Hot Spots of Recombination in Fission Yeast: Inactivation of the *M26* Hot Spot by Deletion of the *ade6* Promoter and the Novel Hotspot *ura4-aim*

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ABSTRACT

The *M26* mutation in the *ade6* gene of *Schizosaccharomyces pombe* creates a hot spot of meiotic recombination. A single base substitution, the *M26* mutation is situated within the open reading frame, near the 5' end. It has previously been shown that the heptanucleotide sequence 5' ATGACGT 3', which includes the *M26* mutation, is required for hot spot activity. The 510-bp *ade6-delXB* deletion encompasses the promoter and the first 23 bp of the open reading frame, ending 112 bp upstream of *M26*. Deletion of the promoter *in cis* to *M26* abolishes hot spot activity, while deletion *in trans* to *M26* has no effect. Homozygous deletion of the promoter also eliminates *M26* hot spot activity, indicating that the heterology created through deletion of the promoter *per se* is not responsible for the loss of hot spot activity. Thus, DNA sequences other than the heptanucleotide 5' ATGACGT 3', which must be located at the 5' end of the *ade6* gene, appear to be required for hot spot activity. While the *M26* hotspot stimulates crossovers associated with *M26* conversion, it does not affect the crossover frequency in the intervals adjacent to *ade6*. The flanking marker *ura4-aim*, a heterology created by insertion of the *ura4⁺* gene upstream of *ade6*, turned out to be a hot spot itself. It shows disparity of conversion with preferential loss of the insertion. The frequency of conversion at *ura4-aim* is reduced when the *M26* hot spot is active 15 kb away, indicating competition for recombination factors by hot spots in close proximity.

HOMOLOGOUS recombination rearranges the genetic information encoded by two DNA molecules with (nearly) identical sequences, usually homologous chromosomes in eukaryotes. Recombination generates genetic diversity, is required for proper meiotic chromosome segregation, and is involved in the repair of damaged DNA in somatic cells. Meiotic recombination is best studied in organisms that allow the analysis of all four nuclei derived from individual meiocytes (tetrad analysis). Analysis of the segregation of genetic markers in tetrads has demonstrated unidirectional transfer of genetic information between chromatids (non-Mendelian or aberrant segregation), as well as classical reciprocal exchange (crossover). Two types of aberrant marker segregations are distinguished: unidirectional transfer resulting in either both strands of the acceptor chromatid being changed (conversions, e.g., 6:2 and 2:6 segregations) or only a single-strand being transferred (postmeiotic segregations, e.g., 5:3 and 3:5 segregations). The segregation nomenclature is based on the presence of eight DNA single-strands in the four haploid products of yeast meiosis. The first number always refers to single-strands with wild-type information, the second to single-strands with mutant information. Thus, in a one-factor cross of *ade6⁺* with *ade6⁻*,

normal Mendelian segregation results in 4:4 tetrads (4 *ade⁺* : 4 *ade⁻*). A 6:2 conversion tetrad carries three *ade⁺* spores and one *ade⁻* spore. Postmeiotic-segregation tetrads contain spores in which both wild-type and mutant information are present (heteroduplex DNA). Fungal recombination in general and the analysis of aberrant segregations are reviewed in ORR-WEAVER and SZOSTAK (1985) and PETES *et al.* (1991).

Homologous recombination events are not randomly distributed throughout the genome. Regions with lower (cold spots) and higher (hot spots) than average frequency of aberrant segregations and crossovers have been identified in many different organisms. Specific short DNA sequences required for hot spot activity have been identified in some cases (reviewed by SMITH, 1994). One manifestation of hot spots in ascomycetes is polarity of aberrant segregation: when mutations across a gene are studied, a gradient of aberrant segregation frequencies is often observed, with either the 5' or the 3' end of the gene displaying the highest frequency (reviewed by NICOLAS and PETES 1994). Hot spots and high aberrant segregation ends of genes have been correlated with preferential initiation sites for recombination. In *Saccharomyces cerevisiae*, initiation can occur by double-strand breaks whose locations correlate with DNase hypersensitive sites in chromatin (OHTA *et al.* 1994; WU and LICHTEN 1994).

One of the most extensively characterized recombination hot spots is *M26* in the fission yeast *S. pombe*.

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The *M26* hot spot mutation is a single base substitution within the *ade6* open reading frame, close to the transcription start at the 5' end (PONTICELLI *et al.* 1988; SZANKASI *et al.* 1988). *M26* stimulates both homologous and ectopic recombination in meiosis; however, it does not stimulate mitotic recombination (PONTICELLI *et al.* 1988; SCHUCHERT and KOHLI 1988; GRIMM *et al.* 1991). *M26* was identified as a recombination hotspot by GUTZ (1971), who observed its marker effect in two-factor crosses. The frequencies of prototrophic recombinants in random spores are 3- to 21-fold higher in crosses involving *M26* than in the corresponding crosses with the control mutation *M375* (also a G to T transversion, located just 3 bp upstream of *M26*). *M26* has a 5% frequency of aberrant segregation in tetrads, 10-fold higher than *M375*. In addition, *M26* shows strong disparity: conversions are predominantly from *M26* to wild type, with 6:2 tetrads being 12-fold more frequent than 2:6 tetrads. In contrast, other mutations in the *ade6* gene show parity of gene conversion (equal frequency of 6:2 and 2:6 segregation) (GUTZ 1971). Thus, in heterozygous crosses, the hotspot-carrying chromatid acts as an acceptor in gene conversion events, as is generally observed for hot spots. The frequency of postmeiotic segregation at *M26* is comparable with that of similar mutations at other locations in fission yeast (SCHAER *et al.* 1993). Crosses homozygous for *M26* have demonstrated independent and additive action of the two *M26*-carrying chromatids (PONTICELLI *et al.* 1988; SCHAER and KOHLI 1994). The physical analysis of conversion tracts did not identify significant differences between *M26*-induced and basal recombination (GRIMM *et al.* 1994). However, it showed that *M26* creates or enhances an initiation site for conversion, rather than acting in the termination of conversion. A recombination initiation site is located at the 5' end of the *ade6* gene, even in the absence of the *M26* hotspot mutation. While the exact location and nature of the DNA lesion initiating *M26* recombination is still unknown, hybrid DNA is frequently formed across *M26*. Furthermore, it is the transcribed strand of the donor chromatid that is preferentially transferred (SCHAER and KOHLI 1994).

