M26 Recombinational Hotspot and Physical Conversion Tract Analysis in the *ade6* Gene of *Schizosaccharomyces pombe*

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ABSTRACT

At the *ade6* locus of *Schizosaccharomyces pombe* flanking markers have been introduced as well as five silent restriction site polymorphisms: four in the 5′ upstream region and one in the middle of the gene. The mutations *ade6-706*, *ade6-M26* (both at the 5′ end) and *ade6-51* (middle of the gene) were used as partners for crosses with the 3′ mutation *ade6-469*. From these three types of crosses, wild-type recombinants were selected and analyzed genetically to assess association with crossing-over and physically to determine conversion tract lengths. The introduced restriction site polymorphisms (five *vs.* only one) neither influenced the pattern of recombinant types nor the distribution of conversion tracts. The hotspot mutation *M26* enhances crossing-over and conversion to the same proportion. *M26* not only stimulates conversion at the 5′ end, but does this also (to a lower extent) at the 3′ end of *ade6* at a distance of more than 1 kb. The majority of meiotic conversion tracts are continuous and postmeiotic segregation of polymorphic sites is rare. Conversion tracts are slightly shorter with *M26* in comparison with its control *706*. The mean minimal length of tracts varies from 670 bp (*M26*) to 890 bp (*706*) to 1290 bp (*51*). It is concluded that *M26* acts as an initiation site of recombination or enhances initiation of recombination. *M26* does not act by termination of conversion. A region of recombination initiation exists at the 5′ end of the *ade6* gene also in the absence of the *ade6-M26* hotspot mutation.

RECOMBINATION events are not evenly distributed throughout the genome. Some regions of the chromosomes are less recombinogenic than others. For example, the centromeric regions (NAKASEKO *et al.* 1986) and the k region, a 15-kb spacer sequence in the mating-type region of *S. pombe* (EGEL 1984), are silent for crossovers. The rDNA repeats show low frequencies of interchromosomal meiotic recombination in *Saccharomyces cerevisiae* (PETES and BOTSTEIN 1977). Other loci are known to show enhanced recombination frequencies. SYMINGTON and PETES (1988) found both contraction and expansion of genetic map length relative to physical length in a 23-kb region of chromosome *III* in *S. cerevisiae*. A hotspot for crossover and gene conversion was studied in more detail (SYMINGTON *et al.* 1991). Already before DNA could be analyzed physically, it was shown that certain mutations increase meiotic recombination frequencies: *cog* in *Neurospora crassa* (CATCHESIDE 1974), *YS17* in *Sordaria brevicollis* (MACDONALD and WHITEHOUSE 1979) and *ade6-M26* in *S. pombe* (GUTZ 1971). The hotspot *HOT1* for mitotic recombination has been characterized in detail (KEIL and ROEDER 1984; STEWART and ROEDER 1989).

The phenomenon of polarity of gene conversion (reviewed by WHITEHOUSE 1982; ORR-WEAVER and SZOSTAK 1985) indicates that preferential initiation and/or termination points exist for meiotic recombination. Recently, a short DNA sequence in the upstream region of the *S. cerevisiae ARG4* gene has been shown to be partly responsible for high frequency of conversion (NICOLAS *et al.* 1989; SCHULTES and SZOSTAK 1991). In the same region frequent double-strand breaks have been observed in meiotic prophase (SUN *et al.* 1989; SUN, TRECO and SZOSTAK 1991). Transient double-strand breaks were also detected at a hotspot for crossover that was created by insertion of *LEU2* sequences at the *HIS4* locus (CAO, ALANI and KLECKNER 1990). Thus, double-strand breaks could be a general feature of initiation of meiotic recombination in budding yeast. This is suggested by a global analysis of double-strand breaks in meiotic chromosomes (ZENVIRTH *et al.* 1992). Strong double-strand breaks have not been reported for the *HIS4* and *HIS2* genes (citations in PETES, MALONE and SYMINGTON 1991). Short sequences responsible for high frequency of conversion have been defined for the *HIS4* gene (WHITE *et al.* 1991). In contrast to *ARG4* and *HIS4* the *HIS2* gene shows opposite polarity of conversion: the initiation site is located in the downstream region of the gene (MALONE *et al.* 1992). So far no double-strand breaks have been detected in the *ade6* region of *S. pombe* (BAEHLER *et al.* 1991).

Physical analysis of conversion tracts has been per-

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formed in a variety of systems. AHN and LIVINGSTON (1986) studied conversion between repeated genes on a plasmid. They found an average tract length of 0.5 kb and that long tracts were more frequently associated with crossing-over than short tracts. JUDD and PETES (1988) compared mitotic and meiotic tracts at the *URA3* gene and found that they are usually continuous. Mitotic tracts were often longer than 4 kb, while most meiotic tracts were shorter. In the 23-kb region on chromosome *III*, SYMINGTON and PETES (1988) found mostly continuous meiotic tracts with minimal lengths varying from 4 bp–12 kb. SCHULTES and SZOSTAK (1990) analyzed conversion tracts at the *ARG4* gene in unselected tetrads and found with increasing distance a gradually decreasing coverage of sites on both sides of the preferred initiation point discussed above. For the analysis of conversion tracts a number of genetically silent restriction-site polymorphisms are introduced into the chromosome region under study. It has been shown that these heterologies may influence the frequency and pattern of recombination events (BORTS and HABER 1987, 1989). Seven to nine heterologies reduced the frequency of crossovers to 50% of the control (no heterologies) and increased the number of tetrads with complex recombination events. The overall number of recombination events was not affected, thus the heterologies did alter the pattern of events. A follow-up study indicated that six or less heterologies did not decrease crossover frequency significantly, but still altered the pattern of events. The average minimal tract length was estimated to be 1.5 kb. So far no physical analysis of conversion tracts has been done in fission yeast.

The hotspot mutation *ade6-M26* of fission yeast is a G to T transversion close to the 5′ end of the open reading frame (SZANKASI *et al.* 1988; PONTICELLI, SENA and SMITH 1988). It shows a 10-fold stimulation of conversion in one-point crosses and a 3- to 20-fold stimulation of adenine-independent recombinants in two-factor crosses with other *ade6* mutations at varying distance. *M26* shows strong disparity; it is preferentially converted to wild-type (GUTZ 1971). In addition to conversion events, crossover events also are stimulated by *M26* in a duplication of the *ade6* gene (SCHUCHERT and KOHLI 1988). The *M26* marker effect operates in heterozygous and homozygous configuration (PONTICELLI, SENA and SMITH 1988). It is not active in mitotic recombination (PONTICELLI, SENA and SMITH 1988; GRIMM *et al.* 1991). The heptanucleotide ATGACGT (**T**= *M26* mutation) is required, but not sufficient for hotspot activity (SCHUCHERT *et al.* 1991). Transplacement of parts or the whole of the *ade6* gene (containing *M26* or a nearby control mutation) to the other arm of chromosome *III* leads to loss of the marker effect. Additionally *M26* enhances plasmid × chromosome recombination only when located on the chromosome (PONTICELLI and SMITH 1992).

