*—	Ribosomal protein L4 (32–43)
	[K] I* E V L* E Q* E L* V R—	ORF YGR086c (165–175)
	[K] N A A G N F G P E L* A R—	ORF YPL004c (44–56)
	[R] A E A E S L* V A E A Q* L* S N I* T R—	YGR086c or YPL004c (175–192)

Tryptic peptide sequences were compared to a database of proteins predicted by the S. cerevisiae genome. An asterisked residue cannot be unambiguously differentiated within its isobaric pair (I/L, molecular weight = 113; Q/K, molecular weight = 128) in mass spectrometric sequencing. For these cases, the residue predicted by the DNA sequence is given. For each peptide, the predicted upstream residue (brackets) is either arginine or lysine, as expected from the cleavage specificity of trypsin. Dashes indicate blank cycles.

Figure 3. Spo11 Associates Specifically with DSB Fragments

(A) Map of the *HIS4LEU2* recombination hot spot (Cao et al., 1990). This site was created by insertion of *LEU2*-containing sequences (shaded box) downstream of the *HIS4* gene on chromosome III. The positions of the two prominent DSB sites are shown.

(B) Bulk genomic DNA was purified through the CsCl step of the procedure shown in Figure 1. Samples were collected from *rad50S* strains carrying either the wild-type *SPO11* gene (*SPO11+*, NKY2059) or an epitope-tagged Spo11 construct (*SPO11-HA*, NKY2969). DNA was digested with PstI, deproteinized, and analyzed by Southern blotting for DSBs at the *HIS4LEU2* locus.

(C) Coprecipitation of DSB fragments with Spo11-HA. DNA samples as in panel B were digested with PstI and immunoprecipitated with or without anti-HA monoclonal antibody as indicated, and the co-precipitating DNA was deproteinized and analyzed by Southern blotting.

For this purpose, DSBs were analyzed at the *HIS4-LEU2* recombination hot spot. The *HIS4LEU2* locus specifies two prominent DSB sites, site I and site II (Cao et al., 1990; Figure 3A). A Southern blot of PstI-digested meiotic DNA from a strain carrying this locus reveals DSB fragments from each of the two hot spots as well as the full-length parental restriction fragment (Figure 3B). To allow specific immunoprecipitation of Spo11 protein, the 3′ end of the *SPO11* coding sequence was modified by insertion of the sequence for a hemagglutinin epitope. The tagged construct (*SPO11-HA*) fully complements a *spo11Δ* mutant (Figure 3B; N. Chen, T. Hillmer, and C. N. G., unpublished data).

Genomic DNA was prepared from a meiotic culture of a *rad50S SPO11-HA* strain, and tagged Spo11 was immunoprecipitated with antibody to the HA epitope. In this experiment, DSB DNA fragments specifically coprecipitated with the Spo11-HA immune complexes (Figure 3C) and were enriched more than 600-fold relative to parental DNA in the immunoprecipitate. In an untreated DNA sample from the *rad50S SPO11-HA* strain, the two DSB fragments were 11.1% and 4.6% of total DNA (sites I and II, respectively; Figure 3B). In the anti-HA immuno-precipitate, in contrast, the DSB fragments were present

at high levels (82% and 17% of total DNA), while the parental fragment was barely detectable. If the anti-epitope antibody was omitted, the immunoprecipitate contained only a trace of DSB DNA, representing a low nonspecific background in the immunoprecipitation reaction. As an additional control for the specificity of the coprecipitation, immunoprecipitations were performed with DNA prepared from a strain in which the *SPO11* gene does not carry the *HA* tag (*rad50S SPO11*). In this case, only the antibody-independent background of DSB fragments was recovered in the precipitate (Figure 3C).

Stable Spo11–DNA Complexes Occur Specifically in Cells That Accumulate Protein-bound DSBs

As final confirmation that Spo11 is the DSB-associated protein, we examined the genetic requirements for detection of Spo11–DNA complexes. Spo11 should be stably bound to DNA from a strain that accumulates the protein–DSB intermediate (e.g., *rad50S*) but not to DNA from a wild-type strain, where only resected DSBs are detected, or from a strain in which DSB formation is completely defective.

To test this prediction, protein–DNA complexes were purified according to the approach outlined in Figure 1 from *SPO11-HA* strains that were also either *rad50S*, wild-type, or doubly mutant for *rad50S* and *mei4Δ* (*MEI4* is one of the meiosis-specific genes required for DSB formation; see Introduction). The DNA in the complexes was then degraded; proteins were resolved on SDS-PAGE and blotted; and Spo11-HA protein was detected specifically with an anti-HA monoclonal antibody (Figure 4). As a positive control for blotting and detection, in vitro-translated Spo11-HA protein was analyzed in parallel on the same gel. The presence of the tag, which adds approximately 1.3 kDa to the protein, causes Spo11-HA to run more slowly than wild-type Spo11 protein. As expected, Spo11-HA protein is detected in the sample purifed from the *rad50S* strain. No Spo11-HA was detected in the samples from the wild-type strain (*RAD50*) or from the *rad50S mei4Δ* double-mutant strain. Therefore, stable association of Spo11 with DNA occurs specifically in the genetic background in which unre-sected, protein-associated DSBs accumulate.

