

Natural Meiotic Recombination Hot Spots in the *Schizosaccharomyces pombe* Genome Successfully Predicted from the Simple Sequence Motif *M26*[†]

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The *M26* hot spot of meiotic recombination in *Schizosaccharomyces pombe* is the eukaryotic hot spot most thoroughly investigated at the nucleotide level. The minimum sequence required for *M26* activity was previously determined to be 5'-ATGACGT-3'. Originally identified by a mutant allele, *ade6-M26*, the *M26* heptamer sequence occurs in the wild-type *S. pombe* genome approximately 300 times, but it has been unclear whether any of these are active hot spots. Recently, we showed that the *M26* heptamer forms part of a larger consensus sequence, which is significantly more active than the heptamer alone. We used this expanded sequence as a guide to identify a smaller number of sites most likely to be active hot spots. Ten of the 15 sites tested showed meiotic DNA breaks, a hallmark of recombination hot spots, within 1 kb of the *M26* sequence. Among those 10 sites, one occurred within a gene, *cds1*⁺, and hot spot activity of this site was confirmed genetically. These results are, to our knowledge, the first demonstration in any organism of a simple, defined nucleotide sequence accurately predicting the locations of natural meiotic recombination hot spots. *M26* may be the first example among a diverse group of simple sequences that determine the distribution, and hence predictability, of meiotic recombination hot spots in eukaryotic genomes.

Homologous recombination occurs at high frequency during meiosis in most sexually reproducing organisms (4). Crossovers resulting from recombination create connections between homologous chromosomes (chiasmata), which are critical for the proper segregation of chromosomes at the first meiotic division. In the absence of recombination, homologs segregate nearly randomly in most organisms, often resulting in aneuploid meiotic products (gametes or spores). Aneuploid progeny in multicellular eukaryotes, such as humans, are usually inviable or suffer from a number of physical and mental abnormalities, as seen, for example, in human trisomy 21 (Down syndrome). In addition to its role in the proper segregation of chromosomes, meiotic recombination also results in the formation of new genetic combinations and is therefore an important mechanism of increasing genetic diversity within a species.

Recombination does not occur at a uniform frequency throughout the genomes of the organisms examined. Rather, there are sites that recombine at a significantly higher or lower frequency than the genomic average, termed hot spots and cold spots, respectively. Hot spots have been described in organisms as diverse as bacteria and their phages, mice, and humans (9, 26, 30). In the two distantly related yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, meiotic hot spots of recombination are sites of programmed double-strand DNA breaks (DSBs) made by the Spo11 protein (Rec12 in *S.*

pombe) (11, 26, 31, 33). Spo11 is a meiotically induced protein with amino acid sequence similarity to an archaeal type II topoisomerase, TopoVI. Spo11 homologs are widely conserved in eukaryotes and are essential for meiotic recombination in several diverse organisms (20, 21). Thus, it is likely that programmed DSBs are a universal mechanism for the initiation of meiotic recombination.

Though much progress has been made in elucidating the mechanism of recombination, it is less clear how this process is regulated in eukaryotes. In particular, little is known about the factors controlling the locations of prominent DNA break sites, i.e., hot spots of recombination. In a global analysis of meiotic DSB sites in *S. cerevisiae*, Gerton et al. (15) found a significant correlation between DSB sites and the local G+C content. When viewed in 5-kb windows, >90% of all DSBs were found within 2.5 kb of regions where the G+C content exceeded the genomic average. The molecular basis of this correlation is not clear. Blumental-Perry et al. (6) reported that DSBs in *S. cerevisiae* were correlated with a 50-bp degenerate motif termed the common homology region or CoHR. However, a recent test of this hypothesis found no significant association of the CoHR with DSB sites, and deletion of the CoHR at *HIS2*, one of the hot spots used to identify the CoHR sequence, affected neither the position nor the intensity of meiotic DNA breakage at that site (19).