The heptanucleotide ATGACGT (*M26* mutation underlined) has been shown through extensive mutation analysis to be required for hot spot activity (SCHUCHERT *et al.* 1991). Recently, a heteromeric protein consisting of the Mts1 (70 kD) and the Mts2 (28 kD) subunits has been purified and shown to bind specifically to the ATGACGT heptanucleotide (WAHLS and SMITH 1994). Binding of the heteromeric protein to the heptanucleotide correlates with hotspot activity. However, the heptanucleotide alone is not sufficient for *M26* hotspot activity. In plasmid-chromosome recombination, hot spot activity was absent when a 3-kb *ade6-M26* bearing fragment was on the plasmid, but present when *M26* was on the chromosome. Transplace-

TABLE 1
S. pombe strains

Genotype (designation)	Source
<i>h</i> ⁺ wild type (975)	Bern collection
<i>h</i> ⁻ wild type (972)	Bern collection
<i>h</i> ⁺ <i>ade6-M26</i>	Bern collection
<i>h</i> ⁻ <i>ade6-M26</i>	Bern collection
<i>h</i> ⁺ <i>ade6-M375</i>	Bern collection
<i>h</i> ⁻ <i>ade6-M375</i>	Bern collection
<i>h</i> ⁻ <i>ade6-406</i>	Bern collection
<i>h</i> ⁺ <i>ura4-D18</i>	Bern collection
<i>h</i> ⁻ <i>ura4-D18</i>	Bern collection
<i>h</i> ⁺ <i>ade6-M26 ura4-D18</i>	Bern collection
<i>h</i> ⁺ <i>ade6-M375 ura4-D18</i>	Bern collection
<i>h</i> ⁻ <i>tps16-26 ura4-aim ura4-D18</i>	Bern collection
<i>h</i> ⁺ <i>ade6-delXB ura4-294</i> (GP193)	PONTICELLI (1988)
<i>h</i> ⁺ <i>ade6-delXB,M26 ura4-294</i> (GP198)	PONTICELLI (1988)
<i>h</i> ⁺ <i>ade6-delXB,M375 ura4-294</i> (GP195)	PONTICELLI (1988)
<i>h</i> ⁺ <i>ade6-delXB</i> (MZ6)	This study
<i>h</i> ⁻ <i>ade6-delXB</i> (MZ5)	This study
<i>h</i> ⁺ <i>ade6-delXB,M26</i> (MZ10)	This study
<i>h</i> ⁺ <i>ade6-delXB,M375</i> (MZ8)	This study
<i>h</i> ⁺ <i>ade6-delXB,M26 ura4-D18</i> (MZ16)	This study
<i>h</i> ⁺ <i>ade6-delXB,M375 ura4-D18</i> (MZ14)	This study

ment of the same 3-kb fragment to the *ura4* locus also abolished *M26* hot spot activity in chromosome by chromosome recombination (PONTICELLI and SMITH 1992). The 3-kb fragment used in these experiments includes 870 bp upstream of the transcription initiation codon and 510 bp downstream of the termination codon (SZANKASI *et al.* 1988), and provides full wild-type gene activity. Obviously, additional sequences beyond the 3 kb tested, or a specific chromatin context, are required for *M26* hot spot activity.

To ascertain whether the promoter region encompassed by the 3-kb *ade6* fragment is required for *M26* hot spot activity, the effect of a 510-bp promoter deletion was studied in detail using tetrad analysis. The inclusion of flanking markers enabled us to investigate the possible effects of *M26* and the promoter deletion on crossovers in the *ade6* region. This led to the discovery of the novel recombination hot spot, *ura4-aim*, whose activity is reduced when the *M26* hot spot is active.

MATERIALS AND METHODS

Strains, media, genetic and statistical methods: The *S. pombe* strains used in this work are listed in Table 1. The original strains carrying the promoter deletion in combination with the *ade6-M26* and *M375* mutations were the kind gift of A. S. PONTICELLI and G. R. SMITH. These strains were constructed by generation of a 510bp *XhoI-BamHI* deletion (designated delXB) in the plasmids pade6-M26 and pade6-M375, deleting the promoter and the first 23 bp of the *ade6* coding sequence (see Figure 1A), followed by transformation into *S. pombe* strains bearing the *ura4-294* mutation (PONTI-

FIGURE 1.—Structure of the *ade6* gene and scheme of crosses for tetrad analysis. (A) The thick line and the box (the open reading frame of the *ade6* gene) correspond to the ~3 kb of DNA sequenced by SZANKASI *et al.* (1988). The beginning and end points of the open reading frame, the positions of the M26 and M375 point mutations, and the end points of the *ade6-delXB* deletion that includes the promoter and extends into the open reading frame are indicated. The broken arrow indicates the length and polarity of the *ade6* mRNA. (B) The basic configuration of the crosses carried out to determine the effect of the promoter deletion is shown. Both cross partners usually carried the *ura4-D18* deletion (1.8-kb fragment) in their genetic background. The upper strain carried the *ade6+* allele in combination with the flanking markers *tps16-26* (mutation for heat-sensitive growth) and *ura4-aim*, an insertion of the same 1.8-kb *ura4+* fragment deleted at the natural locus (*ura4-D18*). Thus there is a 1.8-kb sequence heterology in the four crosses considered in Table 3. The lower strain carried either the M26 point mutation (recombination hot spot) or M375 (control, basal recombination). For the crosses heterozygous for the promoter deletion (not drawn), *delXB* was either combined with the *ade6+* allele (*in trans* configuration with respect to the *ade6* point mutations), or *delXB* was associated with the point mutation alleles M26 and M375 (*in cis* configuration). Both crossing partners carried *delXB* in the cross homozygous for the promoter deletion.