The aim of this work is the mechanistic analysis of the *M26* hotspot and the physical characterization of conversion tracts. For this purpose we have introduced five silent restriction site polymorphisms in the *ade6* region. Ade$^+$ recombinants were selected from random spores of two-factor crosses with auxotrophic *ade6* mutants and the minimal lengths of conversion tracts were determined. The inclusion of flanking markers allowed the distinction between conversion events and events involving crossover. This allowed for the first time the direct assessment of *M26* hotspot activity on reciprocal exchanges.

MATERIALS AND METHODS

***S. pombe* strains:** All strains are from the collection in Bern or have been constructed for this project (Table 1). The *ade6* mutations have been isolated by GUTZ (1963). The DNA sequence of *ade6* and the base substitutions in *ade6-M26* and *ade6-469* have been published by SZANKASI *et al.* (1988). *ade6-51* has been sequenced by SCHAER and KOHLI (1993). The mutation *ade6-706* destroys the *Hae*III site at position 923 and is suppressible by UGA nonsense suppressors. Thus, it is most probably a C to T transition at position 923 (data not shown). The flanking marker *tps16-26* has been described by GYGAX and THURIAUX (1984), whereas *ura4-aim* (artificially introduced marker) was created by insertion of the *ura4*$^+$ gene 15 kb proximal to *ade6* (Figure 1, see below). The strains GC12 and GC13 carry five restriction site alterations described below, while GC15 carries only one.

Standard methods: A description of standard genetic methods and media is given in GUTZ *et al.* (1974). The crosses were carried out and the vegetative cells were inactivated by glusulase treatment as described by MUNZ and LEUPOLD (1979). Adenine-independent recombinants were selected on YEA medium supplemented with 200 mg guanine per liter (PONTICELLI and SMITH 1989; CUMMINS and MITCHISON 1967). The lithium acetate protocol of ITO *et al.* (1983) was used for *S. pombe* transformation. A small-scale method was used for preparation of genomic DNA (WRIGHT, MAUNDRELL and SHALL 1986). DNA was analyzed by gel electrophoresis and filter hybridization as described (AMSTUTZ *et al.* 1985). The Wilcoxon signed rank test was carried out as described (WILCOXON 1945).

Restriction site polymorphisms: pUC plasmids (VIEIRA and MESSING 1982) or pBR322 (BOLIVAR *et al.* 1977) were used for constructions and named pCG followed by a number. The positions of the changed restriction sites are given in Table 1 and Figure 1. All the restriction site alterations were constructed either on plasmid pCG6 (contains the 3.3-kb *Hin*dIII fragment of *ade6* and its 5′ flanking sequences in pUC8, Figure 1) or pCG37 (contains the 1.2-kb *Bam*HI/*Eco*RI *ade6* fragment in pBR322, Figure 1) and were subsequently introduced into yeast according to the gene replacement protocol of GRIMM *et al.* (1988). To introduce the 5′ polymorphisms, the *Eco*RV/*Bam*HI fragment in the 5′ region of *ade6* was replaced by the *ura4* gene in the strains *ura4-D18 ade6*$^+$ and *ura4-D18 ade6-469*. The *ura4* gene then was replaced by *ade6* sequences carrying the altered restriction sites upstream of *ade6* by transformation with linear DNA. With the strain carrying no mutation in *ade6*, selection was for adenine independence. With the

TABLE 1

S. pombe strains

Name	Genotype	*ade6* mutation	Restriction site polymorphisms[a]	Reference
GC150[c]	*h⁻/h⁺ ura4-D18*			GRIMM *et al.* (1988)
GC151[c]	*h⁻/h⁺ ura4-D18 ura4-aim*			This work
GC152[c]	*h⁻/h⁺ ura4-D18 ade6-469 tps16-26*	C to T at 2342		This work
GC12	*h⁻ leu1-32 ura4-D18 ade6-5rpm*		*Eco*RV −1300 *Xho*I-50 *Xho*I 387 *Hin*dIII 1713 *Eco*RI 739[b]	This work
GC13	*h⁻ ura4-D18 ade6-469,5rpm*	C to T at 2342	*Eco*RV −1300 *Xho*I −50 *Xho*I 387 *Hin*dIII 1713 *Eco*RI 739[b]	This work SZANKASI *et al.* (1988)
GC15	*h⁻ ura4-D18 ade6-469,1rpm*	C to T at 2342	*Xho*I 387	This work
GC101	*h⁺ ura4-D18 ura4-aim tps16-26 ade6-706*	C to T 923		This work
GC102	*h⁺ ura4-D18 ura4-aim tps16-26 ade6-M26*	G to T 1010		This work SZANKASI *et al.* (1988)
GC103	*h⁺ ura4-D18 ura4-aim tps16-26 ade6-51*	C to T 2141		This work SCHAER AND KOHLI (1993)

[a] The number after the restriction enzyme symbol indicates the position of the site in *ade6* sequence as published by SZANKASI *et al.* 1988.
[b] This is a newly created restriction site, while the other listed sites have been destroyed (see text).
[c] Each of these strains has been isolated from one cross as segregants with different mating-types (h^- and h^+), but otherwise identical genotypes.

strain carrying *ade6-469*, transformants were selected on 5-FOA plates for uracil dependence (GRIMM *et al.* 1988). To introduce the internal polymorphism, the *ura4* gene was inserted into the *Hin*dIII site in the middle of the gene by transformation of the strains carrying the altered restriction sites in the upstream region of *ade6* with linear DNA fragments. These *ura4* insertions were replaced by *ade6* sequences with the abolished *Hin*dIII site. In this way the strains GC12, GC13 and GC15 were obtained. The isolation and normal growth of GC12 shows that the restriction site alterations do not affect *ade6* expression.