In prokaryotes, hot spots of recombination are often determined by short DNA sequence motifs. For example, in *Escherichia coli* a unique eight-base sequence, Chi, is recognized and activated by the RecBCD enzyme at an early step in recombination (30). Chi hot spots are active in other enteric bacteria (24), and short (5- to 7-bp) sequences also appear to be hot spots in the distantly related gram-positive bacteria

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Bacillus subtilis and *Lactococcus lactis*. Both of these species contain functional homologs of the RecBCD enzyme, whose activity is regulated by their respective Chi sequences in a manner comparable to that of the *E. coli* RecBCD enzyme (7, 10). Thus, discrete sequence motifs defining hot spots of recombination may be a common feature of prokaryotes.

The *M26* hot spot of *S. pombe* is the only reported eukaryotic hot spot comparable to the Chi hot spot in that it is defined by a simple sequence motif. This hot spot was first identified as a mutation in the *ade6* gene, *ade6-M26*, that elevated recombination up to 20-fold compared to other *ade6* alleles (17). The hot spot results from a single G→T nonsense mutation near the 5' end of *ade6* (34). This mutation creates a 7-bp sequence, 5'-ATGACGT-3' (*M26* mutation underlined), essential for hot spot activity (28). This sequence is at least part of the binding site for a heterodimeric transcription factor, Atf1-Pcr1, which is also essential for *M26* hot spot activity (22, 36). When created by mutation of 1 to 4 bp, the *M26* heptamer generates hot spots at multiple positions within the *ade6* and *ura4* genes (12), and all of those sites tested stimulate the formation of Rec12-dependent meiosis-specific DSBs near the site of the *M26* heptamer (31). In addition to *M26*, Fox et al. (13) showed that a closely related sequence, 5'-TGACGT^C/_A-3', termed *CRE* (cyclic AMP response element), is also an Atf1-Pcr1-dependent hot spot at one site within the *ade6* gene. *M26* and *CRE* overlap at six of their seven base pairs, differing only at the ends (Fig. 1).

Collectively, the *M26* and *CRE* heptamers occur 879 times in the sequenced *S. pombe* genome (39), but hot spot activity of these sites has not been reported. One *M26* heptamer that occurs in the *ura1*⁺ gene was tested for its potential as a hot spot, but mutation of this site had no significant effect on recombination within *ura1* (M. Fox and G. Smith, unpublished observations). Thus, not all *M26* or *CRE* heptamers, henceforth referred to as *M26/CRE*, are hot spots. Recently, however, we showed that the *M26/CRE* heptamer alone is not sufficient for full hot spot activity (32). In at least one location within *ade6*, this sequence showed no significant activity and bound Atf1-Pcr1 weakly compared to an overlapping active heptamer in the opposite orientation. This observation led to the discovery that *M26/CRE* forms part of a larger consensus binding sequence, 5'-GNVTATGACGTCATNBNC-3' (V is A, C, or G; B is C, G, or T; N is any nucleotide), termed *M26_{CS}*, required for optimal binding of Atf1-Pcr1. When *M26_{CS}* was created at the site of the inactive *ade6* heptamer, an exceptionally strong hot spot resulted. In addition, creation of *M26_{CS}* at the site of an already active *M26* heptamer, *ade6-M26*, increased recombination about threefold compared to the heptamer alone. This observation suggested that *M26/CRE* is most likely to be active when it forms part of an expanded sequence containing at least some of the additional nucleotides found in the consensus binding sequence. We used this information to narrow our survey of genomic *M26/CRE* sites and found that this simple sequence motif accurately predicted the locations of numerous natural recombination hot spots.

MATERIALS AND METHODS

***S. pombe* strains, growth media, and meiotic crosses.** Solid and liquid growth media were made as previously described (18, 32). For media, 5S refers to the supplements adenine, leucine, lysine (100 µg/ml each), uracil, and histidine (50