CELLI 1988). Strains bearing the *ura4-D18* mutation (GRIMM *et al.* 1988), a complete deletion of the *ura4* gene, were obtained by crossing. Flanking markers were then introduced by additional crosses. The right flanking marker *tps16-26* has been described by GYGAX and THURIAUX (1984) and the left *ura4-aim* marker (*aim*: artificially introduced marker) by GRIMM *et al.* (1994). A description of standard genetic methods and media is found in GUTZ *et al.* (1974). Tetrads were dissected using either a Singer Instruments MSM System or a classical dissection microscope. G-tests of independence were used to compare the recombination frequencies from different crosses (contingency tables) (SOKAL and ROHLF 1981).

Determination of *ade6* genotypes of spores derived from crosses with double mutants: The genetic configuration at the *ade6* locus in adenine-auxotrophic spores of 2:2 segregating tetrads from crosses involving strains MZ14 and MZ16 were determined. The spore clones were backcrossed against two tester strains, one bearing the promoter deletion only (MZ5 or MZ6) and the other bearing the appropriate point mutation only (either M26 or M375; Table 1), and the presence of recombinant prototrophs scored. The conditions for these assays were set in trial experiments. When M26 was

involved, patch-crossing, followed by replica plating, yields a lawn of prototrophs for the cross *ade6-delXB* × *ade6-M26*, but no prototrophs for the two selfings *ade6-M26* × *ade6-M26* and *ade6-delXB* × *ade6-delXB*. When M375 was involved, the patch-crossing method was not sufficiently discriminative, and the cross material was treated with *Helix pomatia* juice (MUNZ and LEUPOLD 1979), and concentrated 10-fold by centrifugation before plating out on minimal medium. The *ade6-delXB* × *ade6-M375* test cross yielded >100 colonies per plate, while the selfings *ade6-M375* × *ade6-M375* and *ade6-delXB* × *ade6-delXB* yielded no prototrophic colonies. In this way it could clearly be distinguished whether an adenine-dependent spore clone carried either the *delXB* deletion, the point mutation (either M26 or M375), or both (double mutant).

The reliability of these assays is assured by comparison with the meiotic reversion frequencies of the mutations (selfings), as well as by the determination of prototroph frequencies for some of the crosses. GUTZ (1971) obtained reversion frequencies in selfings of $<2 \times 10^{-8}$ for M375 and 8×10^{-9} for M26. The reversion frequency for the promoter deletion *delXB* was found to be $<7 \times 10^{-9}$. The frequency of prototrophs for the cross *ade6-delXB*, M375 × *ade6-M375* is $<4 \times 10^{-6}$, while that of the cross *ade6-delXB* × *ade6-M375* is 28×10^{-6} . It is thus possible to distinguish clearly between *ade6-M375* (point mutation alone), *ade6-delXB* (promoter deletion alone), and the double mutation *ade6-delXB*, M375 (point mutation and promoter deletion). Due to the hot spot activity of M26, the corresponding strains with M26 are distinguished even more easily than those bearing the M375 control mutation.

RESULTS

Definition of the *ade6* promoter and the experimental strategy: The *ade6* gene is expressed with low efficiency both in mitotic and meiotic cells as judged from Northern blots (GRIMM *et al.* 1991). No obvious TATA box or other known transcription-related sequences have been identified (SZANKASI *et al.* 1988). A set of nested *ExoIII* deletions of sequences 5' to the open reading frame have been transformed into an *ade6* deletion strain and checked for complementation of adenine auxotrophy. The deletion constructions were expressed from multicopy plasmids, as well as from single-copy integrations into the genome (P. HÄNNI, J. GAFNER and J. KOHLI, unpublished results). Deletions leaving intact 190 bp upstream of the open reading frame do not affect *ade6* expression, while the gene is inactivated when 5' deletions end ~40 bp upstream of the translation initiation codon. Thus, the 510-bp deletion *ade6-delXB* (constructed by PONTICELLI 1988) encompasses the *ade6* promoter. In addition, *delXB* includes the first 23 bp of the open reading frame and ends 112 bp upstream of M26 (Figure 1). While the promoter deletion does not affect spore viability, it leads to adenine dependence for growth and confers red pigmentation to colonies grown on media with limiting adenine concentration. This phenotype is identical to the one observed for *ade6*-inactivating point mutations like M26, M375 and 406 (Table 1).