The *Eco*RV site was deleted with *Exo*III nuclease. The overhangs were digested with S1 nuclease and the plasmid was religated (T4 ligase). The size of the deletion was analysed on a 1.5% agarose gel and found to be about 40 bp (data not shown). Both *Xho*I sites were destroyed by filling in the overhanging ends with Klenow fragment of DNA polymerase I. The two remaining alterations are single base-pair changes engineered by oligonucleotide-directed *in vitro* mutagenesis as described by SCHUCHERT *et al.* (1991). An A to C substitution at positon 739 yielded a new *Eco*RI site 136 bp upstream of the open reading frame. A T to A substitution at position 1717 destroyed the *Hin*dIII site within the *ade6* gene. This change is silent, because a GCT codon is altered to GCA, both specifying alanine. The correctness of the constructions (altered restriction sites) was checked by analysis of the restriction pattern of genomic DNA (data not shown).

Isolation of DNA sequences flanking *ade6*: The plasmid pUC18 was digested either with *Eco*RI, *Sma*I or *Bam*HI. Genomic DNA of wild-type strain 972 h^-, completely digested with the respective restriction enzyme was ligated into the correspondingly cut plasmid (*Eco*RV digested DNA was ligated into *Sma*I cut vector) and immediately transformed into *Escherichia coli* strain JA221 (BEGGS 1978). Clones with *ade6* flanking sequences were identified by colony hybridization. Some of the newly cloned fragments are shown in Figure 1 (plasmids pCG6, pCG73, pCG162 and pCG568). pCG20 and pCG37 were obtained by subcloning.

Flanking markers: The 4.5-kb *Bam*HI/*Sal*I fragment of pCG568 was subcloned into pUC18 yielding pCG20 (Figure 1). A 1.8-kb fragment containing the functional *ura4* gene (GRIMM *et al.* 1988) was then inserted into the *Eco*RV site of pCG20 (approximately −14 kb on the chromosome). Then the strain *ura4-D18* was transformed with the *Bam*HI/*Sal*I fragment of this plasmid. Stable uracil-independent transformants were selected and analyzed by Southern hybridization (data not shown).

The strains GC101, GC102 and GC103 (Table 1) were obtained by standard crosses between various strains carrying *ade6* mutations (*706*, *M26*, *51*), *ura4-D18*, *ura4-aim* and *tps16-26*. The source strains all derive from the original strain that was subjected to genetical analysis by Leupold. To obtain some of the auxotrophy mutations these strains have been subjected to mutagenesis (*e.g.*, GUTZ 1963). The genetic distances between *ade6* and the flanking markers *ura4-aim* and *tps16* were measured by tetrad analysis (see RESULTS).

Physical analysis of conversion tracts: Haploid strains auxotrophic for adenine were crossed as described in RESULTS. After selection, adenine-independent recombinants were transferred to 10 ml of liquid selective medium containing uracil (but no adenine). The cultures were incubated at 25° and DNA prepared as described (WRIGHT, MAUNDRELL and SHALL 1986). Genomic DNA of each recombinant was double digested with *Eco*RV/*Xho*I, as well as with *Hin*dIII/*Eco*RI. The probe for Southern hybridization was plasmid pCG6 (Figure 1). The DNA of some recombinants showed the restriction pattern expected if a restriction site

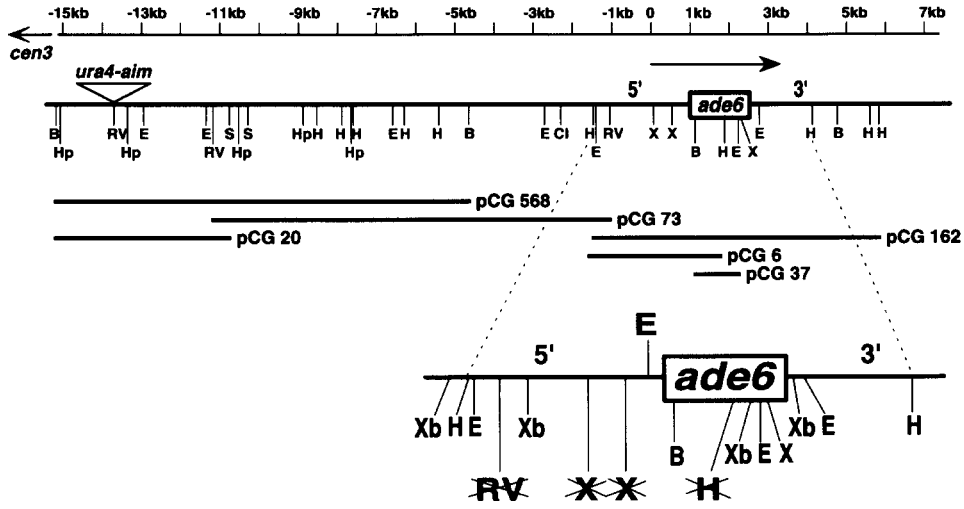

FIGURE 1.—Restriction map of a 21-kb region of chromosome *III* containing the *ade6* gene. The box stands for the open reading frame. The arrow indicates direction of transcription and the extent of the known nucleotide sequence (SZANKASI *et al.* 1988). The inserts of several plasmids carrying fragments of the region are drawn below the chromosome. The integration point of *ura4-aim* is marked with a triangle. The five restriction sites that were used to create polymorphisms are indicated with large letters. The destroyed sites are shown below, the newly constructed *Eco*RI site above the blowup of the chromosome. Restriction sites are abbreviated as follows: B: *Bam*HI, Cl: *Cla*I, E: *Eco*RI, H: *Hin*dIII, Hp: *Hpa*I, RV: *Eco*RV, S:*Sal*I, X: *Xho*I, Xb: *Xba*I.

is present as well as absent in about an equal subset of the cells used for DNA preparation. Because all spores were plated onto selective medium immediately after meiosis, and kept under selective pressure until DNA was prepared, only DNA of *ade*$^+$ recombinants could have been analyzed. Therefore, such a mixed pattern must have arisen from post-meiotic segregation (PMS) of the corresponding restriction site.

RESULTS

Restriction map of the *ade6* region: The physical analysis of conversion tracts requires restriction site polymorphisms in the region under study. In addition, flanking markers are necessary for a more detailed analysis of recombination. At the *ade6* locus of *S. pombe*, however, only a distal marker, *tps16*, has been described so far (GYGAX and THURIAUX 1984). We decided therefore to construct an artificial flanking marker on the proximal side of *ade6*. For these constructions we had to clone additional DNA sequences flanking the *ade6* gene (see MATERIALS AND METHODS). Overlapping DNA fragments were obtained and the sites of several restriction enzymes were mapped (Figure 1). In addition to the known nucleotide sequence of the 3-kb fragment with the *ade6* gene (SZANKASI *et al.* 1988), 15 kb of DNA upstream and 3 kb of DNA downstream of *ade6* on chromosome *III* are now amenable to further analysis.

