A heteromeric protein that binds to a meiotic homologous recombination hot spot: correlation of binding and hot spot activity

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Homologous recombination hot spots are DNA sites that increase the frequency of recombination in their vicinity. The *M26* allele of the *ade6* gene in *Schizosaccharomyces pombe* is the first meiotic hot spot with an identified unique nucleotide sequence. We have purified 40,000-fold a heteromeric protein, containing polypeptides Mts1 (70 kD) and Mts2 (28 kD), that binds to the *M26* site. Binding in vitro strictly correlates with hot spot activity in vivo for numerous single base pair substitutions in the vicinity of the *M26* site, indicating that Mts1/Mts2 activates the *M26* site and promotes a rate-limiting step of meiotic recombination. These and other data suggest that homologous recombination may be regulated primarily by discrete DNA sites and proteins that interact with those sites.

[**Key Words:** Genetic recombination; homologous recombination; meiosis; recombination hot spot; recombination proteins; *Schizosaccharomyces pombe*]

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Homologous recombination, the exchange of genetic information between homologous chromosomes, serves three principal functions. First, recombination generates new combinations of alleles, which may permit evolution to occur more rapidly than would be possible if newly arising mutations maintained their initial linkages. Second, in most organisms recombination is required for the proper segregation of chromosomes at meiosis I (Hawley 1989). Third, recombination is used to repair certain types of DNA damage (Resnick 1976; Szostak et al. 1983).

Although the frequency of recombination between any two genetic markers is roughly proportional to the physical distance between them, some regions of chromosomes, called recombination hot spots, undergo a much higher rate of recombination than others. Mutations or strain differences identifying hot spots were originally found in fungi (Angel et al. 1970; Gutz 1971; MacDonald and Whitehouse 1979) and have been subsequently found in a variety of organisms from bacteriophages (Lam et al. 1974; Stahl et al. 1975) to humans (Wahls et al. 1990a). Known recombination hot spots stimulate recombination up to 30-fold and exert their effects within a few tens of kilobase pairs. Because recombination hot spots must promote rate-limiting steps of recombination, elucidating how hot spots function will provide a better understanding of how recombination occurs. Presumably, as for transcriptional promoters and enhancers,

there are proteins that interact with recombination hot spots to mediate their biological activity.

The *M26* recombination hot spot, located in the *ade6* gene of the fission yeast *Schizosaccharomyces pombe*, is a well-characterized eukaryotic recombination hot spot (for review, see Wahls and Smith 1993). Of the almost 400 mutant alleles of *ade6* that were isolated by Gutz (1971), the *M26* allele is unique; *M26* increases the frequency of gene conversion at the *ade6* locus to ~ 10-fold the level of other *ade6* mutations, including the closely linked *M375* allele (Fig. 1; Gutz 1971). The *M26* and *M375* alleles are attributable to single G → T transversions that create translational stops at codons 46 and 45, respectively (Fig. 1; Ponticelli et al. 1988; Szankasi et al. 1988). Thus, *M375* serves as an excellent negative control for genetic and biochemical studies of recombination promoted by *M26*. The *M26* recombination hot spot functions during meiosis but not during mitosis (Ponticelli et al. 1988; Schuchert and Kohli 1988), exerts its effect in the vicinity of *ade6* but not at other loci (Ponticelli et al. 1988), and increases the frequency of both reciprocal exchange and gene conversion events up to 15-fold (Schuchert and Kohli 1988; Grimm et al. 1994). Finally, a heptameric DNA sequence surrounding the *M26* mutation (5'-ATGACGT-3'; *M26* mutation underlined) is required for hot spot activity (Schuchert et al. 1991). These data suggest that the *M26* mutation creates a recognition site for a recombination-promoting protein.

Figure 1. (A) Diagram of the *ade6* gene of *S. pombe* showing the positions of the M375, M26, and 469 alleles, and the gel mobility retardation probes used in this study. (B) An example of M26 meiotic homologous recombination hot spot activity (Ponticelli and Smith 1992). (C) Sequence analysis of wild-type, M375, and M26 alleles (Ponticelli et al. 1988; Szankasi et al. 1988). Both alleles are single base pair substitutions (circled) that create translational stops. A specific heptanucleotide sequence (boxed) surrounding the M26 mutation is required for hot spot activity in vivo (Schuchert et al. 1991).

Results

Detection of an M26-binding protein

Because the M26 recombination hot spot functions only during meiosis (Ponticelli et al. 1988; Schuchert and Kohli 1988), we reasoned that a putative M26 DNA-binding protein might be meiotically induced. Therefore, we initially searched for M26-binding activity in extracts of cells undergoing meiosis.

