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Genetics

Chromosomal context dependence of a eukaryotic recombinational hot spot

(*Schizosaccharomyces pombe*/*M26* hot spot/meiosis)

ALFRED S. PONTICELLI* AND GERALD R. SMITH†

Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104; and Department of Pathology, University of Washington, Seattle, WA 98195

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ABSTRACT **The single base-pair mutation *M26* in the *ade6* gene of the fission yeast *Schizosaccharomyces pombe* creates a hot spot for meiotic homologous recombination. When DNA fragments containing *M26* and up to 3.0 kilobases of surrounding DNA were moved to the *ura4* gene or to a multicopy plasmid, *M26* had no detectable hot spot activity. Our results indicate that nucleotide sequences at least 1 kilobase away from *M26* are required for *M26* hot spot activity and suggest that, as for transcriptional promoters, a second site or proper chromatin structure is required for activation of this eukaryotic recombinational hot spot. We discuss the implications of these results for studies of other meiotic recombinational hot spots and for gene targeting.**

Special sites enhance homologous recombination within their vicinity on the chromosomes of prokaryotes and eukaryotes (1). These sites, called hot spots, are thought to be recognition sites for recombination-promoting enzymes, which act at high frequency at or near the hot spot. Recombinational hot spots are thus analogous to promoters of transcription and origins of DNA replication, in that they define discrete chromosomal sites that stimulate chromosomal activity in their vicinity.

The *M26* mutation in the *ade6* gene of *Schizosaccharomyces pombe* creates a recombinational hot spot (2) that enhances gene conversion and crossing-over at the *ade6* locus but not at other loci; the *M26* hot spot is active during meiosis but not during mitosis (3, 4). In some respects the *M26* hot spot appears simple: it is a single base-pair change G·C → T·A (3, 5) creating the sequence 5′-ATGACGT-3′, which is required for hot spot activity (6). *M26* acts when it is present on one or both of the recombining chromosomes (3). These results are consistent with the view that *M26* is the recognition site for a sequence-specific recombination-promoting protein. Indeed, an *M26*-specific binding protein has recently been detected (E. Käslin and J. Kohli, personal communication; W. Wahls and G.R.S., unpublished observations). We report here, however, that the requirements for *M26* action are complex: when the *M26* site with up to 3.0 kilobases (kb) of surrounding DNA was moved to other locations in the genome, it failed to enhance recombination. These results imply that the action of *M26* requires another chromosomal site or proper surrounding chromatin structure. This complexity urges caution in the interpretation of large deletions that appear to locate other chromosomal sites, including recombinational hot spots.

MATERIALS AND METHODS

***S. pombe* Strains and Culture Procedures.** The *S. pombe* strains and their genotypes are given in Table 1. Strains were constructed by standard meiotic crosses (7) or by transformation of spheroplasts (8) or LiOAc-treated cells (9) with appropriate DNA.

Table 1. *S. pombe* strains

Strain	Genotype	Source or ref.*
GP6	h^+ *ade6-M375*	3
GP18	h^- *leu1-32*	V. Zakian
GP24	h^+ *ade6-M26*	3
GP66	h^{90} *ade6-M26 ura4-294* (pade6-469)	3
GP67	h^{90} *ade6-M375 ura4-294* (pade6-469)	3
GP185	h^- *ura4-101::M26 leu1-32*	T of GP18
GP186	h^- *ura4-102::M26 leu1-32*	T of GP18
GP187	h^- *ura4-103::M375 leu1-32*	T of GP18
GP188	h^- *ura4-104::M375 leu1-32*	T of GP18
GP191	h^+ *ura4-595 leu1-32*	T of GP18
GP205	h^- *ade6-469*	J. Kohli
GP341	h^{90} *ade6-469 ura4-294*	
GP353	h^- *leu1-32 ade6-D1*	T of GP18
GP401†	h^+ *ade6-D1 ura4-107::ade6-M375*	
GP409‡	h^- *ade6-D1 ura4-106::ade6-469*	
GP411§	h^+ *ade6-D1 ura4-105::ade6-M26*	