Spo11 Defines a Widely Conserved Family of Proteins

When the predicted amino acid sequence of Spo11 was compared to database sequences, three homologous sequences from widely diverged organisms were identified: the S. pombe rec12 protein, the predicted product of a Caenorhabditis elegans ORF of unknown function (T05E11.4), and the predicted product of ORF MJ0369 from the archaebacterium Methanococcus jannaschii. Alignment of these sequences demonstrates substantial conservation over most of the length of each (Figure 5). Pairwise comparisons among them show 22%–28% sequence identity (45%–52% similarity when conservative substitutions are included), although several gaps must be introduced for optimal alignment. Four blocks of particularly strong conservation (40%–65% identity in at least three of the four sequences) are also apparent.
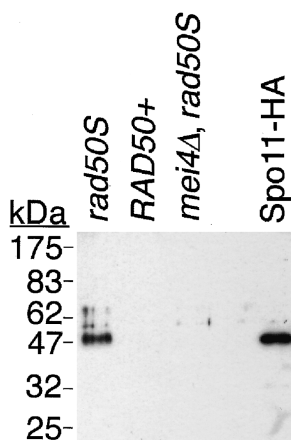
Figure 4. Stable Spo11–DNA Complexes Are Absent in Strains That Do Not Accumulate Protein–DSB Complexes

Protein–DNA complexes were purified as described in Figure 1 from meiotic cultures of several strains carrying the epitope-tagged *SPO11-HA* gene. Purified material was then digested with Benzonase and resolved on SDS-PAGE. Tagged Spo11 protein was detected by Western blot analysis using an anti-HA monoclonal antibody. Spo11-HA is detected in the sample from the *rad50S* strain (NKY2969), in which protein-DSB complexes accumulate, but not in an otherwise wild-type strain (*RAD50+*, NKY2968), in which only resected DSBs are observed, nor in a *mei4Δ rad50S* double mutant strain (NKY2970), in which no DSBs form at all. The far right lane (Spo11-HA) is a positive control for blotting and detection. It contains tagged Spo11 protein prepared by in vitro transcription and translation of *SPO11-HA*.

One of these blocks contains the only tyrosine that is conserved in all four sequences (Figure 5, asterisk). This tyrosine is a strong candidate for the catalytic residue that becomes linked to the DNA (see Discussion).

Discussion

Spo11 Is Most Likely the Catalytic Subunit of the DSB Cleavage Activity

The unresected DSBs that accumulate in *rad50S* and *sae2Δ* mutants have protein bound to their 5′ strand termini (Keeney and Kleckner, 1995; de Massy et al., 1995; Liu et al., 1995). We demonstrate here that this protein is the product of the *SPO11* gene. These results strongly implicate Spo11 as the catalytic subunit of the meiotic DNA cleavage activity and provide the first identification of a biochemical function for any of the gene products known to be required for DSB formation. The only alternative explanation for the presence of the DSB-associated protein would be that it becomes attached to the break after it is formed by a nuclease activity. We have argued previously that this idea is unlikely to be correct (Keeney and Kleckner, 1995). The results presented here reinforce this view because the model for a postcleavage attachment of Spo11 to DSBs fails to account for the fact that Spo11 is required for cleavage.

DSBs Appear to Be Formed by a Topoisomerase-like Transesterification Reaction
A Specific Model for DSB Formation
Identification of Spo11 as the likely catalytic subunit of the meiotic DNA cleaving activity permits more specific refinement of earlier models for the mechanism of DSB formation (Keeney and Kleckner, 1995; de Massy et al., 1995; Liu et al., 1995). As detailed below and in Figure 6, we propose that a tyrosine side chain on Spo11 attacks the DNA phosphodiester backbone, generating a phosphodiester linkage between the protein and the 5′ terminal strand and releasing a free 3′ OH-terminus.