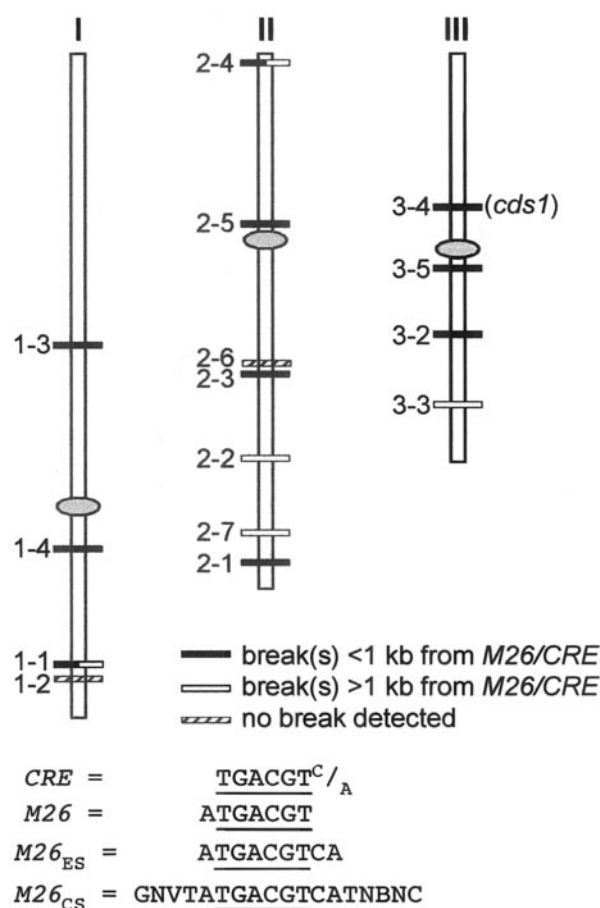


FIG. 1. Positions of *M26* or *CRE* sites (*M26/CRE*) tested for meiotic DNA breakage. Site numbers correspond to those described in the text. Chromosomes are drawn to scale, and centromeres are indicated by ovals. Below the chromosomes are definitions of the sites studied here.

µg/ml each). 4S refers to the same supplements minus adenine. Guanine (G; 100 µg/ml) was added to media as necessary to select for Ade⁺ recombinants (16).

The strains used in this study are listed in Table 1. The *ade6* alleles *M26*, *M375*, and *469* have been described previously (17, 34). The other *ade6* alleles, *pcr1::his7⁺*, and *atf1::ura4⁺* were described by Steiner and Smith (32); *pat1-114* and *rad50S* were described by Young et al. (40); *h⁺ mat1PΔ17::LEU2* was described by Arcangeli and Klar (2). The *cds1* alleles contain the following nucleotide substitutions (numbered from the start of the coding sequence): *cds1-T11A*, A31G (35); *cds1-1*, G543T (nonsense mutation); *cds1-2*, C533T; *cds1-3*, C533T G534T; *cds1-4*, T192C. All *cds1* mutations were generated with a site-directed mutagenesis kit (QuikChange; Stratagene) and, as the template, a plasmid containing a 3.3-kb PstI-SpeI fragment of *cds1* (pBS-*cds1*) (35). Mutations were confirmed by nucleotide sequencing. The mutant plasmids were digested with PstI and SpeI and used for LiCl-mediated transformation (3) of GP4279, containing *Δcds1::ura4⁺* (25). After overnight recovery in YEL-5S, linear transformants were selected on NBA plates (0.67% Difco yeast nitrogen base without amino acids) supplemented with uracil, leucine, and 1 mg/ml 5-fluoroorotic acid (32). Homologous integration was confirmed by Southern blot hybridization, and mutations were confirmed by sequencing or by the expected restriction site alteration.

Diploid strains were constructed by mating appropriate haploid strains for 6 to 12 h on SPA-5S (18) at 25°C and streaking them onto NBA containing appropriate supplements but lacking leucine and histidine to select for diploids. Alternatively, diploid strain WS119 was constructed by protoplast fusion (1).