*T, transformation of the indicated strain (see text). Strains GP401 to GP414 were derived by transformation of GP18 or related strains plus additional meiotic crosses with strains in our collection. Complete genealogies of these strains, as well as that of GP341, are available upon request.
†GP402, an independently constructed strain, has the same genotype but is h^-.
‡GP407, GP408, and GP410, independently constructed strains, have the same genotype, but GP407 and GP408 are h^+.
§GP412, GP413, and GP414, independently constructed strains, have the same genotype, but GP413 and GP414 are h^-.

For culturing *S. pombe* strains liquid media were minimal EMM2 (10) and rich broth YEL (7), and solid media were minimal MMA (7), minimal NBA (11), and rich broth YEA (7). Strains to be crossed were grown in YEL, mixed, concentrated by centrifugation, and spotted on solid sporulation (SPA) medium (7); after 2 days at 25°C meiotic spores were harvested and analyzed for total viable spores and for recombinant spores by differential plating as described (11).

In the experiment described in Table 2, group D, and Fig. 1*D* we used the *ade6-M375-M26* double mutation (6) rather than *ade6-M26* on the multicopy plasmid for the following reasons. Multicopy plasmids containing the 3.0-kb fragment with *ade6-M26* render *ade6-469* cells, but not *ade6-M26* cells, Ade^+ (unpublished observations). This Ade^+ phenotype presumably stems from the combination of high-level expression of the *ade6-M26* gene on the plasmid, low-level suppression of the nonsense mutation created by *M26* (2, 5), and intragenic

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*Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115.
†To whom reprint requests should be addressed at the Fred Hutchinson Cancer Research Center.

complementation frequently observed for *ade6* alleles (2). The *ade6-M375-M26* gene inserted into the plasmid does not confer the Ade$^+$ phenotype to *ade6-469* cells (unpublished observations); this result is expected, since the *M375* mutation, like *M26*, creates a nonsense mutation (5) and suppression of the double nonsense allele should be very rare.

Plasmids. Plasmids pade6-M375, pade6-M26, and pade6-469 contain a 3.0-kb *S. pombe* fragment including the entire *ade6* gene with the indicated mutations, the *Saccharomyces cerevisiae URA3* gene (which complements *S. pombe ura4* mutations), the *S. pombe ars* and *stb* elements (which increase plasmid stability), and the *amp* gene of plasmid pBR322; these plasmids differ from plasmid pAS1 by the indicated *ade6* mutations and the loss of an *Xho* I site in *stb* (5). Plasmid pade6-M375-M26 is identical but contains the double mutation *ade6-M375-M26*. It was constructed as follows. A 1.9-kb *Xho* I fragment from plasmid pRF111, which contains a 2.4-kb insertion of *ade6-M375-M26* DNA (6) in plasmid M13mp18 (J. Kohli, personal communication), and a 7.2-kb *Xho* I fragment from pade6-M26, which lacks the major part of the *ade6* gene, were isolated and ligated. DNA clones isolated in *Escherichia coli* were verified by restriction enzyme digestions to have the *ade6-M375-M26* insertion in the proper orientation and to contain *M375* and *M26* [lack of *Fok* I and *Mnl* I sites (5)].

Plasmid pSPa6 contains a 6.4-kb partial *Sau*3A DNA fragment with the *S. pombe ade6*$^+$ region inserted into the *Bam*HI site of plasmid pFL20 (5). Plasmid pASP1 contains a 3.9-kb *Bam*HI DNA fragment with the major part of the *S. pombe ade6-M26* region inserted into the *Bam*HI site of plasmid pUC19 (3). Plasmid pura4-Sph, also called pUC19SU4, contains a 1.8-kb *Hin*dIII DNA fragment with the *ura4*$^+$ gene (12) inserted, after the addition of linker oligonucleotides, into the *Sph* I site of pUC19 (30). Plasmid pDB248′ is a composite of pBR322, the *S. cerevisiae* 2-μm plasmid, and the *S. cerevisiae LEU2* gene (8).