Previous analysis of protein–DSB complexes suggested that protein is linked to the 5′ terminus of the broken DNA molecule itself (Keeney and Kleckner, 1995; de Massy et al., 1995; Liu et al., 1995). The properties of the complex are most consistent with a covalent linkage through a 5′-phosphodiester bond to an amino acid side chain of Spo11. Proteins that cleave DNA via a phosphodiester intermediate use the OH groups of serine, threonine, or tyrosine as the nucleophile. We can exclude a serine or threonine phosphodiester as the intermediate because the DSB protein–DNA linkage is stable in alkali (S. K., unpublished data; J. Liu and M. Lichten, personal communication). (Tyrosine phosphodiesters are alkali-stable [Rothberg et al., 1978], whereas serine and threonine linkages are alkali-labile [Shabarova, 1970].) The only conserved tyrosine in the Spo11 family (residue 135 of Spo11; Figure 5) is thus a prime candidate for the catalytic residue. Consistent with this assignment, the Spo11 tryptic peptide containing tyrosine-135 could not be detected at its expected position in the mass spectrum (data not shown), as predicted if this residue is DNA-linked. Moreover, site-directed mutagenesis of this residue to phenylalanine in Spo11 confers a DSB-defective phenotype (Bergerat et al., submitted). Finally, the DSB 3′ strands should have free 3′ OH-termini if Spo11 is linked to the 5′ strand through a phosphodiester bond. This is the case, as judged by the exonuclease sensitivity of the 3′ ends (Xu and Petes, 1996; S. K., unpublished data) and their ability to serve as primers for DNA polymerase (B. de Massy, personal communication).