Flanking markers for the *ade6* gene: As a flanking marker on the proximal side of *ade6*, we have inserted the *ura4* gene in strain GC150 carrying the *ura4-D18* deletion (GRIMM *et al.* 1988), resulting in strain GC151 with the genotype *ura4-D18 ura4-aim* (see MATERIALS AND METHODS). The insertion is located 15 kb upstream of *ade6* (Figure 1) and is named *ura4-aim* for *a*rtificially *i*ntroduced *m*arker. The deletion of the *ura4* gene at its original locus (*ura4-D18*) excludes ectopic recombination with *ura4-aim*. Together with *tps16*, two useful flanking markers are now available. Since only the *ura4*$^+$ gene has been integrated, all crosses in this study were done with strains heterozygous for the *ura4* insertion.

The cross of strains GC151 h^+ × GC152 h^- was subjected to tetrad analysis for determination of the genetic distances between *ura4-aim*, *ade6* and *tps16*; 89 tetrads were scored. For the interval *ura4-aim–ade6* 82 PD, 7 T and 0 NPD tetrads were observed. According to the mapping function based on the absence of crossover interference (Poisson distribution) (P. MUNZ, unpublished data), the genetic distance is 4.1 cM. The distance between *ade6* and *tps16* is 6.6 cM (78 PD, 11 T, 0 NPD) and between *ura4-aim* and *tps16* 11.3 cM (72 PD, 16 T, 1 NPD). A comparison of the physical length (15 kb) and the genetic distance (4.1 cM) between *ura4-aim* and *ade6* yields a ratio of 0.27 cM per kb. This value is not far from the average estimate of 0.15 cM per kb as derived from the recent estimates of total genetic (2100 cM, P. MUNZ, unpublished data) and total physical genome length (13.8 Mb, FAN, Grothues and SMITH 1991). It is surprising that 7% of the tetrads showed conversion of *ura4-aim*, in all cases tetrads with one ura$^+$ spore (*ura4-aim*) and three ura$^-$ spores (no *ura4* insertion). This conversion frequency is higher than for any other mutation studied in *S. pombe* and may be due to the extended heterology occurring in this cross that is heterozygous for the *ura4-aim*

insertion. So far there are no data on recombination of large heterologies in *S. pombe*.

Restriction site polymorphisms: For the physical analysis of conversion tracts three restriction sites upstream and one site in the middle of the *ade6* gene were destroyed. In addition a new site was constructed immediately upstream of the reading frame (Figure 1). For a detailed description of the constructions see MATERIALS AND METHODS. They were first carried out in a *ura4-D18 ade6*$^+$ background and resulted in strain GC12 (Table 1). The strain was indistinguishable from its parent concerning growth properties and adenine independence. Thus, the expression of *ade6* and possible other genes upstream of *ade6* does not seem to be affected by the restriction-site polymorphisms. Subsequently the same silent DNA alterations were introduced into a strain carrying *ura4-D18* and *ade6-469* (GC13, Table 1). Since it was reported that restriction-site polymorphisms may influence the frequency and pattern of recombination events (BORTS and HABER 1987, 1989), the control strain GC15 was constructed that carries only one of the five polymorphisms (Table 1). The correct introduction of the polymorphisms into the three strains has been verified by restriction analysis and filter hybridization of genomic DNA (data not shown).

Experimental strategy: The strain GC13 (*ade6-469, 5rpm*, Table 1) was crossed with three other strains (GC101, GC102, GC103; Table 1), all carrying the flanking markers *ura4-aim* and *tps16-26* but different *ade6* mutations (*706, M26, 51*) (see Figures 2-4). Adenine-independent recombinants were selected in the presence of uracil at the permissive temperature of 25°. After determination of the flanking marker configurations, spore clones were picked and their DNA analyzed as described in MATERIAL AND METHODS. Since the three *ade6* mutations *706, M26* and *51* are located 5′ of the mutation *ade6-469*, spore clones with the parental flanking marker configuration *ura4-aim tps16*$^-$ were considered as 5′ conversions (phenotype ura$^+$-ade$^+$-tps$^-$), whereas the other parental flanking marker configuration (phenotype ura$^-$-ade$^+$-tps$^+$) were taken as 3′ conversions. Ade$^+$ spore clones with exchanges of the flanking markers (phenotypes ura$^-$-ade$^+$-tps$^-$ and ura$^+$-ade$^+$-tps$^+$) were considered as conversions associated with crossovers and not analyzed physically, because the extent of conversion tracts cannot be deduced when a crossover is involved. From each cross we analyzed conversion tracts in 60 5′ and 10 3′ convertants. To evaluate whether the number of heterologies in a cross influences the frequency and pattern of recombination, the same analysis was carried out with the control strain GC15 (Table 1) that carries only one restriction-site polymorphism.

Genetical analysis of recombination: First, we determined the frequency of adenine-independent recombinants among random spores for all crosses (Table 2). The first set of crosses was done to obtain genetical data and *ade*$^+$ convertants for physical analysis with five restriction-site polymorphisms. Subsequently, these crosses were repeated (second set) in parallel with the ones with a single restriction-site polymorphism (third set, also used for physical analysis of tracts). We conclude that there are no significant ade$^+$ frequency differences between the crosses with five polymorphisms *vs.* one polymorphism. Up to two-fold frequency differences (set 1 *vs.* set 2) for crosses of the same strains done at different times are routinely observed (see also large standard deviations). They are probably caused by the complexity of frequency determination: ratio of number of prototrophs (small) over spore titer (large number). The experimental determination of these values is sensitive to small serial dilution errors (see also SCHUCHERT *et al.* 1991). Samples of *ade*$^+$ segregants were then assayed for their flanking marker configurations. Conversions without exchange of flanking markers occurred in 35–48% of the examined *ade*$^+$ recombinants. Crossovers were associated with 52–65% of the convertants, depending on the cross (Table 2). No significant influence of the hotspot mutation *M26* (*vs. 706*) on the proportion of crossing-over is discernible ($P > 0.07$, chi-square of 2 × 2 contingency tables for all three sets of crosses). Among the *ade*$^+$ recombinants with flanking marker exchange, the configuration *ura*$^-$ *tps16*$^-$ was strongly preferred. This type of recombinant is easily explained by conversion of either of the *ade6* mutations accompanied by a crossover. It is unlikely that a sizeable fraction of these recombinants occur by an intragenic crossover alone. GUTZ (1971) has demonstrated by tetrad analysis that intragenic crossovers are rare (*ade*$^+$ formation with 2:2 segregation of both markers in a two-point cross). The minority class (*ura4-aim tps*$^+$) is not easily explained. If conversion of one of the *ade6* alleles to wild type is assumed, the accompanying crossover would be situated beyond the nonconverted mutation toward the flanking marker that is nonadjacent to the converted mutation. Noncontinuous conversion tracts associated with a crossover could explain this type of recombinant. Under the assumption of double-strand gap formation covering the 5′ mutation and inclusion of the 3′ mutation in hybrid DNA, the minority class could originate from restoration at the 3′ site associated with a crossover. The presence of five or only one silent polymorphism does not influence the distribution of the four different types of recombinants (Table 2: $P > 0.05$ in all six possible 4 × 2 contingency table tests of corresponding crosses). The influence of the *M26* mutation on the distribution of the four types of recombinants is treated in the DISCUSSION.