***S. pombe* Strains with Chromosomal Rearrangements.** Strains with 354-base-pair (bp) fragments of *ade6* DNA inserted into the *ura4* gene (Fig. 1*B*) were constructed as follows. A 354-bp *Hae* III fragment from the *ade6* gene of plasmid pade6-M26 or pade6-M375 was inserted into the *Eco*RV site in the *ura4* gene of plasmid pura4-Sph; both orientations were obtained for each fragment. The 2.2-kb *Sph* I DNA fragments of these plasmids were isolated and used with plasmid pDB248′ to transform spheroplasts of strain GP18. The population was enriched for Leu$^+$ (pDB248′-containing) transformants by growth in liquid EMM2 medium containing 1.2 M sorbitol and uracil (100 μg/ml). Ura$^-$ transformants were selected on MMA medium containing uracil (50 μg/ml) and 5-fluoroorotic acid (1 mg/ml). Clones were purified on YEA medium to obtain Leu$^-$ segregants and examined by Southern hybridization analysis to verify the correct insertions into the *ura4* gene. Insertions *101* and *103* are in one orientation, and *102* and *104* are in the other orientation.

Strains with 3.0-kb fragments of *ade6* DNA inserted into the *ura4* gene (Fig. 1*C*) were constructed as follows. The 3.2-kb *Pvu* II–*Eco*RV DNA fragments (which include the