It is likely that a pair of Spo11 monomers acts in concert to cut both DNA strands. While DSBs are abundant in meiotic DNA, single-strand nicks are not detected, even at very strong DSB hot spots (Xu and Kleckner, 1995; de Massy et al., 1995; Liu et al., 1995).
Spo11 Is Related to an
Archaebacterial Topoisomerase
The hyperthermophilic archaeon Sulfolobus shibatae contains a topoisomerase activity with features that distinguish it from known eubacterial and eukaryotic type II topoisomerases (Bergerat et al., 1994). The genes encoding this activity have been identified recently, and the catalytic subunit was shown to be a member of the Spo11 family (Bergerat et al., submitted). This may be the only class of type II topoisomerases in archaebacteria, because the genome of M. jannaschii (Bult et al., 1996) does not encode a protein homologous to the eukaryotic or eubacterial enzymes. The similarity of Spo11 to a protein with topoisomerase activity further supports the hypothesis that Spo11 is the catalytic subunit of meiotic DNA cleavage activity.
Implications of a Transesterase Mechanism
The likelihood that meiotic DSBs form via a topoisomerase-like transesterification reaction, rather than by endonucleolytic hydrolysis, has several implications.
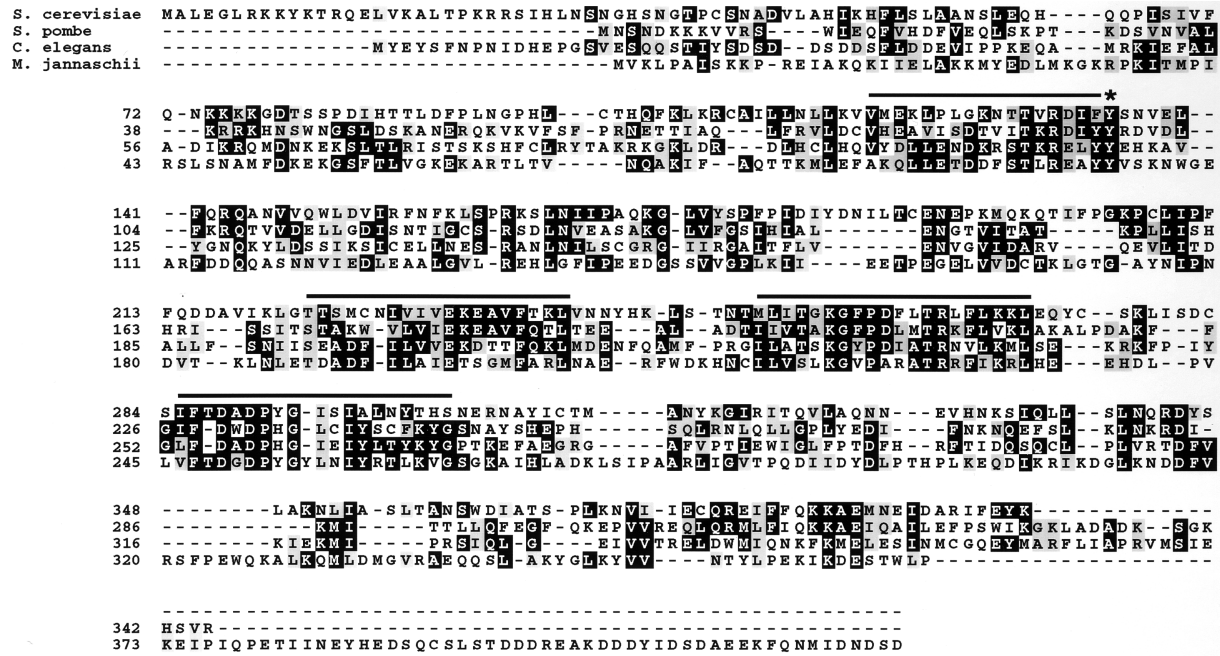
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S. cerevisiae  MALEGLRKKYKTRQELVKALTPKRRSIHLNSNGHSNGTPCSNADVLAHIKHFLSLAANSLEQH----QQPISIVF
S. pombe       -------------------------------MNSNDKKKVVRS----WIEQFVHDFVEQLSKPT---KDSVNVAL
C. elegans     ---------------MYEYSFNPNIDHEPGSVESQQSTIYSDSD--DSDDSFLDDEVIPPKEQA---MRKIEFAL
M. jannaschii  ----------------------------------MVKLPAISKKP-REIAKQKIIELAKKMYEDLMKGKRPKITMPI
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         72  Q-NKKKKGDTSSPDIHTTLDFPLNGPHL---CTHQFKLKRCAILLNLLKVMEKLPLGKNTTVRDIFYSNVEL--
         38  ---KRRKHNSWNGGSLDSKANERQKVKVFSF-PRNETTIAQ---LFRVLDCVHEAVISDTVITKRDIYYRDVDL--
         56  A-DIKRQMDNKEKSLTLRISTSKSHFCLRYTAKRKGKLDR---DLHCLHQVYDLLENDKRSTKRELYYEHKAV--
         43  RSLSNAMFDKEKGSFTLVGKEKARTLTV-----NQAKIF--AQTTKMLEFAKQLLETDDFSTLREAYYVSKNWGE
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        141  --FQRQANVVQWLDVIRFNFKLSPRKSLNIIPAQKG-LVYSPFPIDIYDNILTCENEPKMQKQTIFPGKPCLIPF
        104  --FKRQTVVDELLGDISNTIGCS-RSDLNVEASAKG-LVFGSIHIAL------ENGTVITAT-----KPLLISH
        125  --YGNQKYLDSSIKSICELLNES-RANLNILSCGRG-IIRGAITFLV-------ENVGVIDARV----QEVLITD
        111  ARFDDQQASNNVIEDLEAALGVL-REHLGFIPEEDGSSVVGPLKII----EETPEGELVVDCTKLGTG-AYNIPN
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        213  FQDDAVIKLGTTSMCNIVIVEKEAVFTKLVNNYHK-LS-TNTMLITGKGFPDFLTRLFLKKLEQYC--SKLISDC
        163  HRI---SSITSTAKW-VLVIEKEAVFQTLTEE---AL--ADTIIVTAKGFPDLMTRKFLVKLAKALPDAKF---F
        185  ALLF--SNIISEADF-ILVVEKDTTFQKLMDENFQAMF-PRGILATSKGYPDIATRNVLKMLSE---KRKFP-IY
        180  DVT--KLNLETDADF-ILAIETSGMFARLNAE--RFWDKHNCILVSLKGVPARATRRFIKRLHE---EHDL--PV
```

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        284  SIFTDADPYG-ISIALNYTHSNERNAYICTM-----ANYKGIRITQVLAQNN---EVHNKSIQLL--SLNQRDYS
        226  GIF-DWDPHG-LCIYSCFKYGSNAYSHEPH------SQLRNLQLLGPLYEDI----FNKNQEFSL--KLNKRDI-
        252  GLF-DADPHG-IEIYLTYKYGPTKEFAEGRG-----AFVPTIEWIGLFPTDFH--RFTIDQSQCL--PLVRTDFV
        245  LVFTDGDPYGYLNIYRTLKVGSGKAIHLADKLSIPAARLIGVTPQDIIIDYDLPTHPLKEQDIKRIKDGLKNDDFV
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        348  --------LAKNLIA-SLTANSWDIATS-PLKNVI-IECQREIFFQKKAEMNEIDARIFEYK--------------
        286  -----------KMI-----TTLLQFEGF-QKEPVVREQLQRMLFIQKKAEIQAILEFPSWIKGKLADADK--SGK
        316  --------KIEKMI-----PRSIQL-G----EIVVTRELDWMIQNKFKMELESINMCGQEYMARFLIAPRVMSIE
        320  RSFPEWQKALKQMLDMGVRAEQQSL-AKYGLKYVV----NTYLPEKIKDESTWLP--------------------
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        342  HSVR----------------------------------------------------------------------
        373  KEIPIQPETIINEYHEDSQCSLSTDDDREAKDDDYIDSDAEEKFQNMIDNDSD---------------------
```

Figure 5. Alignment of Members of the Spo11 Family

Predicted amino acid sequences of S. cerevisiae Spo11, S. pombe rec12 protein (Lin and Smith, 1994; G. Smith, personal communication), C. elegans ORF T05E11.4 (Wilson et al., 1994), and M. jannaschii ORF MJ0369 (Bult et al., 1996) were aligned using the PIMA program (Smith and Smith, 1990). Dashes indicate the positions of gaps introduced to maximize alignment scores. Identical residues are highlighted in black; conservative substitutions are shaded in gray. The bars indicate blocks of sequence that are strongly conserved among at least three of the four sequences; the asterisk marks the position of the conserved tyrosine (residue 135 of Spo11).