Induction of meiosis in *S. pombe* strain GP535 (*h⁻pat1-114^{ts} end1-458 ade6-M26*) was achieved by temperature shift as described previously (Szankasi and Smith 1992). Three different assays were used to judge the efficiency and synchrony of the induced meiosis (Szankasi and Smith 1992). First, the bulk of premeiotic DNA synthesis occurred between 2 and 3 hr of meiosis (Fig. 2). Second, the majority of cells completed the second meiotic division between 5 and 6 hr after induction (Fig. 2). Third, we observed appropriate meiotic induction of exonuclease I (Exo I) (Fig. 2), a double-stranded DNA (ds-DNA) exonuclease that is meiotically induced (Szankasi and Smith 1992). In a related study two meiotic recombination genes, *rec7* and *rec8*, were induced at 2 and 3 hr of meiosis (Fig. 2; Lin et al. 1992). We conclude that the meiotic induction was synchronous and that meiotic enzymes were induced appropriately.

We used a gel mobility retardation assay to determine whether extracts of *S. pombe* cultures at various time points of meiosis contained a protein that binds to the M26 recombination hot spot. Probes (Fig. 1) were derived from *ade6* DNA bearing either the single base pair M26 substitution (recombination hot spot) or the single base

pair M375 substitution (normal recombination levels). As shown in Figure 2B, two M26-specific binding activities (complex 1 and complex 2) were detected in extracts of mitotic and meiotic cells. Because the M375 allele maps very close to M26 (Fig. 1) and exhibits normal recombination levels in genetic crosses, it serves as an excellent control to correlate these biochemical analyses with genetic hot spot activity. Probes from wild-type *ade6* DNA also failed to be bound by protein (data not shown; see also Fig. 7, below), indicating that the M26 mutation generated a protein-binding site (and disproving the possibility that the M375 mutation disrupted a pre-existing site in wild-type DNA). The presence of M26 DNA-binding activity was independent of whether or not the cells harbored the M26 allele at *ade6* (data not shown) and could be competed away quantitatively with unlabeled M26 probe but not with unlabeled M375 probe (data not shown). These data indicate that M26 recombination hot spot-binding proteins were present and constitutively expressed in mitotic and meiotic cells. The meiotic specificity of the M26 hot spot in genetic crosses

Figure 2. Detection of M26-specific DNA-binding proteins. (A) Time course of synchronous meiosis in *S. pombe*. Boxes indicate times at which the indicated activity is induced. The *rec7* and *rec8* transcript induction data are from Lin et al. (1992). (B) Gel mobility retardation assay. Extracts were prepared as described (Szankasi and Smith 1992) from mitotic cells (hour 0) and meiotic cells (hours 1–6 after shift of the culture to 34°C). Binding reactions were in 15 μ l and contained 10 μ g of protein, 1 μ g of poly[d(I-C)], and 4 fmole of the 215-bp probe containing either M26 or M375 single base pair substitutions. The position of two M26-specific complexes (1 and 2) are indicated; (Free) The position of unbound DNA.

must therefore result from another factor (see Discussion).

Binding occurs at M26

A specific heptamer, 5'-ATGACGT-3', is required for M26 hot spot activity *in vivo* (Schuchert et al. 1991). We used methylation interference to determine the DNA sequence in our M26 probe that was bound by protein. Methylation of any G residue within the M26 heptamer abolished protein binding, whereas probes methylated outside the heptamer were bound efficiently by protein (Fig. 3). Thus, the methylation interference footprint superimposes on the DNA sequence required for hot spot activity and shows that the protein binds directly to the M26 site.

Low abundance of M26 DNA-binding protein

To estimate the abundance of the M26-specific DNA-binding proteins in each cell, we determined the amount of probe that was bound by serial dilutions of unfraction-

ated cell extract. Under conditions with DNA concentration above K_d ($\sim 1 \times 10^{-9}$ M) and probe excess, the extract from $\sim 1.2 \times 10^6$ cells (12 μ g of fraction I protein) was required to shift the mobility of 0.4 fmole of the probe (data not shown). These data suggest that there are ~ 200 M26-binding proteins per cell. Estimates based on the recovery of purified protein (Table 1) give a similar value.

Purification of M26 DNA-binding activity

We used standard chromatographic techniques including a final M26 DNA affinity column (Fig. 4A) to purify the M26-specific DNA binding activity from extracts of mitotic cells of strain GP61 (*h⁻ leu1-32 end1-458*). As with the meiotic extracts (Fig. 2B), fractions from all stages of purification generated two M26-specific protein-DNA complexes when analyzed on gel mobility retardation gels (Fig. 4C). We calculate that we achieved $\sim 40,000$ -fold purification of the M26 DNA-binding activity (Table 1). As shown in Figure 4B, SDS-PAGE analysis revealed that purified fraction VI was composed primarily ($\geq 50\%$ by weight) of two polypeptides. We call these polypeptides M-twenty-six-binding proteins, abbreviated as Mts1 (70 kD) and Mts2 (28 kD), respectively.

Although the factors that produced the two different protein-DNA complexes cofractionated throughout the purification (Fig. 4C), they also showed some degree of independence. Analysis of sequential fractions from the final column revealed that factors required for complex 1 eluted from the column slightly earlier than factors required for complex 2 (Fig. 5B). Similar behavior was observed with sequential fractions from other columns (data not shown). In each case, the factors responsible for the two complexes always eluted with some overlap (suggesting that they shared common subunits), and they were often, but not always, offset slightly from one another (suggesting that they were distinct).