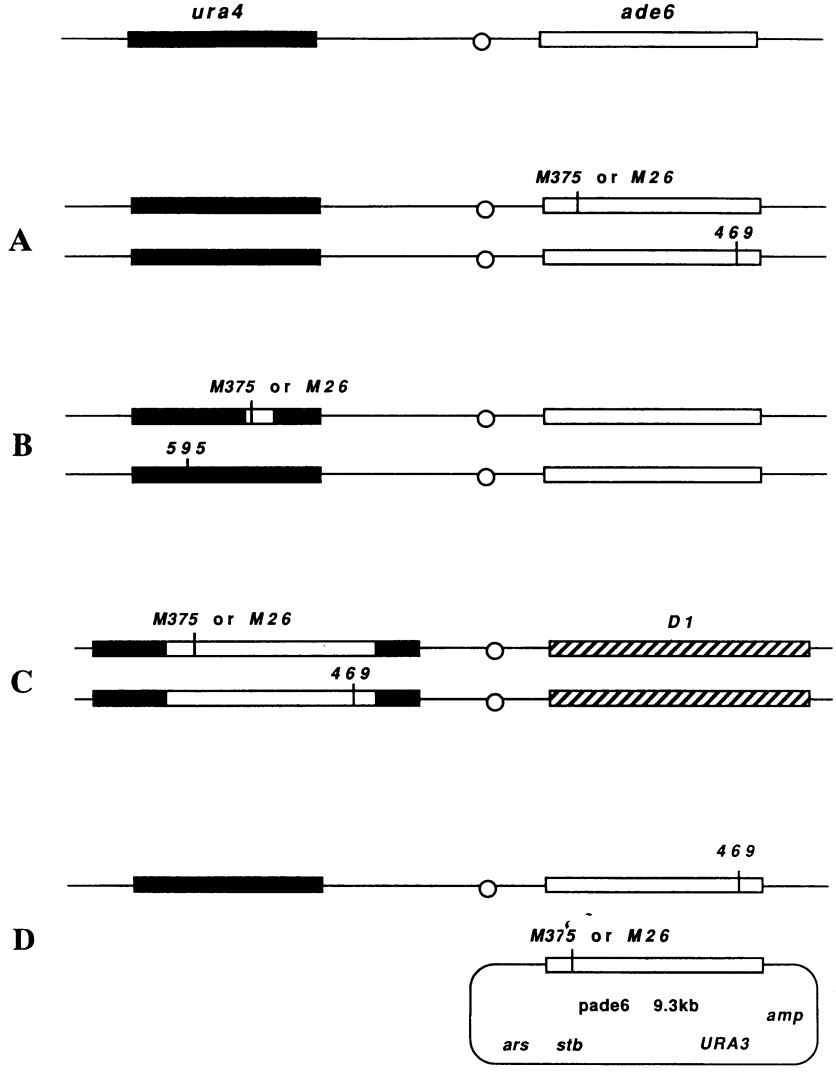

FIG. 1. Structure of the *S. pombe* chromosomes and plasmids tested for *M26* recombinational hot spot activity. The top line shows (not to scale) a part of chromosome III of *S. pombe* with the *ura4* gene (filled box) and the *ade6* gene (open box) separated by the centromere (open circle). These genes are transcribed from left to right, as drawn here, but the orientation of *ura4* on the chromosome is not known. (*A*) Chromosomes with point mutations (*M375, M26*, or *469*) in *ade6* at its endogenous location. (*B*) Chromosomes with insertions, into *ura4*, of the 354-bp *Hae* III fragment of *ade6* containing either *M26* or *M375* located 89 or 86 bp from one end. These fragments were inserted, in each orientation, into the *Eco*RV site of the *ura4*$^+$ gene 620 bp from the site of the *ura4-595* mutation, a "fill-in" mutation of a *Sty* I site. (See refs. 5 and 12 for details of the *ade6* and *ura4* genes, respectively, and the text for details of the constructions used here.) (*C*) Chromosomes with insertions, into *ura4*, of the 3.2-kb *Pvu* II–*Eco*RV fragment. This fragment contains the 190-bp *Bam*HI–*Eco*RV fragment of pBR322 and 3.0 kb of *ade6* DNA bearing *M375, M26*, or *469*, located 1007, 1010, and 2342 bp from the *Pvu* II end. These fragments were inserted into the *Eco*RV site of the *ura4* gene such that *ura4* and *ade6* would be transcribed in the same direction (left to right as drawn here). These chromosomes also contain a 3.7-kb deletion (hatched box), designated *ade6-D1*, extending from the *Pvu* II site defining one end of the *ade6* insertion to the *Sac* I site about 0.7 kb beyond the other end of the insertion. (*D*) Plasmid pade6 containing the 3.0 kb of *ade6* DNA used in *C*. pade6 contains the *URA3* gene of *S. cerevisiae* (which complements *S. pombe ura4* mutations), the *S. pombe ars* DNA and *stb* DNA (which increase plasmid stability in *S. pombe*), and the *amp* gene of plasmid pBR322. The plasmid-borne *ade6* gene, containing either *M375* or *M26-M375*, can recombine with the endogenous *ade6-469* gene during meiosis following self-mating of the homothallic (h^{90}) strain.

190-bp *Bam*HI–*Eco*RV fragment of pBR322) from plasmids pade6-M26, pade6-M375, and pade6-469 were inserted into the *Eco*RV site in the *ura4* gene of plasmid pura4-Sph such that the *ade6* and *ura4* genes were transcribed in the same direction. The 5.0-kb *Sph* I fragments containing the *ura4*::*ade6* insertions were isolated and used to transform *ade6 leu1* strains to Leu^+ Ura^- by the cotransformation scheme described above. In some cases crosses with strains carrying the *ade6-D1* deletion mutation, described below, were needed to generate the desired *ura4*::*ade6 ade6-D1* strains, whose insertions and deletions were verified by Southern hybridization analysis.