To determine the effect of the *delXB* deletion on M26 hot spot activity, the crosses depicted in Figure 1B were

TABLE 2
The promoter deletion *in trans* does not abolish M26 hot spot activity

Cross partners	Frequencies of Ade ⁺ recombinants × 10 ⁶	
	<i>ade6-406</i>	<i>ade6-delXB</i>
<i>ade6-M375</i>	25, 38, 30, 56, 26 (35 ± 13)	25, 30, 28 (28 ± 3)
<i>ade6-M26</i>	324, 216, 207, 336, 232 (263 ± 62)	813, 728, 473, 420 (608 ± 191)
M26 hot spot activity	8	22

Determination of recombinant prototroph frequencies by random spore analysis. Parenthetical values are means ± SD.

carried out. M26 hot spot activity was assayed by comparing the conversion frequencies at the *ade6* locus for analogous crosses involving M26 and M375. The effect of M26 hot spot activity on crossover frequencies in the *ade6* region was determined using the flanking markers *tps16* and *ura4-aim*. The latter is an insertion of the functional *ura4* gene 15 kb upstream of *ade6*, and was on a *ura4* deletion background to exclude ectopic recombination (Table 1) (GRIMM *et al.* 1994). The basic conversion and crossover frequencies were determined in crosses without the *ade6* promoter deletion (exactly as shown in Figure 1B) and serve as a control for the crosses involving the promoter deletion. At the same time they are a repetition of the crosses carried out by GUTZ (1971) but, for the first time, provide information concerning the effect of M26 on crossovers at the tetrad level. Subsequently the effect of heterozygosity of the promoter deletion was studied with *delXB* either *in trans* (present in the upper cross partner in Figure 1B), or *in cis* (lower cross partner in Figure 1B) to the M26 and M375 mutations. Finally, crosses were performed with the promoter deletion in homozygous and the M26 mutation in heterozygous configuration to exclude loss of hot spot activity due to the sequence heterology created by the promoter deletion in the heterozygous cross. The random spore data for the promoter deletion *in trans* configuration are presented first (Table 2), followed by the detailed results of the tetrad analysis (Table 3). The conversion data for all three genetic loci in Table 4 are extracted from Table 3 and show that the *ade6* promoter deletion *in cis* abolishes the M26 conversion hot spot. The novel *ura4-aim* recombination hot spot is also apparent from its elevated conversion frequencies. Finally the crossover data are presented in Table 5 with reference to the presence of M26 (*vs.* M375) and the promoter deletion (*vs.* wild-type promoter).

The promoter deletion *delXB* does not abolish the M26 hot spot *in trans*: Following construction of the *ade6-delXB* deletion, PONTICELLI (1988) showed by ran-

dom spore analysis that crosses of a deletion strain with *ade6-M26* yield an eightfold higher frequency of Ade⁺ recombinants than crosses of the deletion strain with *ade6-M375* indicating that the promoter deletion *in trans* does not abolish the M26 hot spot. To confirm this result, crosses of strains with the *delXB* deletion were compared with crosses carrying *ade6-406* (Table 2), a G to A transition in the initiation codon of *ade6* (SCHAER and KOHLI 1993). This mutation is located 20 nucleotides upstream of the right endpoint of the *delXB* deletion (Figure 1A). The ratio of the frequency of recombinant adenine prototrophs obtained for a cross with M26 and that obtained for the corresponding cross with M375 is termed the M26 hot spot activity. A M26 hot spot activity of 22 is obtained for crosses with the *delXB* promoter deletion, while crosses with the *ade6-406* point mutation show a somewhat diminished hot-spot activity (8). The distance between the 406 point mutation and M26 is 133 bp, compared to 113 bp between the endpoint of the deletion and M26.

The promoter deletion *delXB* abolishes the M26 conversion hot spot *in cis*: Random spore analysis of crosses with the promoter deletion *in cis* point to the inactivation of the M26 hot spot; however, the involvement of the heterology created by deleting the promoter could not be excluded (PONTICELLI 1988). The results of the tetrad analysis of four crosses are shown in Table 3. One of the partner strains in these four crosses was always the same (upper strain in Figure 1B). As a control, the crosses depicted in Figure 1B were performed with an intact *ade6* promoter in the partners. One cross involves M375 (*ade6* hot spot not active), the other M26 (*ade6* hot spot active). The other two crosses again involve either M375 or M26, but this time in combination with the promoter deletion *delXB in cis* (altered lower strain in Figure 1B; refer to Table 1 for the complete genotypes). All the observed tetrad types are shown, with the segregation of the alleles at the three loci *ura4-aim*, *ade6* and *tps16*, and their occurrence in the four crosses given. The genotype of the four spores of each tetrad type is displayed for clarity. The classification concerning the nature (conversion or crossover) and number (single or multiple) of the recombination events that account for each tetrad type was done according to the parsimony principle. Postmeiotic segregation was not detectable at any of the three loci due to the experimental procedure used. Except for G to C transversion mutations, postmeiotic segregation is rare in fission yeast (<5% of total aberrant segregations) (SCHAER and KOHLI 1993). Hence a bias of the data, due to misclassification of 5:3 segregations as 6:2, and 3:5 segregations as normal 4:4, can be considered negligible.

Table 4 summarizes all the conversion events observed in the tetrads considered in Table 3. In the control crosses (intact promoter), a fivefold increase in the

TABLE 3

Tetrad analysis of crosses with the heterozygous promoter deletion in *cis* to M26 and control crosses without the deletion