SDS-PAGE analysis of fractions from the final column revealed that the Mts1 and Mts2 polypeptides cofractionated with each other (Fig. 5A) and with the entire region of M26-specific DNA-binding activity (i.e., both complex 1 and complex 2, Fig. 5B). (The slightly earlier appearance of Mts1 relative to Mts2 in Fig. 5A may be attributable to a nominal gel-filtration effect as the proteins elute at high salt from the M26 DNA affinity column.) These data suggest that Mts1 and Mts2 exist as a heterodimer and, furthermore, that the Mts1/Mts2 heterodimer plays a role in the formation of both complexes.

Mts1 and Mts2 exist and bind as a heteromeric complex

To investigate whether Mts1 and Mts2 existed as a heterodimer in solution, we treated purified fraction VI with glutaraldehyde, which cross-links closely associated primary amines, and analyzed the products on an SDS-polyacrylamide gel (Fig. 6A). We observed a coordinate reduction in Mts1 and Mts2 band intensities and the

Figure 3. Protein-DNA binding occurs at the M26 site. (A) Methylation interference assays of both strands of the M26 probe. (Bottom, Top) Strand that was 3'-end labeled; (F) free probe; (B) bound probe. Arrows indicate positions of guanine bases which, when N7-methylated, interfere with protein-DNA binding. (B) Summary of methylation interference data. The M26 mutation is in boldface type; the heptanucleotide sequence required for hot spot activity *in vivo* is boxed (Schuchert et al. 1991); arrows indicate position of close protein contacts with guanine bases in the major groove. Note that methylation of guanines immediately flanking the heptamer does not interfere with protein-DNA binding.

Table 1. Purification of M26 DNA-binding activity

Fraction	Source	Total protein (mg)	Total activity (units) ^a	Specific activity (U/mg)	Yield per step (%)	Yield total (%)	Purification (fold)
I	cell extract	12,700	230,000	18	100	100	1.0
II	0–41% (NH ₄) ₂ SO ₄ ppt	4,800	210,000	44	91	91	2.4
III	SP-Sepharose	226	150,000	660	71	65	37
IV	ssDNA–cellulose	32.9	30,000	910	20	13	51
V	Mono-S	9.75	21,000	2,200	70	9	120
VI	M26 ₁₈ DNA–Sepharose	0.01	6,900	690,000	33	3	38,000

^aOne unit is the amount of protein required to shift 50% of the M26 probe (4 fmoles) in standard gel mobility retardation assays.

appearance of a novel band of 98 kD (the expected sum of Mts1 and Mts2 molecular masses); higher order aggregates were not observed. These data suggest that Mts1 and Mts2 exist as a heterodimer in solution.

To characterize the protein–DNA complexes, we eluted the Mts1 and Mts2 polypeptides individually

from an SDS–polyacrylamide gel into a denaturing solution; then we renatured the proteins and used mixing experiments to determine the requirements for M26-specific DNA binding (Fig. 6B). As a control, we conducted the same experiments with fraction VI that had not been subject to SDS-PAGE. When fraction VI was denatured and renatured, both protein–DNA complexes were reformed. Under identical assay conditions, no binding was detectable with Mts1 alone or with Mts2 alone. However, when isolated Mts1 and Mts2 were either renatured and mixed together, or mixed together and renatured, M26-specific complex 1 was restored. These results show that a heterodimer or heteromultimer of Mts1 and Mts2 is necessary and sufficient for formation of protein–DNA complex 1. From protein titration in gel mobility retardation assays, we calculate that at least 50% of the purified Mts1/Mts2 heterodimer molecules in fraction VI were active for M26 binding (data not shown).

On much longer exposures of the X-ray film, additional M26-specific protein–DNA complexes were observed in reactions with Mts1 alone (complex 3) and Mts2 alone (complex 4; Fig. 6B). Complex 3 migrated

Figure 4. Purification of M26-specific DNA-binding proteins. (A) Summary of purification procedure. (B) Coomassie-stained, 12% SDS-PAGE analysis of fractions from purification. (MW) Molecular weight standards, 3 μ g; (fraction I) 25 μ g; (fraction II) 10 μ g; (fraction III) 2.3 μ g; (fraction IV) 1 μ g; (fraction V) 0.5 μ g; (fraction VI) 0.5 μ g. (C) Gel mobility retardation assay of fractions from purification. Assay and probes were as in Fig. 2 and contained 30 μ g (fraction I); 11 μ g (fraction II); 1 μ g (fraction III); 0.5 μ g (fraction IV); 0.2 μ g (fraction V); <0.001 μ g (fraction VI). (+) M26 probe (recombination hot spot); (–) M375 probe (normal recombination levels).

Figure 5. Coelution of Mts1 and Mts2 polypeptides with M26 DNA-binding activity in fractions from final column. Sequential column fractions were analyzed on Coomassie-stained, 12% SDS–polyacrylamide gel (A) and by gel mobility retardation assay for binding to M26 probe (B).