The *ade6-D1* mutation, a 3.7-kb deletion of the *ade6* gene and flanking DNA (Fig. 1*C*), was generated by ligating the 3.9-kb *Sac* I fragment of plasmid pASP1 and the 1.4-kb *Pvu* II–*Eco*RV fragment of plasmid pSPa6 to generate plasmid pade6-D1. The 2.6-kb *Bam*HI–*Eco*RI fragment was isolated and used to transform strain GP18 to Leu^+ Ade^- by the cotransformation scheme described above.

The *ura4-595* mutation (Fig. 1*B*) was generated by "filling in" the cut *Sty* I site at position 595 (as numbered in ref. 12) in the *ura4* gene of plasmid pura4-Sph and ligating the ends. The mutation was transferred to the chromosome by isolating the 1.8-kb *Sph* I fragment and cotransforming strain GP18 to Leu^+ Ura^- as described above.

RESULTS

M26 hot spot activity is conveniently measured in comparison crosses using the *ade6-M375* mutation, a G·C → T·A single base-pair change located 3 bp 5′ of *M26* (5). *M375* converts at about the same low frequency as most other *ade6* mutations, whereas *M26* converts at about 10-fold higher frequency (2). *M26* also recombines with other *ade6* mutations, such as *ade6-469*, at about 10-fold higher frequency than does *M375* (2). The ratio of recombinant frequencies in the two crosses, *M26* × *469* and *M375* × *469*, is thus a measure of *M26* hot spot activity. When *M26* was at its endogenous location in the *ade6* gene on chromosome III, it had, by this measure, a hot spot activity of 16 (Table 2, group A). Similar results have been reported previously (2, 3).

To test whether the *M26* hot spot was active when moved to another location in the genome, we inserted 354-bp fragments of *ade6* into the *ura4* gene, which is located about 250 centimorgans from *ade6* on the other arm of chromosome III (13). These fragments contained either *M26* or *M375* about 90 bp from one end of the fragments (Fig. 1*B*). As expected, these insertions rendered the cells Ura^- regardless of their orientation in the *ura4* gene. When these *ura4*::*ade6* mutations were crossed with *ura4-595*, located 620 bp from the insertions (12, 14), Ura^+ recombinants were generated at nearly the same frequencies regardless of the presence of *M26* or *M375* on the insertions, and regardless of the orientation of the insertions (Table 2, group B). The *M26*:*M375* ratio of the recombinant frequencies (1.1) indicates that *M26* had no significant hot spot activity in these crosses. Moreover, the frequency of recombinants between *ura4-294* and *ura4-595* (450 Ura^+ per 10^6 viable spores) was comparable to that between *ade6-M375* and *ade6-L52* at the endogenous *ade6* locus (310 Ade^+ per 10^6 viable spores; ref. 2); these pairs of mutations are about the same distance apart (14). Therefore, the lack of *M26* hot spot activity cannot be attributed to the basal frequency of recombination being significantly different at the *ura4* locus than at the *ade6* locus.

Table 2. Test of *M26* recombination hot spot activity at endogenous and transplaced locations