Ade⁺ recombinant frequencies from zygotic and azygotic meioses (see Tables 3 and 4) were determined by random spore analysis as previously described (32). For diploid strains, freshly grown single colonies were used to inoculate 5-ml

TABLE 1. Strains used in this study

Strain	Genotype ^a
GP3111	<i>h⁺ ade6-M26 his7-366 ura4⁺ pcr1::his7⁺ pat1-114 rad50S</i>
GP3307	<i>h⁻ ade6-3044 leu1-32</i>
GP3316	<i>h⁺ ade6-M375 leu1-32</i>
GP3667	<i>h⁺ ade6-3044 leu1-32 his7-366</i>
GP3698	<i>h⁺ ade6-M375 leu1-32 his7-366 pcr1::his7⁺</i>
GP3700	<i>h⁺ ade6-3044 leu1-32 his7-366 pcr1::his7⁺</i>
GP4279	<i>h⁻ leu1-32 cds1::ura4⁺</i>
GP4376	<i>h⁻ cds1-1 leu1-32</i>
GP4378	<i>h⁺ cds1-T11A leu1-32</i>
GP4451	<i>h⁺ mat1PΔ17::LEU2 cds1-T11A leu1-32 atf1::ura4⁺</i>
GP4452	<i>h⁻ cds1-1 leu1-32 atf1::ura4⁺</i>
GP4453	<i>h⁻ cds1-1 his7-366 pcr1::his7⁺</i>
GP4454	<i>h⁻ cds1-T11A leu1-32 his7-366 pcr1::his7⁺</i>
GP4457	<i>h⁻ ade6-3070 leu1-32</i>
GP4505	<i>h⁻ ade6-3083 leu1-32</i>
GP4630	<i>h⁻ leu1-32 cds1-1,2</i>
GP4669	<i>h⁺ mat1P Δ17::LEU2 leu1-32 cds1-T11A,2</i>
GP4872	<i>h⁺ mat1P Δ17::LEU2 leu1-32 cds1-T11A,3</i>
GP4873	<i>h⁺ mat1P Δ17::LEU2 leu1-32 cds1-T11A,2,4</i>
GP4874	<i>h⁻ leu1-32 cds1-1,3</i>
GP4875	<i>h⁻ leu1-32 cds1-1,2,4</i>
GP4879	<i>h⁺ ade6-3074 ura4⁺ pat1-114 rad50S end1-458</i>
GP4880	<i>h⁺ ade6-3083 ura4⁺ pat1-114 rad50S end1-458</i>
GP4937	<i>h⁻ ade6-3070 leu1-32 pcr1::his7⁺</i>
GP4938	<i>h⁻ ade6-3083 leu1-32 pcr1::his7⁺</i>
WS101	<i>h⁺ ade6-M375 + his7-366</i> <i>h⁻ ade6-3070 leu1-32 +</i>
WS102	<i>h⁺ ade6-469 + his7-366</i> <i>h⁻ ade6-3083 leu1-32 +</i>
WS103	<i>h⁺ ade6-3070 leu1-32 + atf1::ura4⁺</i> <i>h⁻ ade6-M375 + his7-366 atf1::ura4⁺</i>
WS111	<i>h⁺ ade6-469 + his7-366</i> <i>h⁻ ade6-M375 leu1-32 +</i>
WS118	<i>h⁺ ade6-M375 his7-366 +</i> <i>h⁻ ade6-3044 + leu1-32</i>
WS119	<i>h⁺ ade6-469 + his7-366 atf1::ura4⁺</i> <i>h⁻ ade6-3083 leu1-32 + atf1::ura4⁺</i>

^a All strains are also *ura4-D18* unless otherwise indicated. See Materials and Methods for references to specific alleles.

YEL-5S cultures, which were grown overnight at 32°C. Appropriate dilutions of these cultures were plated onto YEA-5S or YEA-4SG in order to determine the frequency of Ade⁺ mitotic recombinants prior to sporulation. The frequency of Ade⁺ cells in these cultures was <50/10⁶ in all cultures tested. One milliliter of each diploid culture was centrifuged; the cells were washed twice with 0.85% NaCl and plated onto SPA supplemented with adenine and/or uracil. After 2 days of incubation at 25°C, spores were treated with Glusulase and ethanol to kill the remaining vegetative cells as previously described (32).

cds1 mutants were also mated and sporulated on SPA and treated as described above. Spores were plated on YEA-5S and YEA-5S plus 5 mM hydroxyurea (HU) (35) to determine total and *cds1*⁺ spore yields, respectively.