Physical analysis of conversion tracts: The physi-

TABLE 2

Recombination frequencies and flanking marker configurations

Cross	Frequency of Ade$^+$ spores ($\times 10^6$)[a]	Ade$^+$ recombinants tested (100%)	Flanking marker configuration[b]													
			Conversion							Conversion and crossover						
			ura4-aim tps16$^-$				*ura4$^-$ tps16$^+$*			*ura4$^-$ tps16$^-$*				*ura4-aim tps$^+$*		
5rpm (set 1)																
706 × 469	264 (85)	749	210	28	74		90	12	32	427	57	150		22	3	8
						13.2			3.9				13.2			7.9
M26 × 469	3143 (313)	892	278	31	974		37	4	126	561	63	1980		16	2	63
51 × 469	14 (5)	549	97	18	3		166	30	4	244	44	6		42	8	1
5rpm (set 2)																
706 × 469	447 (208)	384	110	29	128		35	9	41	222	58	258		17	4	18
						12.4			5.1				10.5			11.6
M26 × 469	4726 (1203)	384	129	34	1588		17	4	209	221	58	2720		17	4	209
51 × 469	11 (2)	384	82	21	2		94	24	3	182	47	5		26	7	1
1rpm (set 3)																
706 × 469	591 (159)	384	95	25	146		44	11	68	230	60	354		15	4	23
						15.3			7.6				13.1			9.4
M26 × 469	7587 (2824)	384	113	29	2233		26	7	514	234	61	4623		11	3	217
51 × 469	14 (4)	384	68	18	2		117	30	4	172	45	6		27	7	1

All strains were *ura4-D18*. The strain 469 harboring the mutation *ade469* always had the flanking marker combination *ura4$^-$tps16$^+$*, whereas the others had the flanking marker configuration *ura4-aim tps16$^-$*. 5rpm (1rpm) means five (one) restriction site polymorphisms.

[a] The mean of three or more independent crosses is given with standard deviations in parenthesis.

[b] The numbers given in each box from left to right are:
— Count of ade$^+$ colonies with corresponding flanking marker configuration.
— Percent of total ade$^+$ colonies analysed for flanking marker configuration.
— Frequency of ade$^+$ colonies with corresponding flanking marker configuration normalized to 10^6 spores.
— Enhancement of frequency by *M26*: ratio of frequencies for *M26* and *706*.

cal analysis has been restricted to *ade$^+$* recombinants originating from conversion alone (parental flanking markers) of sets 1 and 3. Presence or absence of a restriction site was scored at the five polymorphic sites shown in Figure 1. The results for the crosses with five polymorphisms are given in Figures 2–4. The conversion tracts are drawn as bars that cover the converted mutation and those restriction sites that have changed their state in comparison with the parental chromatid. The bars are minimal estimates of the length of the tracts, since the exact end cannot be located.

The great majority of tracts is continuous and represents full conversion of the polymorphic sites. In a few cases some sites showed postmeiotic segregation (PMS). These recombinant clones showed mixed restriction patterns best explained as deriving from hybrid DNA at the involved restriction site.

Only 15 tracts (8.3%) among the 180 5′ tracts studied reached the most upstream restriction site (Figures 2–4). Thus, the majority of the tracts ended less than 2 kb upstream of the gene. No preferred 5′ endpoint is discernible (Figure 5). Very similar patterns were observed for the 3′ ends of the 5′ conversion tracts for the two crosses *706 × 469* (Figure 2) and *M26 × 469* (Figure 3). The majority of conversion events of markers at the 5′ end of the gene did not reach beyond a stretch of 800 bp into the gene: 75% (*706 × 469*) and 80% (*M26 × 469*) of the tracts did not reach the *Hin*dIII site in the middle of the gene. These values are not different ($P > 0.5$, chi-square 2 × 2 table). But there is a trend for shorter tracts with *M26* than with *706* (see DISCUSSION). The frequencies of noncontinuous tracts and PMS events are not different in the crosses with *706* and *M26*.

Since it is not possible to localize the exact endpoints of the tracts in the interval between two restriction sites, the values for the whole tract lengths given in Figures 2 to 4 are always minimal estimates. The value for *M26 × 469* (670 bp ± 660 bp, Figure 3) is in the same range as for the control *706 × 469* (890 bp ± 720 bp, Figure 2). The cross *51 × 469* (Figure 4) differs from the other two crosses by the location of *51* beyond the middle of the open reading frame. It was used as an alternative for the study of the 5′ endpoints of conversion tracts. Forced by the experimental circumstances the 3′ ends of the 5′ conversion tracts must lie within the 200 bp between *51* and *469*. The great majority of tracts (51 of the 60) had their 5′ end in the 5′ flanking sequence or in the 5′ half of the open reading frame (Figure 4). The difference between the first two crosses and the cross *51 × 469* is reflected in the average minimal lengths of conversion tracts. The value is clearly higher for this cross (1290 bp ± 1080 bp, Figure 4). This difference in the average minimal lengths of the conversion tracts is