Group	Mutations (GP strain numbers) crossed	*n**	Recombinants per 10^6 viable spores: Selected type	Range	Mean	*M26* hot spot activity†
A	*M26* at the endogenous *ade6* locus‡					
	ade6-M26 × *ade6-469* (24 × 205)	2	Ade^+	10,400–10,900	10,600	16
	ade6-M375 × *ade6-469* (6 × 205)	2	Ade^+	550–800	680	
B	*M26* on a 354-bp insertion into *ura4*					
	ura4-101::*M26* × *ura4-595* (185 × 191)	1	Ura^+	540	610	1.1
	ura4-102::*M26* × *ura4-595* (186 × 191)	1	Ura^+	680		
	ura4-103::*M375* × *ura4-595* (187 × 191)	1	Ura^+	520	580	
	ura4-104::*M375* × *ura4-595* (188 × 191)	1	Ura^+	640		
C	*M26* on a 3.0-kb insertion into *ura4*					
	ura4-105::*ade6-M26* × *ura4-106*::*ade6-469* (411-414 × 407-410)	8	Ade^+	1,400–2,200	1,900	0.54
	ura4-107::*ade6-M375* × *ura4-106*::*ade6-469* (401-402 × 407-410)	4	Ade^+	3,000–3,900	3,500	
D	*M26* on a 3.0-kb insertion into a multicopy plasmid§ (plasmid-by-chromosome recombination)					
	pade6-M375-M26 × *ade6-469*	9	Ade^+	220–480	310	0.84
				(240–440)	(300)	(0.83)
	pade6-M375 × *ade6-469*	3	Ade^+	340–390	370	
				(310–420)	(360)	
	pade6-469 × *ade6-M26*¶ (66)	7	Ade^+	12,000–18,000	14,000	14
	pade6-469 × *ade6-M375*¶ (67)	7	Ade^+	1,000–1,400	1,100	

The structures of the chromosomes and plasmids analyzed for recombination are diagramed in Fig. 1. Strains with *M26* or *M375* were constructed and mated on supplemented SPA medium, and meiotic spores were harvested and analyzed (see text). Ade^+ or Ura^+ recombinants were determined by plating spore suspensions on NBA minimal medium, supplemented with uracil (100 μg/ml) for the crosses in group C and some in group D; total viable spores were determined on YEA rich medium or supplemented NBA medium. From 100 to 1200 colonies were counted for each determination.

*Number of crosses analyzed.

†Frequency of recombinants with *M26* divided by that with *M375*.

‡Similar results have been reported previously (2, 3).

§Independent transformants of strain GP341, nine with pade6-M26 and three with pade6-M375, were self-mated. Recombinant frequencies in parentheses are values for Ura^+—i.e., plasmid-containing—spores; other values are for total viable spores.

¶Data from ref. 3.

We next inserted into the *ura4* gene DNA fragments containing 3.0 kb of *S. pombe* DNA with the entire *ade6* gene, marked with *M26, M375*, or *469* (Fig. 1*B*). The inserted *ade6* genes were transcribed in the same direction as *ura4*. Crosses between *ura4::ade6-M26* (or *M375*) and *ura4::ade6-469* therefore had complete homology, except for the single base-pair *ade6* mutations, along the recombining chromosomes. The strains crossed contained the *ade6-D1* deletion of the endogenous *ade6* locus; *ade6-D1* deletes the DNA corresponding to the 3.0-kb insertion into *ura4* plus an additional 0.7 kb (ref. 14; Fig. 1*C*). Thus, in these strains Ade^+ recombinants could be generated only by recombination between the *ade6* alleles inserted into *ura4*. Four independent insertions of *M26*, two of *M375*, and four of *469* were made and verified by Southern hybridization analysis (data not shown). Eight crosses of *469* with *M26* and four with *M375* showed that the *M26* hot spot had no detectable activity when the recombining *ade6* genes were remote from their endogenous location (Table 2, group C). [The 2-fold lower frequency with *M26* than with *M375* is within the range previously reported (2) for apparent "marker effects", possibly due to differential correction of mismatches, in *ade6*.]

Although the frequency of recombinants in the crosses between *M375* and *469* at the *ura4* locus was higher (3500 per 10^6 viable spores; Table 2, group C) than that between these alleles at the endogenous locus (680 per 10^6 viable spores; Table 2, group A), the frequency was not so high as to preclude observing a stimulation by *M26*. Higher Ade^+ recombinant frequencies are readily measured: 10,600 per 10^6 viable spores with *M26* at the endogenous *ade6* locus (Table 2, group A); 14,000 per 10^6 viable spores with *M26* recombining with a multicopy plasmid bearing *ade6-469* (see below and Table 2, group D); and 16,000 per 10^6 viable spores with *M26* and *469* coupled to the *adh1* promoter (15). In our assays for *M26* hot spot activity at *ura4* the measured recombinant frequencies were lower than these values: 610 vs. 580 per 10^6 viable spores for *M26* and *M375*, respectively, on the 0.35-kb insertions and 1900 vs. 3500 for *M26* and *M375*, respectively, on the 3.0-kb insertions (Table 2, group B and group C). We conclude that *M26* has no significant recombinational hot spot activity when it is inserted on a 0.35-kb or 3.0-kb DNA fragment into the *ura4* locus.