First, it has been suggested that homologous DNA molecules contact one another during meiosis prior to DSB formation, possibly in a progression of interactions (Weiner and Kleckner, 1994; Xu and Kleckner, 1995; Keeney and Kleckner, 1996; Rocco and Nicolas, 1996; Bullard et al., 1996). Since type II topoisomerases can interact simultaneously with two DNA duplexes (Roca et al., 1993), it is possible that, prior to DNA cleavage, Spo11 mediates direct contact between two molecules that are destined for a recombinational interaction. Such contact might ensure that a DSB is much less likely to occur unless a partner is nearby.

Second, in a DNA transesterase reaction, the energy of the phosphodiester backbone is retained in the protein–DNA linkage, and direct reversal of the cleavage reaction can restore the integrity of the broken strand (Figure 6). Nucleolytic cleavage, in contrast, releases the energy of the phosphodiester bond such that strand integrity cannot be restored without input of energy in a reaction that is not the simple reversal of the original scission. Reversibility of the transesterase reaction could provide additional opportunities for monitoring (and even aborting) DSB formation.

Third, why should DSB formation be subject to mutational disruption specifically at the covalent protein–DNA intermediate stage? One possibility is that specific protein factors block the reaction at this point, with additional factors then alleviating the block. An example of such a blocking factor is the E. coli F-factor protein CcdB, which stabilizes a gyrase–DNA cleavable complex (Bernard et al., 1993). Another possibility, not exclusive with the first, is that the DSB pathway is driven

irreversibly forward by release of the protein (or resection, or both), with the rad50S and sae2∆ mutational defects directly affecting this release step. Other observations have led independently to the notion that some proteins are required for assembly of pre-DSB "potential," while others, notably Red1, are required for activation of that potential at a later stage (Xu et al., 1997). Activation of the pre-DSB complex without protein release might result in a block at the protein–DNA complex stage.

Fourth, two types of protein release mechanism are possible, in principle (Figure 6). The protein–DNA bond might be hydrolyzed, releasing Spo11 and an unresected DSB with 5′ terminal phosphates. A yeast enzyme that hydrolyzes tyrosine 3′-phosphodiester linkages has recently been identified (Yang et al., 1996), raising the possibility that an analogous 5′-phosphodiesterase might exist as well. Alternatively, protein release and resection could be directly coupled: single-strand nucleolytic cleavage at some distance from the protein–DNA linkage could release oligonucleotide-bound Spo11 plus a partially (or fully) resected 5′ strand. These mechanisms can be distinguished experimentally by the presence or absence of an oligonucleotide bound to Spo11. The latter mechanism uniquely predicts that Spo11 acts in a "suicide" reaction: after cleavage and release, Spo11 would still have DNA bound to its active site and thus could not catalyze another DSB.

Recent studies provide good candidates for the proteins responsible for DSB resection (and, perhaps, Spo11 release). The SbcCD complex of E. coli has both
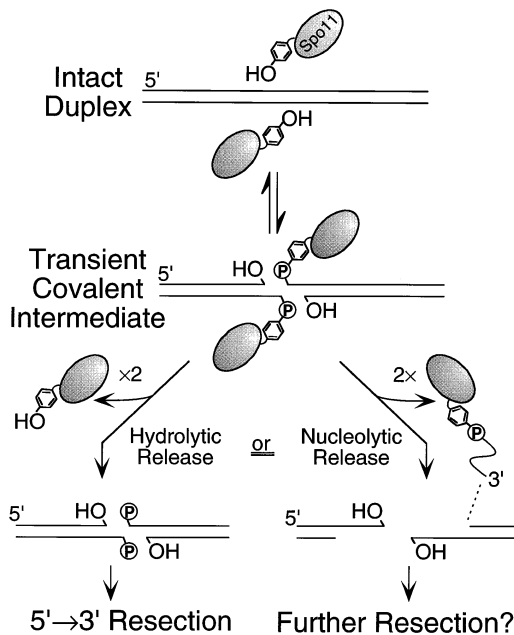
Figure 6. Model for the Mechanism of Meiotic DSB Formation

Spo11 (represented by shaded elipses) is proposed to cleave DNA by a reversible transesterase reaction in which a tyrosine side chain on the protein attacks the phosphodiester backbone, forming a covalent phosphodiester linkage between the protein and the 5′ terminus of the broken strand and releasing a free 3′ OH. Two Spo11 monomers act in concert to generate a pair of closely spaced nicks on opposite strands. The cleavage reaction is driven irreversibly forward by the release of Spo11, either by hydrolysis of the tyrosine phosphodiester, which would release intact Spo11 monomers and a 5′-phosphate terminus on the cleaved strand, or by a downstream single-strand nucleolytic cleavage, which would release oligonucle-otide-bound Spo11 monomers and a (partially or fully) resected DSB.