Figure 6. Evidence for heterodimer. (A) Glutaraldehyde cross-linking of Mts1 and Mts2. Cross-linking of purified fraction VI was for 10 min at 37°C under the indicated conditions. The samples were then fractionated on a Coomassie-stained, 12% SDS–polyacrylamide gel. (B) Reconstitution of M26-specific band shift using purified components. Fraction VI, gel-purified Mts1, and gel-purified Mts2 were individually denatured, renatured, mixed as indicated, and tested for M26 DNA-binding activity. (Complex 1) Heterodimer of Mts1 and Mts2; (complex 2) heterotrimer of Mts1, Mts2, and third factor?; (complex 3) homodimer of Mts1–low affinity binding; (complex 4) homodimer of Mts2–low affinity binding. (a) Renatured and mixed; (b) mixed and renatured. Gel mobility retardation assay conditions and probes were as in Fig. 2.

more slowly than complex 1 (Mts1/Mts2 heterodimer bound to M26 probe), suggesting that it was composed of a homodimer of Mts1 bound to the M26 probe. Likewise, the position of complex 4 suggested that it consisted of a homodimer of Mts2 bound to the probe. (These presumed multimeric states have not been directly demonstrated.) Thus, while Mts1 and Mts2 exist as a heterodimer (or heteromultimer) in solution (Fig. 6A) and a heterodimer of Mts1 and Mts2 is required for optimal binding to the M26 site (Fig. 6B), the individual Mts1 and Mts2 polypeptides are capable of some, albeit lower affinity, M26-specific DNA binding.

In similar experiments, we tested the ability of the purified proteins to bind to single-stranded M26 probes. No single-stranded DNA (ssDNA) binding was observed (data not shown), suggesting that the Mts1/Mts2 heterodimer binds exclusively to dsDNA containing the M26 site.

Evidence for a third factor

Although fraction VI could be denatured and renatured to reconstitute both complex 1 and complex 2, the identical experiment using purified Mts1 and Mts2 reconstituted only complex 1 (Fig. 6B). Thus, there must have been some third factor (Mts3), not cofractionating with 70- and 28-kD polypeptides, that contributed to formation of complex 2; that factor was fractionated away when Mts1 and Mts2 were gel purified. Glutaraldehyde cross-linking indicated that Mts1 and Mts2 exist as a heterodimer in solution (Fig. 6A). Furthermore, in fractions from columns, Mts1 and Mts2 coeluted with each other and with both of the M26-specific DNA-binding activities (complex 1 and complex 2; Fig. 5), suggesting that the Mts1/Mts2 heterodimer is required for both complexes. On the basis of those data, we assume that

Mts3 interacts with the Mts1/Mts2 heterodimer to give rise to protein–DNA complex 2.

Polypeptides of ~40 kD seemed to coelute with protein–DNA complex 2 and are possible candidates for the third factor Mts3 (Fig. 5). If so, SDS-PAGE analyses suggest that Mts3 must either stain poorly or be a low-abundance protein that increases the affinity of the Mts1/Mts2 heterodimer for the M26 site. However, the 40-kD polypeptides are not present in equimolar concentrations with Mts1 and Mts2 (Fig. 5), they do not efficiently cross-link to the Mts1/Mts2 heterodimer when treated with glutaraldehyde (Fig. 6), and they are apparently absent from some purifications (data not shown), suggesting that the 40-kD polypeptides are unlikely candidates for Mts3. Because there are no other obvious polypeptide candidates for Mts3, it may not be composed of protein. Mts3 could be a small molecule, metal ion, or other cofactor that changes the conformation (and electrophoretic mobility) of the protein–DNA complex. Alternatively, as has been found for some other proteins involved in macromolecular nucleic acid metabolism (Kole and Altman 1981; Greider and Blackburn 1987; Young et al. 1991), Mts3 might be composed of RNA. RNA has been found in the 40,000-fold purified fraction VI, but the available evidence suggests that it is not a functional subunit of the Mts1/Mts2 protein–DNA binding activity (Wahls 1994). Further investigation may reveal the nature of Mts3 and its role in binding of the Mts1/Mts2 heterodimer to the M26 recombination hot spot.

Mts1/Mts2 binding at M26 correlates with hot spot activity

Because Mts1/Mts2 bound only probes bearing the M26 recombination hot spot (Figs. 2, 4, and 6) and footprinted to the heptameric sequence required for hot spot activity

(Fig. 3), it was likely that Mts1/Mts2 protein binding was required for hot spot activity. To confirm this, we tested the ability of purified fraction VI to bind to DNA molecules that contained single base pair substitutions in the vicinity of the M26 site. As shown in Figure 7, substitutions within the M26 heptamer abolished both protein binding in vitro and hot spot activity in vivo, although substitutions outside the M26 heptamer abolished neither protein binding nor hot spot activity. Furthermore, one substitution that decreased hot spot activity ~40%

also decreased protein binding ~40%, whereas another substitution that increased hot spot activity 40% also increased protein binding a similar amount. Thus, the amount of recombination hot spot activity observed in vivo (Schuchert et al. 1991) correlated precisely with the amount of protein–DNA binding in vitro for every single base pair substitution. Identical results were obtained with protein fraction II from all time points of meiosis (data not shown). From this strict correlation of in vivo genetic hot spot activity and in vitro Mts1/Mts2 protein binding, we conclude that binding of Mts1/Mts2 protein to the heptanucleotide M26 DNA sequence is required for the recombination hot spot activity.