Tetrad class	Description of the recombination event(s) ^a	Genotypes of the four spores ^b	Wild-type <i>ade6</i> promoter		<i>ade6</i> promoter deletion in <i>cis</i>	
			M375	M26	M375	M26
1	None (PD, PD, PD)	++-/+ +-/- -+/- -+ -	459	865	203	801
Single conversions						
2	<i>ura4-aim</i> 6:2	++-/+ +-/- -+/- -+ -	3	5	1	9
3	<i>ura4-aim</i> 2:6	++-/- +-/- -+/- -+ -	24	16	4	28
4	<i>ade6</i> 6:2	++-/+ +-/- -+/- -+ -	0	7	0	0
5	<i>ade6</i> 2:6	++-/+ +-/- -+/- -+ -	0	0	0	0
6	<i>tps16</i> 6:2	+++ / +-+ / - -+ / - -+ -	0	1	1	0
7	<i>tps16</i> 2:6	++- / +-+ / - -+ / - -+ -	0	7	0	0
Multiple conversions						
8	<i>ura4-aim</i> 6:2, and <i>ade6</i> 6:2	+++ / +-+ / - -+ / - -+ -	0	1	0	0
9	<i>ura4-aim</i> 2:6, and <i>tps16</i> 6:2	++- / +-+ / - -+ / - -+ -	0	0	0	1
10	<i>ade6</i> 6:2, and <i>tps16</i> 2:6	++- / +-+ / - -+ / - -+ -	0	0	0	1
11	<i>ura4-aim</i> 6:2, <i>ade6</i> 6:2, and <i>tps16</i> 2:6	++- / +-+ / - -+ / - -+ -	0	0	0	1
Single crossover						
12	interval <i>ura4-aim-ade6</i> (T, PD, T)	++- / +-+ / - -+ / - -+ -	44	72	11	72
13	interval <i>ade6-tps16</i> (PD, T, T)	+++ / +-+ / - -+ / - -+ -	83	134	23	153
Multiple crossovers						
14	double in <i>ade6-tps16</i> interval (PD, NPD, NPD)	+++ / +++ / - -+ / - -+ -	7	5	0	3
15	double, one in each interval, 2-strand (T, T, PD)	++- / +-+ / - -+ / - -+ -	0	1	2	2
16	double, one in each interval, 3-strand (T, T, T)	+++ / +-+ / - -+ / - -+ -	1	4	0	0
17	double, one in each interval, 3-strand (T, T, T)	++- / +-+ / - -+ / - -+ -	0	2	1	2
18	double, one in each interval, 4-strand (T, T, NPD)	+++ / +-+ / - -+ / - -+ -	0	1	1	0
19	triple, two in <i>ura4-aim-ade6</i> , one in <i>ade6-tps16</i> (NPD, T, T)	++- / +-+ / - -+ / - -+ -	0	1	0	0
Conversion and crossover						
20	<i>ade6</i> 6:2, assoc. crossover (-, -, T)	+++ / +++ / - -+ / - -+ -	1	21	2	2
21	<i>ade6</i> 2:6, assoc. crossover (-, -, T)	++- / +-+ / - -+ / - -+ -	2	0	0	0
22	<i>ade6</i> 6:2, assoc. crossover, and crossover <i>ade6-tps16</i> (-, -, NPD)	+++ / +++ / - -+ / - -+ -	0	1	0	0
23	<i>ura4-aim</i> 6:2, crossover <i>ura4-aim-ade6</i> (-, PD, -)	++- / +-+ / - -+ / - -+ -	0	0	1	0
24	<i>ura4-aim</i> 2:6, crossover <i>ura4-aim-ade6</i> (-, PD, -)	++- / +-+ / - -+ / - -+ -	0	1	0	3
25	<i>ura4-aim</i> 6:2, crossover <i>ade6-tps16</i> (-, T, -)	+++ / +++ / - -+ / - -+ -	1	0	0	2
26	<i>ura4-aim</i> 6:2, crossover <i>ade6-tps16</i> (-, T, -)	+++ / +++ / - -+ / - -+ -	0	0	0	2
27	<i>ura4-aim</i> 2:6, crossover <i>ade6-tps16</i> (-, T, -)	++- / +-+ / - -+ / - -+ -	4	0	0	1
28	<i>ura4-aim</i> 2:6, crossover <i>ade6-tps16</i> (-, T, -)	+++ / +-+ / - -+ / - -+ -	3	2	0	3
29	<i>tps16</i> 6:2, crossover <i>ade6-tps16</i> (PD, -, -)	+++ / +++ / - -+ / - -+ -	0	1	0	0
Total tetrads			632	1148	250	1086

Viability in all crosses was very high (with an average of 82% tetrads with four spores growing) and did not differ significantly between the crosses (data not shown). Only tetrads with four viable spores were considered in the analysis.

^a The tetrad types (PD, parental ditype; NPD, nonparental ditype; T, tetratype) are, from left to right, for the three intervals *ura4-aim-ade6*, *ade6-tps16*, and *ura4-aim-tps16*.

^b The spore genotypes given are in the order *ura4-aim*, *ade6*, *tps16*; +, prototrophy (*ura4-aim*⁺, *ade6*⁺) or temperature resistance (*tps16*⁺); -, auxotrophy or temperature sensitivity (refer to Figure 1B for details).

conversion frequency at the *ade6* locus was observed with M26 (2.6%) as compared with the cross with M375 (0.5%). This difference is significant ($P = 0.0004$). The same holds for the strong disparity of conversion at M26: 30 6:2 tetrads *vs.* no 2:6 tetrads. These characteristics of the M26 hot spot have already been described by GUTZ (1971). Deletion of the *ade6* promoter has no discernible effect on conversion frequency at the *ade6* locus in crosses with M375 (0.8% conversion compared

with 0.5% conversion with the intact promoter). In contrast, deletion of the promoter reduces the conversion frequency for the cross with M26 to 0.4% (significantly different from the 2.6% conversion frequency in the control, $P = 0.000004$) and comparable to the levels observed with M375 either with or without promoter deletion (0.8 and 0.5%, respectively).