single-strand endonuclease and double-strand exo-nuclease activities (Connelly and Leach, 1996). The yeast Rad50 and Mre11 proteins are homologous to SbcC and SbcD, respectively (Sharples and Leach, 1995), and have been shown to form a complex with one another and with a third protein, Xrs2 (Johzuka and Ogawa, 1995), suggesting that the three proteins to-gether might form a nuclease. This possibility is sup-ported by the observation of *rad50*, *mre11*, and *xrs2* mutant defects in resection of both mitotic and meiotic DSBs (Alani et al., 1990; Sugawara and Haber, 1992; Ivanov et al., 1994; Prinz et al., 1997; K. Nairz and F. Klein, personal communication). Rad50 (Raymond and Kleckner, 1993) and Mre11 (Johzuka and Ogawa, 1995) individually show DNA binding activity, but nuclease activity has not yet been reported.

**Possible Role(s) for Spo11 in Processes
Other Than Recombination**
A *spo11* null mutant exhibits a number of phenotypes that may not be directly attributable to the loss of DSB formation. For example, a *spo11* null mutant is defective in maturation of silver-staining axial chromosome struc-tures known as axial cores, which, in wild-type cells, are the lateral element components of the synaptonemal complex (Giroux et al., 1989; D. Rieger and C. N. G.,

unpublished data), although it does not appear to be defective in chromatin condensation or axial chromo-some compaction (Giroux et al., 1989; Weiner and Kleck-ner, 1994; Loidl et al., 1994; D. Rieger and C. N. G., unpublished data). In addition, a *spo11*Δ mutation accel-erates cell cycle progression through meiotic prophase (Klapholz et al., 1985; Giroux et al., 1993; D. Rieger and C. N. G., unpublished data). The basis for this effect is unknown. A *spo11*Δ mutant also exhibits a severe reduction in the number of interstitial connections that normally occur between homologs during early meiotic prophase independent of, and likely prior to, DSB forma-tion (Weiner and Kleckner, 1994; Loidl et al., 1994). It is unlikely that Spo11 is involved in homology recognition per se, because analogous pairing interactions in mitotic cells, and probably also in subtelomeric regions of mei-otic cells, are independent of Spo11 (Weiner and Kleck-ner, 1994). Thus, Spo11 may be required for facilitating or stabilizing pairing interactions in interstitial regions of meiotic chromosomes.

The results presented here demonstrate conclusively that Spo11 acts directly at DSB sites—the positions that give rise to stable recombinational interactions between homologous chromosomes. In fact, Spo11 may act ex-clusively at these positions since it appears not to be a very abundant protein (C. N. G., unpublished data). Because Spo11 is required for events of meiotic pro-phase other than recombination (see above), the demon-stration that Spo11 acts directly at the sites of meiotic interhomolog interactions provides support for the view that these positions are functionally complex and are involved in integrating interhomolog interactions with other aspects of meiosis, such as chromosome pairing, axial structure development, and cell cycle progression (Kleckner and Weiner, 1993; Giroux et al., 1993; Kleck-ner, 1996). Similar complexity is seen for Rad50 (Alani et al., 1990; Weiner and Kleckner, 1994).

Is Spo11 Involved in Recombinationless Meiosis?
As discussed above, recombination plays a crucial role in the classic meiotic program. However, in certain ex-ceptional situations, meiosis occurs in the absence of recombination. The paradigmatic exception of this kind is meiosis in Drosophila melanogaster males (see Orr-Weaver, 1995 for review). In this case, autosome disjunc-tion at meiosis I appears to be guided by general DNA homology in euchromatic regions (McKee et al., 1993) but occurs accurately in the absence of either cross-overs or "gene conversions." How do homologous chro-mosomes remain connected to one another through prometaphase without crossovers? The current findings raise the intriguing possibility that a Drosophila homolog of Spo11 might exert its effects in males as an interho-molog topoisomerase rather than as a catalytic subunit of a DNA cleaving activity. Catenations between DNA segments in any topologically closed domain (gener-ated, for example, by the organization of each sister pair into an array of loops) could suffice to hold homologs together.

**Spo11 Structure and Function Are
Evolutionarily Conserved**
Sequences from several widely diverged eukaryotes share significant similarity with that of Spo11. One of

these, the product of the *rec12* gene of S. pombe, is also required for meiotic recombination (Lin and Smith, 1994), strongly suggesting that Spo11 and rec12 are functional as well as structural homologs. This conservation across the wide evolutionary distance between these two fungal species strongly suggests that the mechanism of meiotic recombination initiation is itself highly conserved. To date, meiotic DSBs have been directly demonstrated only in S. cerevisiae, but the observations presented here make it likely that DSBs also occur in S. pombe. It will be interesting to see whether the putative Spo11 homolog from C. elegans plays a role in meiotic recombination in that organism as well.