Figure 7. Hot spot activity in vivo correlates with Mts1/Mts2 protein binding in vitro. (A) DNA sequences of probes used. (Circle) M26 mutation; (box) heptameric sequence required for hot spot activity in vivo (Schuchert et al. 1991). The 215-bp probes differed only by the indicated single base pair substitutions. (B) Gel mobility retardation assay using purified Mts1/Mts2 heterodimer (fraction VI) and the probes indicated in A. (C) Correlation of hot spot activity in vivo (Schuchert et al. 1991) with Mts1/Mts2 protein binding in vitro for single base pair substitutions in the vicinity of the M26 site. For the in vivo data, the values are normalized so that 100% equals the M26 (hot spot) recombinant frequency and 0% equals the M375 (normal) recombinant frequency. For quantitation of the in vitro data, the gel in B was analyzed using a Molecular Dynamics model 400A PhosphorImager and ImageQuant version 3.2 (Molecular Dynamics) software. The relative amount of each probe bound was normalized to the relative amount of binding of the unsubstituted M26 probe in lane 1.

Discussion

Recombination hot spots, sequences, and factors

Recombination hot spots have been studied extensively, particularly in *Saccharomyces cerevisiae*, to gain insight into individual steps of recombination pathways. For example, double-strand breaks appear during meiosis in regions that have high recombination frequencies (Sun et al. 1989; Cao et al. 1990; Sun et al. 1991; Wu and Lichten 1994), supporting models in which double-strand breaks initiate recombination (Resnick 1976; Szostak et al. 1983). Furthermore, certain mutations that reduce meiotic recombination also block or change double-strand break formation (Cao et al. 1990; Alani et al. 1990; Sun et al. 1991; Bishop et al. 1992), again supporting a role for double-strand breaks in recombination. However, specific DNA sequences (and interacting factors) responsible for the formation of double-strand breaks and the initiation of recombination have not, to our knowledge, been identified in *S. cerevisiae*.

In contrast, a specific nucleotide sequence required for the M26 hot spot activity of *S. pombe* has been identified: 5'-ATGACGT-3' (M26 mutation underlined) confers full hot spot activity, whereas alleles lacking this precise sequence (such as M375) also lack hot spot activity (Schuchert et al. 1991). The availability of alleles such as M26 that function as a hot spot, and alleles such as M375 that exhibit normal recombination levels, allowed us to use a gel mobility retardation assay to identify M26-specific binding proteins and to monitor the purification of those proteins.

Function of M26 site and binding proteins

Because binding of the Mts1/Mts2 heterodimer strictly correlates with hotspot activity, it seems likely that its role is to promote an early, rate-limiting step in the initiation of recombination. Three possible modes of action seem plausible. First, the protein may act catalytically at the M26 site to generate recombinogenic lesions such as nicks or double-strand breaks. Second, the bound protein could act as a signal to recruit recombination enzymes in much the same way that transcription factors serve to target RNA polymerase complexes to promoters. Third, binding of Mts1/Mts2 to the M26 site could result in an

"open" chromatin structure, or some other localized perturbation of DNA structure, which could be more accessible to the meiotic recombination machinery. The last mechanism has been proposed recently for *S. cerevisiae*; there is a correlation between nuclease-sensitive sites in chromatin and sites that are "hot" for meiotic recombination (Wu and Lichten 1994). Furthermore, binding of any of three different transcription factors, which could alter local chromatin structure, has about a twofold effect upon recombinant frequencies at the *HIS4* locus (White et al. 1991, 1993).

Several results suggest that other factors interact with the Mts1/Mts2 protein, perhaps as a multicomponent complex, to mediate hot spot activity. First, whereas Mts1 and Mts2 copurify as a heterodimer in solution (Fig. 6), and a heterodimer (or heteromultimer) is required for optimal binding to the M26 site (Fig. 6), the individual subunits are capable of some binding (Fig. 6). This is reminiscent of heterodimeric transcription factors (Blackwood et al. 1992) and provides a possible level of control: Other as-yet-unidentified dimerization partners may contribute to the regulation of hot spot activity. Second, evidence suggests that a third factor (Mts3) is associated with the heterodimer (see Results; Fig. 6). Third, although the M26 hot spot functions only during meiosis (Ponticelli et al. 1988; Schuchert and Kohli 1988), the M26 DNA-binding proteins are present in extracts from both mitotic and meiotic cells (Fig. 2). Some other factor must confer the meiotic specificity. This factor might be encoded by one of the 16 known meiotic *rec* genes of *S. pombe* (Ponticelli and Smith 1989; DeVaux et al. 1992), at least some of which are strongly induced during meiosis (Lin et al. 1992; Lin and Smith 1994).