The possibility that the M26 hot spot activity is not abolished, but only masked by the promoter deletion,

TABLE 4
Summary of conversion events

Conversion events	Control crosses: wild-type <i>ade6</i> promoter						Crosses with <i>ade6</i> promoter deletion <i>in cis</i>					
	<i>M375</i>			<i>M26</i>			<i>M375</i>			<i>M26</i>		
	<i>ura4-aim</i>	<i>ade6</i>	<i>tps16</i>	<i>ura4-aim</i>	<i>ade6</i>	<i>tps16</i>	<i>ura4-aim</i>	<i>ade6</i>	<i>tps16</i>	<i>ura4-aim</i>	<i>ade6</i>	<i>tps16</i>
6+:2-	4	1	0	6	30	2	2	2	1	14	4	1
2+:6-	31	2	0	19	0	7	4	0	0	36	0	2
Total	35	3	0	25	30	9	6	2	1	50	4	3
Frequency (%)	5.5	0.5	0	2.2	2.6	0.8	2.4	0.8	0.4	4.6	0.4	0.3

Number of tetrads: control *M375*, 632; control *M26*, 1148; *ade6* promoter deletion *in cis M375*, 250; and *ade6* promoter deletion *in cis M26*, 1086.

must be considered. In this case, *M26* would be converted to wild type without coconversion of the promoter deletion to wild type, resulting in spores carrying the promoter deletion alone. Such events are not detectable using the simple classification of spores for prototrophy *vs.* auxotrophy used for the tetrads in Tables 3 and 4. To exclude this possibility, the two auxotrophic spore clones from tetrads with 2:2 segregation for adenine auxotrophy were assayed. A total of 221 tetrads for the *M26* cross and 239 tetrads for the *M375* cross were checked for retention of both the point mutation (*M26* or *M375*, respectively) and the promoter deletion by appropriate backcrossing (see MATERIALS AND METHODS). None of these adenine-dependent spores showed conversion to wild type of either the point mutation alone, or the deletion alone. Had the *M26* hot spot been active as in the control cross without the promoter deletion, five conversions of the *M26* mutation to wild type would have been expected among a sample of 221 tetrads. The absence of such conversions is significant ($P = 0.001$), and argues that the *M26* hot spot activity has truly been abolished through deletion of the promoter *in cis*.

Finally, it may be maintained that the sequence heterology created by the promoter deletion prevents the completion of conversion events initiated by *M26* and that such events might be completed in the absence of

the sequence heterology. Therefore, a cross homozygous for the promoter deletion *delXB*, but heterozygous for *M26*, was carried out (without flanking markers). The four auxotrophic spores of 266 tetrads were analyzed by backcrossing (see MATERIALS AND METHODS), with no conversion at *M26* being found. This clearly differs from the 13 conversions expected (5%) (GUTZ 1971) with an active *M26* hot spot ($P = 7 \times 10^{-7}$). *M26* hot spot activity is thus abolished in crosses homozygous for the promoter deletion.

The novel *ura4-aim* hot spot and interaction between hot spots: The overall conversion frequencies at *tps16* (13 per 3116 tetrads, 0.4%) and *ade6-M375* (five per 882 tetrads 0.6%) shown in Table 4 are comparable with those of the majority of genetic loci in fission yeast (<1% conversion) (GYGAX and THURIAUX 1984; MUNZ 1994). In contrast, the *ura4-aim* heterology is converted at high frequency: 116 conversion tetrads (3.7%) out of a total of 3116 tetrads analyzed (Table 4). In addition there is significant disparity: 2:6 tetrads (conversion arising from the loss of the 1.8-kb *ura4-aim* insertion) are more frequent than 6:2 tetrads (90 2:6 *vs.* 26 6:2, $P = 10^{-9}$). This hot spot property of the inserted *ura4* gene near *ade6* is due to the chromosomal context, as concluded from the absence of an enhanced frequency of conversion at the original *ura4* locus at the left end of chromosome III. Among the 265 tetrads analyzed for

TABLE 5
Summary of crossover events not associated with conversion at any locus

Crosses	Constitution with respect to interval <i>ura4-aim-ade6</i> (top) and <i>ade6-tps16</i> (bottom)									Total tetrads
	PD PD	PD NPD	PD T	NPD PD	NPD NPD	NPD T	T PD	T NPD	T T	
Control										
<i>M375</i>	459	7	83	0	0	0	44	0	1	594
<i>M26</i>	865	5	134	0	0	1	72	0	8	1085
Promoter deletion <i>in cis</i>										
<i>M375</i>	203	0	23	0	0	0	11	0	4	241
<i>M26</i>	801	3	153	0	0	0	72	0	4	1033
Total	2328	15	393	0	0	1	199	0	17	2953

the cross *ura4⁺ × ura4-D18* (1.8-kb deletion corresponding to the 1.8-kb insertion at *ura4-aim*), only one 2:6 tetrad was found (0.4%).

The discovery of the *ura4-aim* hot spot allows the study of possible interactions between neighboring hot spots. The physical distance separating the *ura4-aim* hot spot and *ade6* with its M26 hot spot is ~15 kb (GRIMM *et al.* 1994). Conversion at *ura4-aim* is significantly more frequent when the *ade6* hot spot is inactive. Two of the relevant comparisons showing significant differences between crosses in Table 4 are control M375 (5.5% conversion at *ura4-aim*) *vs.* control M26 (2.2% conversion at *ura4-aim*, $P = 0.0003$), and M26 with promoter deletion (4.6% conversion at *ura4-aim*) *vs.* control M26 (2.2% conversion at *ura4-aim*, $P = 0.001$). Thus, the active *ade6*-M26 hot spot reduces conversion frequency at *ura4-aim* by half. The value for the cross M375 with promoter deletion is also low (2.4% conversion at *ura4-aim*), but may be due to the small number of tetrads analyzed for this cross (250). This notion is confirmed by the statistical homogeneity of *ura4-aim* conversion frequencies in the three crosses in which the *ade6*-M26 hot spot is inactive (control M375, M375 with promoter deletion, and M26 with promoter deletion, $P = 0.105$).