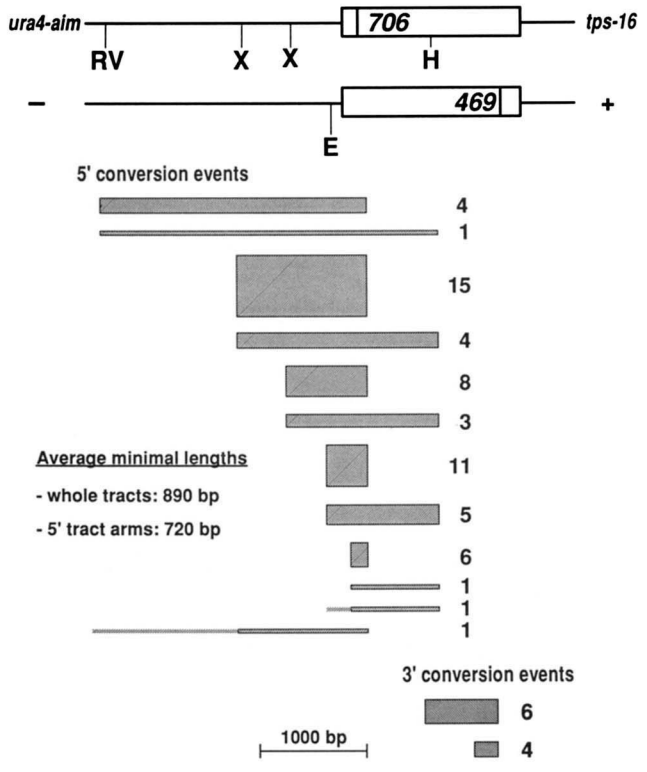

FIGURE 2.—Physical analysis of meiotic gene conversion tracts in convertants of the cross GC101 × GC13. Sixty tracts associated with conversion of the 5′ mutation (*706*) and 10 tracts associated with conversion of the 3′ mutation (*469*) have been analyzed. The *ade6* regions of the crossed strains are drawn at the top. The positions of the mutations and of the polymorphic restriction sites are indicated. The classes of different conversion tracts are shown with hatched boxes and the numbers of tracts within each class are indicated. Sites covered by outlined boxes showed full conversion. Post-meiotic segregation of sites is indicated by hatching only. Interrupted boxes stand for noncontinuous tracts. The width of the boxes is proportional to the number of events.

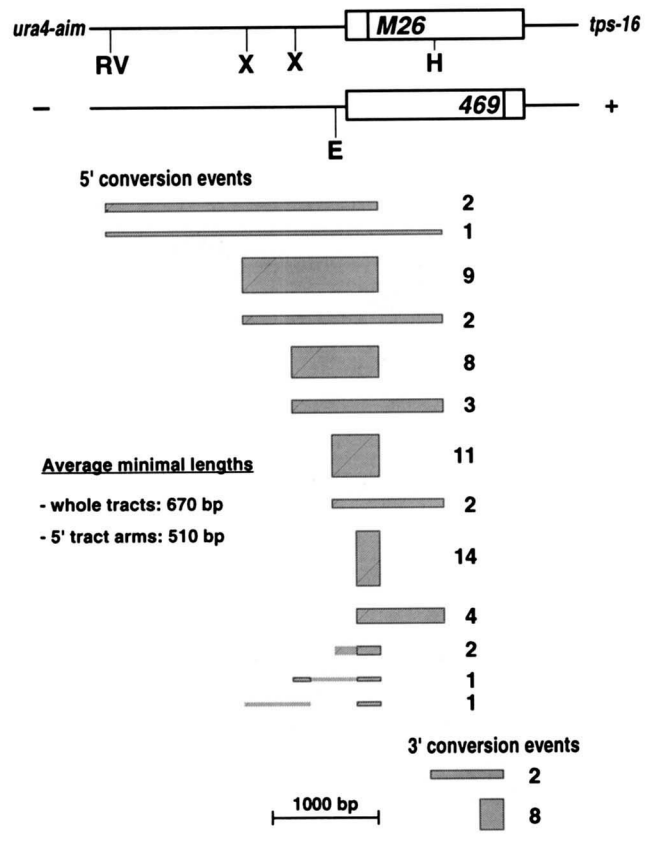

FIGURE 3.—Physical analysis of meiotic gene conversion tracts in convertants of the cross GC102 × GC13 (*M26* × *469*). For explanation see legend of Figure 2.

even more pronounced, if the values are calculated for the unselected tract arms only. In all three crosses the 3′ ends of the 5′ tracts were forced to the region upstream of *469*, whereas the 5′ ends were not forced. The average minimal lengths of the unselected 5′ arms are 720 bp ± 680 bp for *706* × *469* (Figure 2), 510 bp ± 560 bp for *M26* × *469* (Figure 3), and still 1290 bp ± 1080 bp for *51* × *469* (Figure 4).

The concern that the restriction-site polymorphisms used in this analysis could influence the pattern of conversion tracts is unfounded, as far as it was checked. We carried out the same analysis of 5′ conversion tracts from the three crosses of strain GC15 (which has the single polymorphism at the *Xho*I site 486 bp upstream of the open reading frame, see Figure 1) with the strains carrying *706*, *M26* and *51*. Coverage of this *Xho*I site by the tracts was scored and compared with coverage of the same site in the crosses involving five polymorphisms (for the latter data see Figures 2–4) by chi-square 2 × 2 contingency table tests. For *706*, 28 tracts covered the site and 12 did not in the cross with one polymorphism ($P = 0.31$). The corresponding values for *M26* are 17, 23 ($P = 0.81$), and for *51* they are 14, 26 ($P = 0.75$).

DISCUSSION

Meiotic recombination at the *ade6* locus of *S. pombe* has been studied mainly in connection with the *M26* hotspot (GUTZ 1971; GOLDMAN and SMALLETS 1979; PONTICELLI, SENA and SMITH 1988; SZANKASI *et al.* 1988; GRIMM *et al.* 1991; SCHUCHERT and KOHLI 1988; SCHUCHERT *et al.* 1991; PONTICELLI and SMITH 1992). More recently, a detailed analysis of intragenic recombination at *ade6* with emphasis on mismatch repair has been added (SCHAER, MUNZ and KOHLI 1993; SCHAER and KOHLI 1993). To facilitate further studies, the introduction of flanking markers and restriction-site polymorphisms was carried out. The results obtained from selected wild-type recombinants in two-point crosses at *ade6* and the physical analysis of conversion tracts is discussed with respect to the *M26* hotspot and intragenic recombination in general.

The recombinational hotspot mutation *ade6-M26:* It has to be borne in mind that this discussion is based on analysis of *ade*$^+$ recombinants from two-point crosses. Only the laborious analysis of unselected tetrads could yield a complete description. While this may be feasible for crosses involving *M26* (5% aber-