Meiotic DNA break analysis. Meiotic DNA breaks were assayed in *pat1-114 rad50S* strains GP4879, GP4880 (*pcr1*⁺), and GP3111 (*pcr1*Δ). Meiotic inductions, preparation of genomic DNA, restriction enzyme digestion, pulsed-field gel electrophoresis, and Southern blot hybridizations were performed as previously described (31, 40). Under the conditions used, premeiotic DNA replication occurs 2 to 3 h after induction; meiosis-specific DSBs are usually visible by 3.5 to 4 h, and these breaks are not repaired due to the presence of the *rad50S* allele. Samples were taken at multiple time points after meiotic induction, and DNA was purified in agarose plugs to minimize mechanical breakage. Restriction enzymes were chosen that produced fragments of 8 to 29 kb containing the *M26* or *CRE* sites, and DSBs were assayed by Southern blot hybridization with probes specific to one end of each fragment.

DNA breakage was quantitated with a Molecular Dynamics PhosphorImager. Volume reports were determined for broken and unbroken fragments with the local average background subtracted. After quantitation, blots were stripped by two 15-min washes at 95°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate and rehybridized with a different probe. Occasionally, a trace amount of radioactivity in the main restriction fragment remained on the blot after this procedure. Thus, for each blot, probes hybridizing to the largest DNA fragment were used first, followed by probes recognizing successively smaller fragments. Any radioactivity remaining from a previous hybridization would not interfere with the results of the next.

Probes for each of the *M26/CRE* sites in this study were prepared by PCR with primer pairs specific to each of the *M26/CRE* sites as follows: 1-1, 5'-CGACGTCGACGACAAGAGAGATT-3' and 5'-GGCATCTCTGAATTTCAAACCTTCGCA-3'; 1-2, 5'-TAGCAGCCTCAATCTGAACATCGG-3' and 5'-GTGGG AAGACGATTTAGCGAATGC-3'; 1-3, 5'-TGTCTTGGCATCAGCATAACAG TT-3' and 5'-TATGAACCTCGCTCAGGGAATGGAG-3'; 1-4, 5'-GAAGCTTC AAAGGATCGAATTGAGTTGTG-3' and 5'-GATAACCTTTCCAAAGACC GCCTTC-3'; 2-1, 5'-AGGAGCTTACGCCGTCTCAAGTAT-3' and 5'-TTCG TGATCTTCTACCATCGCGTC-3'; 2-2, 5'-CAACACGTTATTATGGTAAAC CGAACG-3' and 5'-ACGGTCGAGATGAGTACAAAGTCA-3'; 2-3, 5'-GCT CAAGTCCACCGATTGTGAAG-3' and 5'-GCAAGCGCAGTTTGAGGTC TGTTA-3'; 2-4, 5'-CCCTGCGAAAGTAAATGAAGTGC-3' and 5'-CCCTC TTCTGTATGAAGTTGTGGATCA-3'; 2-5, 5'-CCAATAGAAGGGAACCTT CATCATAGGC-3' and 5'-GCTCTCATCGCTGTTATGTCTGAGT-3'; 2-6, 5'-TTTGAGGCAACTATCGCTGGACG-3' and 5'-GAGAGGAAATCCATTCATTA GTACCGTTAAA-3'; 2-7, 5'-GCCTAATCAGCAACAATTAGCCCA-3' and 5'-CCGAATTATGGCATTAGCAAGTAAAGCA-3'; 3-2, 5'-AACAAACACC TACCATGTCAGGGTCTC-3' and 5'-TCCGAACGGAACGCTCAAGAGA A-3'; 3-3, 5'-CACACCAACTGAATATTGTATCCCATCC-3' and 5'-AACGG CATGCTACCAGAAAGT-3'; 3-4, PacI, 5'-TGTGAAAGTGTCTAATGCCTT ATCTGA-3' and 5'-GGAAATTAAGACAAGCAAAGCGTGC-3'; 3-4, HaeII, 5'-CCAGCATACACAGCCATTAACCGA-3' and 5'-ACGTCAACTTGGAC GGAGGTTATG-3' or 5'-GGCTTGTCAGATATTACGGCATC-3' and 5'-A CAGTCGACACAAACATCTAAATAAGC-3'; 3-5, 5'-GCTCAAGCGATCCTT CCATCAAAC-3' and 5'-TGATTACTCGCCAATGCITTC-3'.