The presence of Mts1/Mts2 in mitotic cells has two implications. First, the protein may have an as-yet-unidentified role in mitotic cellular metabolism in addition to its role in activating the meiotic recombination hot spot. Second, binding of Mts1/Mts2 to the M26 site is necessary but not sufficient to activate the meiotic recombination hot spot. Other steps are obviously required in the pathway from substrates to recombinant products. Thus, although binding of Mts1/Mts2 to the M26 site stimulates a rate-limiting step in the induction of meiotic recombination, failure of the hot spot to function in mitotic cells could be easily explained by a different step in the pathway being rate-limiting in mitotic cells.

When 3 kbp of *ade6* DNA containing the M26 site was moved to the *ura4* locus, the hot spot failed to function, suggesting that either a second *cis*-linked site or a particular chromatin structure at *ade6* was required for hot spot activity (Ponticelli and Smith 1992). Other experiments, however, show that the M26 site can function as a hot spot when it is moved or when the local chromosomal structure surrounding *ade6* is perturbed: The M26 heptamer has hot spot activity when it is moved 60 or 750 bp downstream of its normal location at *ade6* (J. Metzger and J. Virgin, pers. comm.); in some constructs, M26 does function as a hot spot when moved to *ura4* (J. Virgin, pers. comm.); and the hot spot functions when

the chromosomal structure surrounding *ade6* is altered by inserting a duplication (Schuchert and Kohli 1988). Thus, other naturally occurring M26 sites in the *S. pombe* genome may also function as recombination hot spots.

To estimate the abundance of naturally occurring M26 sites in the *S. pombe* genome, we determined the number of M26 heptamers in *S. pombe* DNA of known sequence. If nucleotides were randomly associated, one would expect to find ~71 copies of any particular heptamer in the two strands of the 583 kbp of *S. pombe* DNA that has been sequenced (GenBank v. 77 and EMBL v. 35). Our homology search detected the M26 heptamer only 13 times, whereas 10 randomized M26 heptamers were found an average of 69 times each (range of 44–101, $\sigma_n = 18$). We conclude that the M26 heptamer is about sixfold under-represented in the *S. pombe* genome. This suggests that there are ~300 heptameric M26 sites in the 14-Mbp (Fan et al. 1989) *S. pombe* genome.

These observations allow a speculative calculation of the fraction of meiotic recombination attributable to M26; 300 M26 sites per genome might be sufficient to account for the observed rates of meiotic recombination. The *S. pombe* genome contains ~14 Mbp (Fan et al. 1989) with a meiotic map size of ~2000 cM (Munz et al. 1989); ~40 reciprocal exchange events occur per meiosis. If the calculated 300 genomic M26 heptamers are all active as hot spots and each promote recombination in 5% of meioses [the level of conversion at *ade6*-M26 (Gutz 1971)], then ~15 conversion events per meiosis would be attributable to M26 sites. About 65% of the apparent conversion events of M26 are accompanied by reciprocal exchange (Grimm et al. 1994). Thus, ~10 reciprocal exchange events per meiosis would be attributable to M26 sites. That is, the predicted 300 M26 sites in the *S. pombe* genome (0.015% of genomic DNA) could conceivably be responsible for up to 25% of all meiotic homologous recombination events.

Recombination hot spots as regulatory elements

Recombination hot spots are active throughout the genomes of organisms from bacteriophages (Lam et al. 1974; Stahl et al. 1975) to humans (Wahls et al. 1990a). Some hot spots (such as M26) function only during meiosis (Ponticelli et al. 1988; Schuchert and Kohli 1988), some function only during mitosis (Keil and Roeder 1984; Voelkel-Meiman et al. 1987), and some function during both meiosis and mitosis (Treco and Arnheim 1986; Wahls et al. 1990b). Thus, in addition to altering the spatial distribution of recombination events (i.e., promoting recombination within a few tens of kilobase pairs of their locations), recombination hot spots differentially influence the amount of recombination occurring during mitosis and meiosis.

These observations imply that a significant fraction of recombination may be regulated by a finite number of discrete sites such as M26. In this scenario, the amount of recombination that occurs in any physical interval depends more on the relative proximity (or local density)

of active recombination hot spots than on its size. Larger physical intervals would generally, but not always, contain a greater number of active hot spots and would thus have a correspondingly greater genetic size. One class of hot spots might be active during mitosis to provide a certain basal level of recombination. Other initiators of recombination, such as DNA damage, clearly contribute to that basal level. During meiosis, when recombination rates must be highly induced to ensure appropriate chromosomal disjunction and to generate genetic diversity, meiotic hot spots would be activated. Thus, discrete sites and their binding proteins could account for the observed regulation of recombination both along the chromosome and during the life cycle.