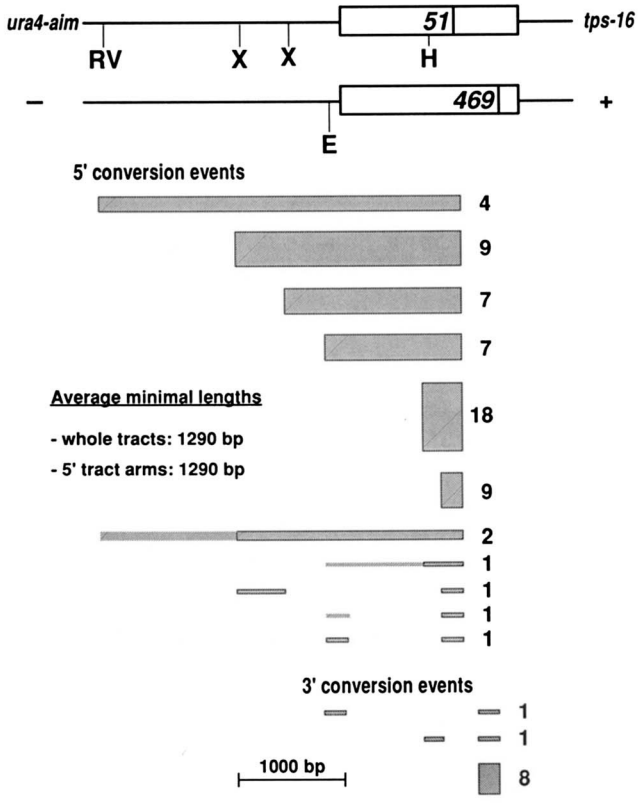

FIGURE 4.—Physical analysis of meiotic gene conversion tracts in convertants of the cross GC103 × GC13 (*51* × *469*). For explanation see legend of Figure 2.

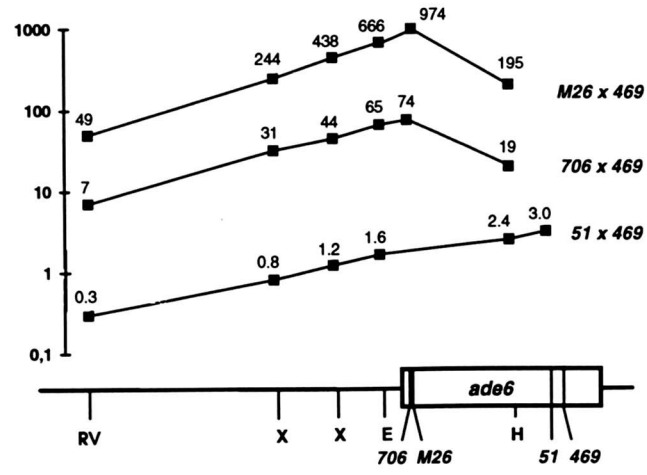

FIGURE 5.—Frequencies of the involvement of the polymorphic sites in tracts associated with conversion of the 5′ mutation of the indicated crosses. The frequencies are given per 10^6 viable spores and plotted on a logarithmic scale. They have been calculated by multiplication of the normalized 5′ conversion frequency for each cross (Table 2) by the number of coverages of each polymorphic restriction site by the tracts in Figures 2–4, and by division by the total number of tracts studied (60). The positions of the *ade6* mutations and of the important restriction sites are shown at the bottom. The distances between the sites are to scale.

rant segregation: GUTZ 1971), it would be too much work for crosses with other *ade6* mutations (0.5% aberrant segregation or less: GUTZ 1971; SCHAER, MUNZ and KOHLI 1993).

M26 enhances the frequency of *ade6*$^+$ recombinants 10–13-fold over the control with *706* in the three sets of crosses (Table 2). It has been shown before that crossing-over is stimulated by *M26* in a heteroallelic duplication of the *ade6* gene (SCHUCHERT and KOHLI 1988). This is now confirmed by analysis of classical meiotic recombination with help of flanking markers. Between 52% and 65% of the *ade6*$^+$ recombinants show crossing-over for flanking markers in the nine crosses presented in Table 2. *M26* does not alter the ratio of association of conversion and crossing-over.

There are three possibilities how the hotspot mutation *ade6-M26* could enhance recombination frequencies in the observed way (see introduction section). (1) *M26* stimulates the normal recombination activity at *ade6*, (2) it creates a novel site for recombination initiation, or (3) it preferentially terminates conversion events.

Termination hotspot: *M26* action on the basis of conversion termination (hypothesis 3) can be rejected on the basis of our data. There are two possibilities how *M26* could stimulate *ade6*$^+$ recombinant frequencies by conversion termination without affecting initiation. Either *M26* attracts events that are initiated somewhere else and are normally not reaching the *ade6* gene, or it terminates events that are normally covering the whole gene and are therefore not yielding wild-type recombinants in two-point crosses. A comparison of the conversion tracts obtained with *M26* (Figure 3) with those of *706* (Figure 2) shows that the first possibility can be excluded. The endpoints of the *M26* tracts are neither further upstream nor downstream than in the control and the tracts do not significantly differ in size ($P > 0.2$, Wilcoxon signed rank test). Thus, *M26* does not attract conversion events from outside. Termination of tracts can also be excluded. If tracts would initiate randomly in the 5′ upstream region of *ade6* and normally cover the whole gene with *M26* terminating these tracts frequently, two predictions would hold: (1) A lower percentage of the 3′ endpoints of the tracts would be downstream of the *Hin*dIII site than in the control; and (2) both *M26* and nearby upstream mutations (*e.g.*, *706*) would be covered by conversion tracts with high frequency and show frequent aberrant segregation in one-point crosses. Neither prediction is supported by our data. Twenty percent of the tracts reach the *Hin*dIII site in the cross with *M26* (Figure 3) and 25% reach this site in the cross with *706* (Figure 2). The difference is not significant ($P > 0.5$, chi square 2 × 2 table). In comparison with *M26*, GUTZ (1971) has reported sixfold and 12-fold reduced aberrant segregation frequencies for *ade6-M375* and *ade6-M216* that are 3 bp and 89 bp, respectively, upstream of *M26* (SZANKASI *et al.* 1988). Thus *M26* must act at the level of initiation of conversion.

Enhancement of background recombination *vs.* creation of a new initiation site by *M26*: A discrimination between hypotheses 1 and 2 for *M26* action is not possible with the available data. In many respects there is no difference between the crosses with *706* and *M26*. The association of crossing-over with conversion is not different. The frequencies of postmeiotic segregation of restriction markers as well as the formation of noncontinuous tracts are similar. The minimal average tract lengths are not significantly different ($P > 0.2$, Wilcoxon signed rank test), although *M26* tracts are somewhat shorter than *706* tracts (Figures 2 and 3). The lack of gross differences between *M26* and *706* concerning conversion tracts and distribution of types of recombinants supports hypothesis 1: stimulation of normal recombination by *M26* instead of creation of a new site. However, it is not possible to exclude the latter possibility. As discussed below, a natural initiation region appears to lie at the 5′ end of the gene, where also *M26* is located. If a new initiation point is created by *M26* near the natural one, it may well be that conversion tracts show no significant differences. The shortening of tracts by *M26* is apparent, when coverage of the polymorphic *Xho*I site closer to the gene is considered in detail: 36 tracts cover this site, 24 do not, in the cross *706* × *469*. The numbers for *M26* × *469* are 27 *vs.* 33 (cross with five polymorphisms, see Figures 2 and 3). The same reversal of proportion is seen in the analysis of the tracts from the crosses with one polymorphism: The *Xho*I site is covered (not covered) by 28 (12) tracts in the cross *706* × *469*. The corresponding values for *M26* × *469* are 17 (23). Chi square calculations in 2 × 2 contingency tables yield statistical significance of the reversal for the crosses with one polymorphism ($P = 0.02$), while the same trend is not significant in the crosses with five polymorphisms ($P = 0.14$). The same phenomenon is visible in Figure 5. The comparison of site coverage by tracts leads to a steeper peak for *M26* than for *706*.