RESULTS AND DISCUSSION

Meiotic DSBs at natural *M26/CRE* sites. The Atf1-Pcr1 consensus binding sequence *M26_{CS}*, noted above, contains 15 non-random bases (32) (Fig. 1) and does not occur in the sequenced *S. pombe* genome (http://www.sanger.ac.uk/Projects/S_pombe/). However, the central 10-bp *M26* palindrome appeared to be the most critical element for strong binding of Atf1-Pcr1 in vitro and therefore most likely for hot spot activity as well. This sequence occurs four times in *S. pombe* (Fig. 1, sites 2-4, 2-5, 2-6, and 2-7). In order to broaden our search for hot spots beyond just these four sites, we also included a

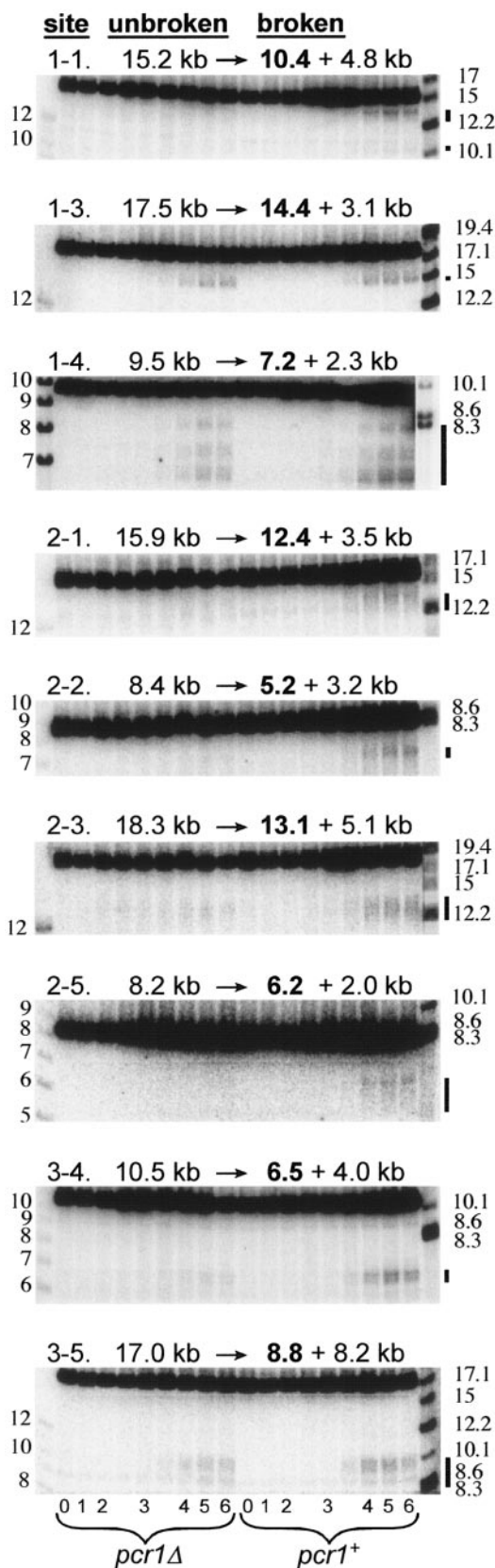


FIG. 2. Meiosis-induced DSBs are produced at the *M26/CRE* motif at several sites in the genome. *pcr1+* or *pcr1Δ* strains were induced for meiosis, and genomic DNA was purified at the indicated time points.

shorter sequence, 5'-ATGACGTCA-3', which appeared essentially as active as the full 10-bp palindrome (32). This extended *M26* sequence, termed *M26_{ES}*, occurs an additional 11 times in the genome (Fig. 1). We also included in our analysis one palindromic *CRE* sequence, 5'-CTGACGTCA-3' (site 1-4), that forms a strong hot spot in *ade6* (13). Since engineered *M26* hot spots in the *ade6* gene are sites of meiotic DSBs and since DSB formation at the one site tested (*ade6-M26*) requires the Pcr1 protein (31), we tested whether the naturally occurring sites (*M26_{ES}* or *CRE*) are also sites of Pcr1-dependent meiotic DNA breaks.