In the last experiments (Table 2, group D) we obtained derivatives of the multicopy plasmid pFL20 with the 3.0-kb *ade6* fragments containing *M375* or the *M375-M26* double mutation (refs. 5, 16, 17; Fig. 1*D*) and introduced these plasmids into a homothallic (h^{90}, mating-type switching) strain containing the chromosomal *ade6-469* mutation. The *M375-M26* double mutation retains full *M26* hot spot activity when in the endogenous *ade6* gene (6) and was used in this experiment for technical reasons (see *Materials and Methods*). The homothallic strains were allowed to self-mate and undergo meiosis. The frequency of plasmid-by-chromosome recombinants with the hot spot allele *M375-M26* was not significantly different from that with the *M375* allele (Table 2, group D). In contrast, when the *ade6* alleles were reversed—i.e., with *469* on the plasmid and *M26* or *M375* on the chromosome—the *M26* hot spot showed its full activity: recombination was stimulated 14-fold. Thus, although *M26* can stimulate plasmid-by-chromosome recombination, it did so only when at its endogenous location on the chromosome but not when moved to the plasmid with 3.0 kb of surrounding DNA.

DISCUSSION

The data reported here show that the *M26* hot spot was active at its endogenous *ade6* locus but not when moved on a 0.35- or 3.0-kb *ade6* DNA fragment to the distant *ura4* locus or to a multicopy plasmid. Since the *M26* mutation creating the hot spot was 1.0 kb from the nearer end of the 3.0-kb fragment, we conclude that some feature of the chromosome near the endogenous *ade6* gene but located at least 1.0 kb from *M26* is necessary for its hot spot activity.

We consider two plausible, not mutually exclusive, explanations for these observations. (*i*) *M26* might be part of a two-element (two site) system that promotes homologous recombination. (*ii*) The activity of *M26* might depend upon the context of the surrounding DNA. The two-site system of the first explanation is exemplified by the Chi recombinational hot spot of *E. coli* (1). This 8-bp sequence, 5′-GCTGGTGG-3′, is recognized by the recombination-promoting RecBCD enzyme, which cuts one DNA strand about five nucleotides to the 3′ side of Chi. Cutting occurs only as the enzyme is unwinding DNA and only when the enzyme approaches Chi from the right (as the sequence is written here) (18, 19). Chi enhances RecBCD-dependent recombination of phage λ but does so only when properly oriented with respect to a second site, the λ *cos* site (20, 21). Terminase protein of λ cuts the intracellular circular form of λ DNA at *cos*, to create the viral linear form (22). Terminase or the λ prohead remains bound to one side of the cut *cos* site, allowing RecBCD enzyme to enter λ DNA from one end but not the other. Thus, the orientation of *cos* dictates the active orientation of Chi (23). Like Chi, *M26* might be a site at which a recombination-promoting enzyme cuts DNA, but that enzyme might enter DNA only at a second site. This hypothetical site might be located near, or properly oriented with respect to, the endogenous *ade6* gene but not *ura4* or *ade6* on the plasmid used here. A search for *S. pombe* DNA fragments that activate the translocated, inactive *M26* site might reveal this second site.