***M26* acts at a distance:** Comparison of the distribution of the four types of recombinants in the crosses *706* × *469 vs. M26* × *469* in Table 2 by 4 × 2 contingency table tests yields the following values: set 1 (five polymorphisms) $P = 2 \times 10^{-8}$; set 2 (five polymorphisms) $P = 0.077$; set 3 (one polymorphism) $P = 0.052$. This shows that *M26* alters the distribution of the four types of *ade*$^+$ recombinants. But large numbers of recombinants must be checked to obtain statistical significance (set 1: 749 + 892 = 1641, sets 2 and 3: only 768 recombinants each). If the statistical analysis is restricted to the two conversion types (no exchange of flanking markers) the 2 × 2 contingency table tests yield the following significant *P* values: 4×10^{-8}, 0.009, 0.02 for sets 1, 2 and 3, respectively. This could mean that *M26* stimulates single-site conversion more strongly at the 5′ end (where it is located itself) than at the 3′ end of the *ade6* gene (where *469* is located). This is best seen from the values normalized to 10^6 spores in Table 2. Stimulation is 12–17-fold for 5′ conversion *vs.* 5–8-fold for 3′ conversion in the three sets. The 3′ mutation *469* is separated from *M26* by 1332 bp. If the operational definition of *ura4*$^-$ *ade6*$^+$ *tps16*$^+$ recombinants as single-site conversions of the 3′ marker *469* is valid, this finding indicates that *M26* stimulates recombination at a distance. For a correct estimate of the relative degrees of stimulation of conversion at the 5′ end and the 3′ end of *ade6* the analysis of unselected tetrads of two-factor crosses is necessary. The effects of *M26* known so far could all be explained mechanistically with the *M26* site acting exclusively as an acceptor for gene conversion. The apparent stimulation of conversion of *469* to wild type on the chromatid not carrying *M26* requires more complexity in mechanistic models.

Conversion tracts and intragenic recombination at *ade6*: The conversion tracts observed with *ade6-706* (Figure 2) can be explained in two ways. First, the tracts initiate randomly along the DNA and elongate symmetrically in both directions or asymmetrically only in one direction. Second, the tracts initiate in a defined region and elongate in both directions. To distinguish between these two possibilities, we analyzed the conversion tracts resulting from the conversion of *ade6-51* to wild-type in the cross *51* × *469*. *51* lies 1218 bp downstream of *706*. Because of this distance, the 5′ endpoints of the tracts should be shifted in the 3′ direction, if the first possibility is correct. However, if the second is true, the 5′ endpoints should not be different, but the mean tract length should be altered. It should either increase (if the postulated initiation point lies in the 5′ region of the gene) or decrease (if this point lies close to *51*). The results are consistent with a defined region of initiation at the 5′ end of *ade6*. The 5′ ends of *51* tracts did not drastically differ from the *706* control (Figures 2 and 4) and the average minimal tract length was increased. Since the 3′ arms of the tracts were forced to terminate between the two mutations (selection for *ade6*$^+$ recombinants), we can compare only the 5′ arms of the tracts. The difference of 5′ arm lengths for *51* (Figure 4, 1290 bp) and *706* (Figure 2, 720 bp) is statistically significant ($P < 0.02$, Wilcoxon signed rank test). Most of the tracts in the cross *51* × *469* actually do or may (endpoint cannot be localized) cover the 5′ end of the gene. Only 9 of 60 5′ arms terminate downstream of the *Hin*dIII site (Figure 4). The hypothesis of preferred initiation of conversion at the 5′ end of *ade6* is also supported by data from one-point crosses that show a slight polarity from the 5′ end toward the 3′ end (GUTZ 1971; SCHAER, MUNZ and KOHLI 1993).

Higher frequency of aberrant segregation for mutations at one end of the gene is generally attributed to fixed initiation points at this end (reviewed by WHITEHOUSE 1982). Recently, polarity of aberrant segregation in the *HIS4* gene of *S. cerevisiae* has been explained by bias of mismatch repair: altering ratio of conversion *vs.* restoration along the gene (DETLOFF, WHITE and PETES 1992). Our analysis of mismatch repair at the *ade6* gene has produced no evidence for a bias of conversion *vs.* restoration (SCHAER, MUNZ and KOHLI 1993; SCHAER and KOHLI 1993).

In contrast to initiation of gene conversion, termination of tracts seems to be random in the *ade6* gene. The frequency of coconversion of a particular restriction site with the *ade6* mutation under study (*706*, *M26* or *51*) decreased exponentially with increasing distance from the mutation (Figure 5).

It is difficult to argue about continuity *vs.* discontinuity of conversion tracts, because the experimental set up allows only the monitoring of a few points in the whole chromosome region under study. Truly discontinuous tracts may be scored erroneously as continuous. In any case, less than 5% of the observed conversion tracts are discontinuous (Figures 2–4). This is in agreement with data from budding yeast on meiotic (BORTS and HABER 1989; JUDD and PETES 1988; SYMINGTON and PETES 1988; SCHULTES and SZOSTAK 1990) and mitotic conversion tracts (JUDD and PETES 1988; AHN and LIVINGSTON 1986). Fission and budding yeasts are also comparable with respect to the length of conversion tracts. The length of individual tracts is very heterogenous, but our estimate for average minimal lengths at *ade6* is in the range of 1 kb (with the reservation that we have studied selected tracts only). The reported values for *S. cerevisiae* depend on the region of the genome: BORTS and HABER (1987, 1989) used plasmid insertions at the *MAT* locus and published values of about 1.5 kb. JUDD and PETES (1988) reported lower values for the *URA3* region. The observed lengths of uninterrupted conversion tracts in *ade6* are in stark contrast to the shortness of mismatch repair excision tracts in *ade6* that have been proposed to be not more than 200 bp (SCHAER and KOHLI 1993). To resolve this discrepancy it may become necessary to postulate a mechanism for the direction of independent excision events to the same DNA strand in long heteroduplexes.

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