By Southern blot hybridization, we tested the *M26*- or *CRE*-containing genome fragments for meiotic DSBs (Fig. 2; the complete set of uncropped images is in Fig. S1 of the supplemental material). DSBs were observed in 13 of the 15 fragments probed. Nine of the 13 observed break sites were prominent ($\geq 1\%$ of the total DNA was broken; Table 2) and often appeared as clusters of breaks spread over regions of up to 2 kb (e.g., site 1-4, Fig. 2). Since approximately 200 kb of the genome was surveyed in our analysis, this corresponds to an average of one prominent break site per ~ 22 kb. This density of breaks appears to be somewhat greater than that observed in previous analyses, where prominent break sites were estimated to occur ~ 25 to 200 kb apart (40). If so, this implies that the presence of *M26/CRE* is positively correlated with DNA breaks. Of course, a more important consideration is the positions of the break sites relative to the *M26/CRE* sequence in each fragment. Fifteen DSB sites were found in 15 DNA fragments averaging ~ 14 kb in length (two fragments, 1-1 and 2-4, had two clusters of breaks each; Fig. 2; see Fig. S1 of the supplemental material). Ten of those breaks were ≤ 1 kb from the *M26/CRE* sequence (Table 2; see Fig. S1 of the supplemental material). In any fragment, the probability of a break occurring ≤ 1 kb from *M26/CRE* by chance is $2 \text{ kb}/14 \text{ kb} = 0.14$, and the probability of ≥ 10 of 15 breaks falling at least that close to *M26/CRE* can be calculated from the binomial distribution to be 4.4×10^{-6} . Thus, most, and perhaps all, of the breaks ≤ 1 kb from the *M26/CRE* sequence likely result from the presence of that motif at those sites.

We looked for a common feature among the *M26/CRE* sites with detectable DSBs. Of the 10 *M26/CRE* sites with DSBs, 9 were located in intergenic regions and 1 (site 3-4) occurred within a gene intron. Seven of the 10 DSB-associated sites were located < 750 bp upstream of open reading frames (potential gene promoter regions), 5 of which show Atf1-dependent induction in response to stress (sites 1-1, 1-3, 1-4, 2-2, and 2-7; http://www.sanger.ac.uk/PostGenomics/S_pombe/projects/stress/). These results are comparable to observations on *S.*

The DNA was digested and analyzed by Southern blot hybridization with probes specific to each of the indicated sites. The predicted sizes in kilobase pairs of the unbroken restriction fragment and of fragments generated by a break at the *M26/CRE* motif are indicated above each blot. The size of the broken fragment recognized by the probe is shown in boldface type. The positions of broken DNA fragments are indicated by black bars to the right of each gel. DNA size markers in kilobases are shown to the left and right of the gels. The exposure of these markers was sometimes adjusted separately from the rest of the blot for clarity.

TABLE 2. Meiotic DSBs at *M26/CRE* sites in wild-type *S. pombe*

Site	Enzyme used for DNA digestion	Unbroken fragment size ^a (kb)	Predicted size of broken fragment ^b (kb)	Break ≤1 kb from predicted position ^c	Break ≥1 kb from predicted position ^c	% breakage <i>pcr1</i> ⁺ ^d	% breakage <i>pcr1</i> Δ ^d
1-1	PacI	15.2	10.4	+		0.5	0.5
1-1	PacI	15.2	10.4		+	2.3	1.1
1-2	NruI	15.0	10.8				
1-3	NruI	17.5	14.4	+		3.2, 2.7	2.3, 2.2
1-4	NcoI	9.5	7.2	+		7.0	5.0
2-1	PacI	15.9	12.4	+		3, 4.3	1.5, 2.8
2-2	PacI	8.4	5.2		+	1.9	0.6
2-3	PacI	18.3	13.1	+		3.3, 5.0	1.8, 2.9
2-4	NruI	7.9	5.1	+		<0.5	<0.5
2-4	NruI	7.9	5.1		+	<0.5	<0.5
2-5	NruI	8.2	6.2	+		0.8, 0.5	<0.5, <0.5
2-6	NruI	14.0	10.7				
2-7	NruI	18.7	12.8		+	<0.5, <0.5	<0.5, <0.5
3-2	NruI	10.3	4.4	+		<0.5, <0.5	<0.5, <0.5
3-3	NruI	28.7	16.0		+	2.0	1.0
3-4	PacI	10.5	6.5	+		1.8, 1.6	0.9, <0.5
3-5	NruI	17.0	8.8	+		3.4	2.1