The proposal that most recombination may be regulated by a finite number of discrete sites is likely to remain speculative for some time. It is clear, however, that recombination hot spots and their associated proteins promote rate-limiting steps of homologous recombination. By understanding how hot spots function, we expect to elucidate discrete steps in pathways of homologous recombination.

Materials and methods

DNA

Gel mobility retardation substrates were gel purified, 215-bp fragments of *ade6* DNA isolated from plasmid or M13 replicative form DNA after digestion with *StyI*. The M26 site is centrally located in these fragments. M13 clones harboring single base pair substitutions in the vicinity of the M26 site were described (Schuchert et al. 1991). DNA fragments for methylation interference were isolated from pUCD4 (354-bp *HaeIII* fragment of *ade6* cloned into the *HincII* site of pUC19; A.S. Ponticelli and G.R. Smith, unpub.) by digestion with *XbaI* and *StyI*. Probes were labeled with [α - 32 P]dNTPs using the Klenow fragment of DNA polymerase I. Standard techniques were used for all DNA manipulations (Sambrook et al. 1989).

Gel mobility retardation assays

The gel mobility retardation assays were as described previously (Wahls et al. 1991). The 15- μ l binding reactions contained 10 μ g of protein, 1 μ g of poly[d(I-C)], and 4 fmoles of 215-bp *StyI*-*StyI* fragment of *ade6* DNA.

Methylation interference assay

The assay was as described (Baldwin 1988) using protein fraction II.

Cell culture and meiotic induction

Meiotic and mitotic extracts were prepared from *S. pombe* strains GP535 (*h⁻ ade6-M26 pat1-114 end1-458*) and GP61 (*h⁻ leu1-32 end1-458*), respectively. Strain genealogies are available upon request. Media, culture techniques, and induction of meiosis by thermal inactivation of the *pat1-114^{ts}* repressor of meiosis have been described (Szankasi and Smith 1992; Wahls et al. 1993). Preparation of extracts from meiotic cultures and analysis of the meiotic time course, DNA replication, and Exo I enzymatic activity were as described (Szankasi and Smith 1992). Induction of meiotic proteases was inferred from the increased

mobility of protein-DNA complexes on mobility retardation gels when extracts were made without adequate protease inhibitors (data not shown). For large-scale purification GP61 cells were grown mitotically in yeast extract liquid at 32°C to late log phase ($A_{595} = 3.5$, $\sim 3.5 \times 10^7$ cells/ml) in a 30-liter capacity fermentor (New Brunswick). The cells were harvested, resuspended in 0.25 volume (vol/wt) of disruption buffer containing 4 $\times$ inhibitors (see below), frozen by drizzling into liquid N₂, and stored at -70°C in sealed containers until they were processed further.

M26₁₈ DNA affinity column

Two complementary, overlapping 31-nucleotide oligonucleotides (M26-1, 5'-TTGGAAATTGATGGATGACGTGAGCA-CATTG-3' and M26-2, 5'-CCAACAATGTGCTCACGTCATC-CATCAATTT-3', M26 mutation is underlined) were annealed to generate a duplex M26 fragment, with 4-bp cohesive ends, which can only ligate in head-to-tail multimers. Tandem arrays were generated and processed as described previously to construct an M26 DNA-Sepharose CL2B column (Wahls et al. 1990a, 1991). The matrix contained ~ 20 μ g of M26₁₈ fragment per milliliter of settled Sepharose.

Purification of M26 DNA-binding activity

Unless stated otherwise centrifugations were for 15 min at 15,000g at 0°C. Column chromatography was at 4°C. Column buffers were adjusted to pH at 4°C, passed through 0.2- μ m filters, and degassed. All column buffers contained 50 mM HEPES-NaOH buffer, 10% glycerol, 0.2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, and NaCl at a concentration designated by shorthand notation. For example, CB7.5/150 was column buffer at pH 7.5 containing 150 mM NaCl. Unless indicated otherwise, buffers contained 1 $\times$ protease inhibitors (1 μ g/ml of aprotinin, 10 μ g/ml of bestatin, 1 μ g/ml of leupeptin, 1 μ g/ml of pepstatin, 20 μ g/ml of N $^{\alpha}$ -p-tosyl-L-lysine chloromethyl ketone-HCl). Fractions from the columns were assayed immediately for DNA-binding activity. A portion of each sample was adjusted to 50% glycerol, dispensed into aliquots, frozen in liquid N₂, and stored at -70°C.

Frozen cells (500 g, frozen in 0.25 vol of CB7.9/500 containing 20% glycerol, 1 mM PMSF, and 4 $\times$ inhibitors) were placed in a 1-gallon Waring Blendor containing ~ 500 ml of liquid N₂. The cells were opened by blending continuously for 30 min at 80% of full power (liquid N₂ was added periodically to maintain a liquid slurry while blending). The powdered frozen lysate was thawed, adjusted to 500 mM NaCl conductivity equivalents by addition of 5 M NaCl, and centrifuged at 100,000g for 30 min, and the supernatant was collected. Nucleic acids were precipitated by stirring in 10% polyethyleneimine-HCl (pH 7.5) to 0.05%, incubating on ice for 15 min, and centrifuging. This procedure yielded 12,700 mg of fraction I (343 ml at 37 mg/ml).