The second explanation supposes that the activity of *M26* depends upon the general context of the surrounding DNA, not just a second discrete site. The structure of chromatin, the protein-bound form of DNA in chromosomes, at a particular site has been hypothesized to depend in a complex way upon the surrounding DNA (24, 25). For example, the sensitivity to limited DNase I digestion of DNA in chromatin differs from that of naked DNA, and the sensitivity of a particular DNA sequence in chromatin depends upon its location (24, 25). The rules governing this differential sensitivity are unknown. The *M26* site might be differentially sensitive to the *M26*-recognizing protein depending upon its location in the genome and the chromatin structure at that location.

The recombinant frequency of non-hot spot alleles also was dependent upon their chromosomal context. The *ade6-M375* and *ade6-469* alleles produced five times more recombinants when inserted into the *ura4* locus than when at their endogenous locus (Table 2, group A and group C). The chromosomal feature(s) responsible for this 5-fold increased recombinant frequency must lie beyond the 1.0 kb and 0.7 kb of *ade6* DNA flanking the *M375-469* interval. These features might be either discrete sites or special chromatin structures that dictate recombinant frequencies at a distance.

We suppose that moving *M26* from its endogenous locus to another locus dissociates it from an element (or proper chromatin structure) that activates *M26*. Alternatively, there may be repressive elements at the loci to which *M26* was moved. Such elements at the new loci would have to repress recombination with *M26* more than that with *M375*. This hypothesis requires that there be a repressive element at *ade6*, but specific for *M375*, to account for the lower recombinant frequency with *M375* at *ade6* than at *ura4* (Table 2, group A and group C). We believe that the hypothesis of locus- and site-specific repressive elements is more complicated than that of an *M26*-activating element.

The observations reported here bear on the interpretation of previous experiments seeking to locate meiotic recombi-

national hot spots. Such hot spots have been inferred from the monotonically decreasing gradients of gene conversion frequencies of alleles across numerous genes in fungi (1). For example, in the budding yeast *S. cerevisiae* the frequencies of conversion range from about 9% to about 1% at the *ARG4* locus (26–28) and from about 15% to about 2% at the *PYK1* locus (29). Such data imply the existence of a hot spot at or near the high-conversion end of the gene. The locations of these hot spots have been sought by deleting, inserting, and translocating DNA segments ranging from about 0.2 to 5 kb. Analysis of 10 deletions near the high-conversion end (that containing the transcriptional promoter) of *ARG4* implicated a critical site in the 250-bp region defined by the endpoints of two deletions (27, 28). This region is immediately upstream of the transcriptional start site. However, an 11th deletion, which removed all of this 250-bp region plus an additional 60 bp, had no significant effect on the frequency of conversion (9%) of the allele monitored. Furthermore, 7 deletions collectively removing all parts of the implicated 250-bp critical region reduced the conversion frequency only partially, and 3 deletions *increased* the conversion frequency (27, 28). Similar results yielding no simple pattern have been obtained with deletions and translocations of the *PYK1* locus (29).

The observations discussed here indicate that eukaryotic recombinational hot spots are more complex than single sites. The spatial and orientation requirements between multiple sites or the nature of the chromosomal context necessary for hot spot activity remain to be elucidated. Such requirements could explain some of the apparently disparate results from previous studies. For example, a large deletion could inactivate a recombinational hot spot by altering the chromatin structure or the relation between the hot spot and a second site, without deleting the hot spot itself. Therefore, we believe it is important to consider these possibilities when interpreting the results of deletion studies. Single base-pair mutations may be necessary to identify recombinational hot spots (6).

These considerations also bear on attempts to increase the efficiency of gene targeting in multicellular organisms. Currently, targeting of exogenous DNA by homologous recombination into the endogenous chromosomal locus occurs at much lower frequency than integration of the DNA by nonhomologous recombination into a different chromosomal locus (31). The factors governing the ratio of homologous targeting to nonhomologous integration are unclear. Understanding the DNA sequence and chromosomal context requirements of eukaryotic recombinational hot spots may lead to improved methods for gene targeting.

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