M26 enhances only conversion-associated crossovers in the *ade6* region: The flanking markers *ura4-aim* and *tps16* were first used in random spore experiments (GRIMM *et al.* 1994), which demonstrated that M26 stimulates conversions with an associated crossover to the same extent as conversions alone (as compared with the control with M375). Depending on the cross, 52–65% of the convertants were found to be associated with a crossover. The results from the comparably small sample of tetrads in our study (Table 3) are consistent with the earlier results: seven of the nine conversion events (78%) observed in crosses with the inactive *ade6* hotspot were associated with a crossover, while 22 of the 30 conversions (73%) in the cross with the active M26 hot spot were associated with a crossover event.

Table 5 is an extract of Table 3 and summarizes the crossover events for the tetrads without conversion event(s) for the four crosses. Statistical evaluation using contingency tables indicates no significant differences in the number and distribution of crossovers in the four crosses as regards the two intervals *ura4-aim-ade6* and *ade6-tps16*. Thus the data were pooled and the genetic distances determined using PERKINS' formula (PERKINS 1949), which, due to the shortness of the intervals and the apparent absence of chromatid interference (based on analysis of tetrads showing tetratype configuration for both intervals) is most appropriate. The *ura4-aim-ade6* interval is 3.8 cM and the *ade6-tps16* interval 8.5 cM. Statistical analysis used to compare the frequency of multiple crossover events and that expected based on the overall crossover frequencies in the *ura4-aim-ade6* interval shows no significant deviation from a Pois-

son distribution of crossovers. When this analysis is performed for the *ade6-tps16* interval, a significant deviation from Poisson distribution of crossovers is observed ($P = 0.003$). Closer inspection of the data in Table 5 reveals that the overabundance of NPD tetrads observed for the *ade6-tps16* interval (which would indicate negative crossover interference) is only observed in the control cross with M375 and not in the other three crosses. For this reason, we hesitate to ascribe any biological significance to this finding. Thus, positive crossover interference is not observed in the *ade6* region, as is true for the genome of fission yeast in general (MUNZ 1994). Consequently, the genetic distance of the compound interval *ura4-aim-tps16* corresponds to the sum of the subintervals (11.8 cM). This distance is not strongly affected by the M26 hot spot, as inclusion of the M26 conversion-associated crossovers in the determination of the *ura4-aim-tps16* distance yields only the slightly higher value of 12.5 cM (data from the control cross with M26, Table 3).

DISCUSSION

Deletion of the *ade6* promoter in *cis* abolishes the M26 hot spot: Following the discovery of the M26 recombination hot spot in *S. pombe*, interest has focused on the DNA sequences required and sufficient for *ade6*-M26 hot spot activity. Systematic exchange of nucleotides in the vicinity of the M26 mutation has demonstrated that the heptanucleotide 5' ATGACGT 3' (M26 mutation: G to T transversion) is required for hot spot activity (SCHUCHERT *et al.* 1991). PONTICELLI and SMITH (1992) then showed that transplacement of a 3-kb fragment carrying *ade6*-M26 to either another site in the fission yeast genome or to a plasmid leads to loss of the M26 hot spot activity. A plausible explanation of this result, namely the requirement of one or more additional special, discrete DNA sequences beyond the standard 3-kb fragment, has been made unlikely by further experiments (J. VIRGIN and G. SMITH, personal communication). It may well be that a specific, context-dependent chromatin structure is required, rather than a few defined DNA sequences alone. This holds true for other recombination hot spots and the location of double-strand breaks acting as initiation events in *S. cerevisiae* (DE MASSY and NICOLAS 1993; GOYON and LICHTEN 1993; MALONE *et al.* 1994). It has recently been demonstrated for the *ARG4* and other hot spots in budding yeast that these occur in the promoter region at sites that are hypersensitive to nuclease digestion, and that this hypersensitivity increases early in meiotic prophase (OHTA *et al.* 1994; WU and LICHTEN 1994).

The experiments presented address the question whether sequences within the 3-kb *ade6* fragment other than the heptanucleotide 5' ATGACGT 3' are required for hot spot activity. We have confirmed the observation

of PONTICELLI (1988) that the promoter deletion *delXB* does not eliminate the *M26* hot spot activity *in trans* (Table 2). However, when *M26* is located *in cis* to *delXB*, the hot spot is abolished both in crosses heterozygous and homozygous for the promoter deletion, as concluded from extensive tetrad analysis (Table 4) and additional experiments. Preliminary evidence in support of this conclusion has been published by PONTICELLI (1988). This loss of *M26* hot spot activity can be explained in several different ways. The deletion may partially or completely remove a defined DNA sequence required for high frequency of recombination, and thus resemble the heptanucleotide 5' ATGACGT 3' that serves as a binding site for a DNA-sequence specific protein (WAHLS and SMITH 1994). Such a defined sequence in the promoter region may be the recognition site for a transcription factor or a protein affecting processes other than transcription. Mutation of the Rap1 binding site upstream of the *HIS4* gene in budding yeast has been shown to reduce meiotic conversion at *HIS4* (WHITE *et al.* 1991). Alternatively the deletion may disturb the local chromatin structure required for *M26* hot spot activity. Micrococcal nuclease hypersensitive sites have been observed in the *ade6* promoter region of chromatin isolated from vegetative cells, while the open reading frame including the *M26* site was covered by nucleosomes (BERNARDI *et al.* 1991). Another explanation is that deletion of the promoter brings a negatively acting element in proximity to *M26*. Finally, transcription of the *ade6* gene may be necessary for *M26* hotspot activity. Replacement of the weak *ade6* promoter by the strong *adh1* promoter boosts mitotic and meiotic recombination frequencies at *ade6*, as well as transcription. When the hot spot mutation *M26* was included in such constructs, meiotic recombination was stimulated further, albeit weakly (GRIMM *et al.* 1991). As for the promoter deletion *delXB*, it has not been shown that the change in recombination frequency is caused by alteration of transcription *per se*, rather than due to other alterations caused by the DNA sequence rearrangements. The influence of altered transcription rates on mitotic recombination has been demonstrated in *S. cerevisiae* (VOELKEL-MEIMAN *et al.* 1987; THOMAS and ROTHSTEIN 1989). No clear correlation between transcription and recombination rates has been reported for meiotic recombination. A 31-bp deletion of the TATAA element of the *HIS4* promoter reduces transcription and mitotic, but not meiotic, recombination (WHITE *et al.* 1992). Two long deletions (815 and 136 bp) in the *ARG4* promoter region strongly reduce transcription and meiotic recombination, but a series of short deletions (13–42 bp) in the same promoter region show no consistent correlation between mRNA levels and conversion frequencies (SCHULTES and SZOSTAK 1991).