Fraction I was brought to 41% of saturation with (NH₄)₂SO₄, incubated on ice for 15 min, and centrifuged. The pellet was resuspended in 50 ml of CB7.25/150, dialyzed to completion against CB7.25/150, and clarified by centrifugation to yield 4800 mg of fraction II (80 ml at 60 mg/ml).

Fraction II was applied at 360 ml/hr to a 500 ml SP-Sepharose (Pharmacia) column (5 cm diameter). The column was washed with 1500 ml CB7.25/150, and the bound proteins were eluted with a 2000 ml gradient of 150–600 mM NaCl in CB7.25. Fractions containing M26 DNA binding activity eluted in a peak centered at 425 mM NaCl. The active fractions were pooled, dialyzed to completion against CB7.25/200, and clarified by

centrifugation to yield 226 mg of fraction III (485 ml at 465 μ g/ml).

Fraction III was applied at 100 ml/hr to a 100-ml ssDNA-cellulose (U.S. Biochemical) column (2.5 cm diam.). The column was washed with 300 ml of CB7.25/200, and the bound proteins were eluted with a 300-ml gradient of 200–600 mM NaCl in CB7.25. Fractions containing M26 DNA-binding activity eluted in a peak centered at 425 mM NaCl. The active fractions were pooled, dialyzed to completion against CB7.25/200, and clarified by centrifugation to yield 32.9 mg of fraction IV (90 ml at 365 μ g/ml).

Fraction IV was applied at 30 ml/hr to a 1-ml Mono-S column (Pharmacia). The column was washed with 10 ml of CB7.25/200, and the bound proteins were eluted with a 60-ml gradient of 200–600 mM NaCl in CB7.25. The active fractions, which eluted in a peak centered at 340 mM NaCl, were pooled, dialyzed to completion against CB7.25/200, and clarified by centrifugation to yield 9.75 mg of fraction V (13 ml at 750 μ g/ml).

Fraction V was mixed with 200 μ g of poly[d(I-C)]; incubated on ice for 10 min, and applied at 15 ml/hr to an 8-ml M26₁₈ DNA-Sepharose column (1 cm diam.). The column was washed with 60 ml of CB7.25/200, and the bound proteins were eluted with an 80-ml gradient of 200–600 mM NaCl in CB7.25. Fractions containing M26 DNA-binding activity eluted in a peak centered at 425 mM NaCl. The active fractions were pooled to yield 10 μ g of fraction VI containing $\geq$ 50% Mts1 and Mts2 polypeptides by weight (judged by the intensity of bands on Coomassie-stained, SDS-polyacrylamide gels).

Glutaraldehyde cross-linking of Mts1 and Mts2

Aliquots (500 μ l) of protein fraction VI (~250 ng of protein) were mixed with 55- μ l aliquots of freshly diluted glutaraldehyde and incubated at 37°C for 10 min. Samples were placed on ice, 65 μ l of 100% TCA was added, and the proteins were recovered by centrifugation for 30 min at 4°C. The pellets were rinsed with 200 μ l of ice-cold acetone/1 mM HCl and spun for 5 min, and the supernatant was discarded. The rinse was repeated a second time, and the pellets were then dried briefly, resuspended in SDS-PAGE loading buffer, heated, and fractionated on a 12% SDS-polyacrylamide gel (Laemmli 1970), which was stained with Coomassie.

Reconstitution of binding activity from purified components

Approximately 4 μ g of purified fraction VI was concentrated by TCA precipitation and fractionated on a 12% SDS-polyacrylamide gel, the Mts1 and Mts2 bands were excised, and the proteins were eluted in 500 μ l of dilution buffer [(DB) 50 mM HEPES at pH 7.25; 10% glycerol; 150 mM NaCl; 100 μ g/ml of BSA; 1 mM DTT] containing 0.1% SDS by rotating the tubes at 21°C for 15 hr. The eluted proteins were filtered (Millipore Millex-GV4 0.22 μ m), precipitated by the addition of acetone to 80% and centrifuged at 4°C for 30 min. The pellets were rinsed with 200 μ l of 80% acetone/20% DB, dried briefly, resuspended in 45 μ l of DB containing 6 M guanidine-HCl, and incubated for 30 min at 21°C. As a control, purified fraction VI was treated in an identical fashion (but without having been fractionated on SDS-polyacrylamide gel). The denatured proteins were mixed as indicated in Figure 6, diluted 50-fold in DB, and allowed to renature for 6 hr at 21°C. The proteins were concentrated by filtration to ~75 μ l (Millipore Centricon 10), and 5 μ l of each reaction was analyzed by the gel mobility retardation assay (Wahls et al. 1991) for the presence or absence of M26 DNA-binding activity.

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