It also will be interesting to determine how Spo11 came to play its role(s) in sexually reproducing organisms. One possibility is that the primordial Spo11 protein arose in a precursor organism that gave rise to both archaebacteria and eukaryotes. Alternatively, Spo11 might have evolved in the archaeal world and then been transferred horizontally into the eukaryotic world and recruited specifically to promote meiotic interhomolog interactions. A scenario for the evolution of meiosis that proposes the progressive acquisition of DSB activity has been presented elsewhere (Kleckner, 1996).

Experimental Procedures

Yeast Strains, Plasmids, and Culture Techniques

All yeast strains in this study are isogenic diploid derivatives of strain SK1 (Kane and Roth, 1974), homozygous for *ho::LYS2, lys2, ura3,* and *leu2::hisG*. Additional alleles are as follows: NKY2059 (*his4XLEU2/his4BLEU2, rad50-KI81::URA3/′′*); NKY2967 (*his4XLEU2/ his4BLEU2, spo11∆::hisG-URA3-hisG/′′*); NKY2968 (*trp1::hisG/′′, his4BLEU2/′′, spo11∆::hisG-URA3-hisG/′′*), containing pNKY1218 (*ARS, CEN, TRP1, SPO11-HA*); NKY2969 (NKY2968 plus *rad50-KI81::ura3-/′′*); and NKY2970 (NKY2969 plus *mei4∆::URA3/′′*). Alleles are as described (Giroux et al., 1989; Alani et al., 1990; Cao et al., 1990; Menees et al., 1992).

To generate *SPO11-HA*, a XhoI site was first incorporated into the *SPO11* sequence immediately preceding the TGA stop codon. Next, a duplex oligonucleotide fragment encoding the HA epitope, YPYDVPDYA, was inserted into the XhoI site, creating an in-frame C-terminal fusion. When carried on a YCp50-based plasmid, pGB933, *SPO11-HA* fully complements the meiotic defects of a *spo11∆* strain (N. Chen, T. Hillmer, and C. N. G., unpublished data). *SPO11-HA* was subcloned from pGB933 into pOL130 (= pRS314; Sikorski and Hieter, 1989) to generate pNKY1218.

Synchronous meiotic cultures were prepared by pregrowth in YPA (1% [wt/vol] yeast extract, 2% Bacto Peptone, 1% potassium acetate, 0.01% antifoam 289 [Sigma]) followed by transfer to sporulation medium (SPM; 0.3% potassium acetate, 0.02% raffinose) as described (Alani et al., 1990; Padmore et al., 1991). For plasmid-bearing strains, YPA was innoculated from saturated cultures grown in synthetic complete medium lacking tryptophan (Kaiser et al., 1994). Meiotic cultures were harvested 4.5–6 hr after transfer to SPM.

Purification of Meiotic Protein–DNA Complexes

For preparative-scale isolation of protein–DNA complexes, 6 liters of NKY2059 culture were harvested, and nuclei were isolated each day for 10 days. Cells from each batch of meiotic culture were resuspended in 180 ml SCE (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA [pH 7.0]) and preincubated for 15 min at 37°C. Six milliliters of zymolyase 100T (ICN; 6 mg/ml in SCE), 1.2 ml β-mercaptoethanol, and 180 μl of 0.4 M pefabloc-SC (Boehringer-Mannheim) were added and incubated for 30–40 min at 37°C. Spheroplasts were pelleted, washed twice with 200 ml of ice-cold SCE plus protease inhibitors (1 mM sodium bisulfite, 0.4 mM pefabloc-SC, 1 mM ε-aminocaproic acid, 1 mM p-aminobenzamidine, 1 mM PMSF, 1 μg/ml each of leupeptin, pepstatin A, and chymostatin), and then

resuspended in 180 ml of ice-cold HLB (20 mM MES-NaOH [pH 6.4], 0.2% [vol/vol] Triton X-100, 5 mM EDTA, plus protease inhibitors as above) and allowed to swell for 10 min on ice. The suspension was Dounce-homogenized and then layered onto six 10 ml sucrose cushions (1 M sucrose in HLB) and centrifuged for 15 min, 20,000 × g, at 4°C. Supernatants were discarded, and each pellet (containing the nuclei) was washed once with HLB and stored at −80°C.

Genomic DNA was extracted as follows. Nuclei from 24 liters of meiotic culture were thawed at 4°C and then homogenized in 24 ml of HLB. The suspension was added dropwise with vigorous stirring to 216 ml of room-temperature guanidine lysis buffer (8 M guanidine HCl, 10 mM DTT, 10 mM EDTA, 0.5% sarkosyl, plus pefabloc-SC, PMSF, leupeptin, pepstatin A, and chymostatin, as above), and stirring continued for 30 min. In smaller-scale preparations, guanidine extraction was performed at 65°C, which reduces the number of proteins copurifying with the genomic DNA (compare Figures 2A and 2B).