The *M26* hot spot and crossover distribution in the *ade6* region: It has previously been reported that conversion-associated crossovers occur with similar fre-

quencies in crosses with *M375* and *M26*. Crossover association of *ade6* conversion was 73 and 78%, respectively, for crosses with inactive *vs.* active *M26* hot spot. These estimates are based on small numbers and thus judged to be less reliable than the earlier values (52–65%) obtained from preselection of *ade*⁺ recombinants from random spores (GRIMM *et al.* 1994). No effect of *M26* or the promoter deletion on the frequency and distribution of crossovers unassociated with *ade6* conversion in the intervals adjacent to *ade6* were observed. There is also no evidence for positive crossover interference in the *ade6* region, a property of most eukaryotes, but not of fission yeast (MUNZ 1994). A genetic distance of 11.8 cM was calculated for the interval encompassing the *ade6* gene and flanked by the *ura4-aim* and *tps16* markers. This value is only slightly increased (to 12.5 cM), when the *ade6* conversion-associated crossovers are included. The ratio of the genetic (3.8 cM) and physical length (15 kb) for the interval *ura4-aim* - *ade6* is 0.25 cM per kilobase, a value which does not differ greatly from the average of 0.16 cM per kilobase derived from recent estimates of total genetic (2200 cM) (MUNZ 1994) and physical genome length (13.8 Mb) (FAN *et al.* 1991).

Conversion frequency at the novel hot spot *ura4-aim* is reduced by the active *M26* hot spot: To create an *ade6* flanking marker, the *ura4* gene was inserted 15 kb upstream of *ade6* in a strain in which *ura4* is deleted at its natural locus (GRIMM *et al.* 1994). Surprisingly high conversion frequencies were observed at this *ura4-aim* locus in the four crosses shown in Table 4 (3.7% average). When the equivalent configuration of wild-type *ura4* and deletion of the 1.8 kb *ura4* fragment was tested at the natural *ura4* locus, only 0.4% conversion was observed, a value typical for most fission yeast loci (GYGAX and THURIAUX 1984; MUNZ 1994). Thus, the 1.8-kb *ura4* heterology *per se* is not responsible for the high frequency of conversion and the *ura4* fragment itself exhibits average recombinational activity. It remains to be determined whether the hot spot activity of *ura4-aim* is a result of insertion of the *ura4* fragment into a natural hot spot area on chromosome III or whether the novel hot spot is a cooperative effect of inserted and resident DNA sequences. An increase in recombination frequencies resulting from the transplacement of defined DNA sequences has been described for the *URA3* and *LEU2* genes in *S. cerevisiae* (BORTS *et al.* 1984; CAO *et al.* 1990). In addition, *ura4-aim* displays significant disparity of conversion with 2:6 tetrads more frequent than 6:2 tetrads (78 *vs.* 22%, respectively). This prevalence of recombination events resulting in loss of the (1.8 kb) insertion fragment has also been observed for a 409-bp insertion in the *b2* gene of *Ascobolus immersus* (HAMZA *et al.* 1987; J.-L. ROSSIGNOL and V. COLOT, personal communication). A large deletion showing disparity of gene conversion

has been described by MCKNIGHT *et al.* (1981). Other heterologies have shown no disparity of conversion (FINK and STYLES 1974; LAWRENCE *et al.* 1975; FOGEL *et al.* 1978; HAMZA *et al.* 1987).

The data in Table 4 argue for an interaction between the *ade6-M26* and the *ura4-aim* hot spots. When *M26* is active (control cross without promoter deletion), the conversion frequency at *ura4-aim* is significantly reduced from an average conversion frequency of 4.6% (three crosses with inactive *M26*) to 2.2%. An effect of *ura4-aim* on *M26* cannot be directly assessed as a low recombination marker at *ura4-aim* (absence of *ura4-aim* hot spot activity) is not yet available. The conversion frequency at *M26* in the control cross with the active *ura4-aim* hot spot (2.2%, 1148 tetrads, Table 4) is significantly lower ($P = 0.004$) than that obtained by GUTZ (1971) (3.9%, 3110 tetrads) in crosses in which the *ura4-aim* marker was absent. This is consistent with the hypothesis that no hot spot exists at the locus prior to insertion of *ura4-aim*. The partial suppression of the *ura4-aim* hot spot by *M26* may be due to competition of the two sites for rate-limiting factors involved in meiotic recombination. Further analysis of the *ura4-aim* hot spot is in progress and evidence for hot spot activity in crosses homozygous for the *ura4-aim* insertion has been obtained (M. BAUR, E. HARTSUIKER, E. LEHMANN and J. KOHLI, unpublished results).

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