The lysate was passed twice through a 22-gauge needle and then centrifuged for 15 min, 30,000 × g, at 4°C. The supernatant was layered onto 12 step gradients, each consisting of 5 ml of Fluorinert FC40 (Sigma, 1.85 g/ml) and 10 ml of a 1.50 g/ml CsCl solution containing 0.5% (wt/vol) sarkosyl and 1 mM EDTA. Gradients were centrifuged for at least 30 hr in a Beckman SW28 rotor, 26,000 rpm, at 25°C. Genomic DNA was collected from the Fluorinert–CsCl interface and dialyzed against two changes of 10 mM Hepes-NaOH (pH 7.5), 50 mM NaCl, 2 mM EDTA, 0.1% sarkosyl, 0.1 mM pefabloc-SC. The extraction was repeated for the remainder of the nuclear preparations, and the DNA was pooled. A total of 72.4 mg genomic DNA was recovered from 60 liters of culture.

The pool was adjusted to 0.3 M NaCl and 0.2% Triton X-100, sonicated, and then passed at 1 ml/min through a series of four Whatman GFC filters (24 mm diameter) preequilibrated in TEN (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl). The filters were washed with 20 ml of TEN, and then the bound protein and protein–DNA complexes were eluted from each filter separately with 600 μl of 0.1% SDS in 10 mM Tris-HCl (pH 8.0). Most (∼80%) of the protein was recovered from the first filter in the series.

Sequencing of Genomic DNA-Associated Protein

The GFC filter–purified material from 60 liters of meiotic culture was diluted with 1 volume of 2 mM $MgCl_2$, 0.8 mM pefabloc-SC. Benzonase (EM Science; 500 units) was added, and the sample was incubated for 4 hr at 37°C. The sample was then electrophoresed on a preparative 7.5%–15% SDS- polyacrylamide gradient gel. Proteins were visualized with Coomassie, excised from the gel, and submitted to the Harvard Microchemistry Facility for digestion and amino acid sequence analysis. Tryptic peptides were sequenced by collisionally activated dissociation on a Finnigan TSQ 7000 triple quadrupole mass spectrometer.

Immunological Techniques

Immunoprecipitations were according to a modification of published methods (Walter et al., 1994). Genomic DNA samples (10 μg each) were digested with PstI in 400 μl using the buffer supplied by New England Biolabs, supplemented with 50 μg/ml BSA, 0.1% Triton X-100, and 0.4 mM pefabloc-SC. After digestion, 20 μl of 0.5 M EDTA, 2 μl of 5 M NaCl, 2.5 μl of 20% sarkosyl, 23 μl of 20% Triton X-100, and 52.5 μl of water were added. Samples were heated to 65°C for 15 min, chilled on ice, and then centrifuged for 15 min at 4°C in a microfuge. Supernatants were transferred to fresh tubes, and 1 μg anti-HA monoclonal antibody HA.11 (BAbCO) was added. After mixing at 4°C overnight, 1 μl of rabbit anti-mouse IgG was added and mixed for 1 hr. Fixed Staphylococcus aureus cells (15 μl of a 20% suspension; Calbiochem) were added and mixed for 15 min. The S. aureus cells were pelleted, washed three times with 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.2% Triton X-100, 0.2 M $(NH_4)_2SO_4$, and then protein–DNA complexes were eluted for 10 min at 58°C with 100 μl of 50 mM sodium bicarbonate (pH 10.0), 1% SDS, 1.5 μg/ml salmon sperm DNA. The elution was repeated twice more, and the eluates were pooled and neutralized with 5 μl of 1 M Tris-HCl (pH 7.5), plus 200 μl 0.3% SDS, 50 mM Tris-HCl (pH 7.5), 10 mM EDTA. The eluates were digested with 100 μg proteinase K

for at least 1 hr at 58°C. Samples were then extracted with phe-nol:CHCl₃:isoamyl alchohol (25:24:1) and processed for Southern blotting as previously described (Keeney and Kleckner, 1995).

For Western blotting, genomic DNA was purified from 6 liters of meiotic culture per strain, through the CsCl step as described above. Protein–DNA complexes were then purified on GFC filters from 3 mg of DNA per strain, digested with Benzonase, resolved on 10% SDS-PAGE, and blotted to nitrocellulose. The blot was probed with antibody HA.11 and then with goat anti-mouse IgG conjugated to horseradish peroxidase. Chemiluminescent detection of HRP was performed according to the instructions of the manufacturer (Pierce). As a positive control, Spo11-HA protein was prepared by in vitro transcription and translation of *SPO11-HA* (Novagen STP2 system).

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