^a Predicted size of unbroken fragment from *S. pombe* genomic sequence (http://www.sanger.ac.uk/Projects/S_pombe/).
^b Size of hybridizing fragment predicted from a break within the *M26/CRE* sequence.
^c Fig. 2; see Fig. S1 of the supplemental material.
^d Percentage of total DNA in broken fragments. Pairs of values are from separate blots of one induction. *pcr1*⁺ and *pcr1*Δ strains were compared side by side on the same blot. The first value of each pair (*pcr1*⁺ or *pcr1*Δ) is from one blot, and the second value is from the second blot.

cerevisiae, in which meiotic DSBs are usually found in intergenic regions, often in gene promoters (5, 15). As noted in the introduction, Gerton et al. (15) also reported a correlation between DSBs in *S. cerevisiae* and the G+C content in 5-kb windows surrounding the break site. In our more limited survey of break sites, we found a similar correlation, but only in a much smaller window. Viewed in 100-bp windows, 9 of the 10 *M26/CRE* sites showing breaks were in regions where the G+C content exceeded the genomic average, and 4 of 5 sites showing no detectable breaks were in regions of lower-than-average G+C content. There was also a positive, but weak, quantitative correlation between G+C content and break intensity (linear regression analysis, $R^2 = 0.30$; unpublished observations). These correlations declined as larger windows were considered. Thus, the pattern of nucleotide composition around hot spots in *S. cerevisiae* noted by Gerton et al. (15) may be similar to but more extensive than that around the active *M26/CRE* sites in *S. pombe*.

Partial Pcr1 independence of *M26*_{CS} hot spot activity. As previously mentioned, the *M26* hot spot was first identified by the *ade6-M26* mutation (17). The activity of that hot spot is completely dependent on the Atf1-Pcr1 transcription factor. Mutation of *pcr1* eliminates detectable breakage at the hot spot (31), and mutation of either *atf1* or *pcr1* reduces recombination to non-hot spot levels (22). A *CRE* hot spot created at the same position as the *ade6-M26* mutation also showed complete dependence on both Atf1 and Pcr1 (13). Thus, we expected that other *M26/CRE* sites would have similar requirements for hot spot activity, and DSBs, if they occurred at these sites, would not be observed in strains lacking Pcr1. We were therefore surprised to see substantial Pcr1 independence of breakage at some of the *M26/CRE* sites whose positions coincided with DSBs (e.g., Fig. 2, sites 1-3, 1-4, and 3-5). In our DSB analysis (Fig. 2), an *atf1* mutant was not tested because it cannot be induced for meiosis in *pat1-114* (ts) strains (31).

Consequently, we used genetic tests for Atf1 and Pcr1 dependence of the *M26/CRE* sites examined here. Previous studies have shown that although mutations in *atf1* or *pcr1* have similar phenotypes, they are not identical. Both mutations result in poor mating and sporulation efficiency, but an *atf1* mutation is more deficient in these processes (23; our unpublished observations). Furthermore, Atf1, but not Pcr1, is required for growth at high osmolarity (22, 23). And mutation of *pcr1*, but not *atf1*, results in cold-sensitive growth on rich medium (37; our unpublished observations). Thus, both of these polypeptides may also act alone or in conjunction with other protein partners in vivo. Since many of the DSBs at or near *M26/CRE* sites were not eliminated in the *pcr1* mutant, we tested whether Atf1 and Pcr1 were required for hot spot activity of the *M26* consensus sequence (*M26*_{CS}) at two sites in the *ade6* gene (Fig. 3, *ade6-3083* and *ade6-3070*) (32). Both of these hot spots showed substantial activity in the absence of Pcr1: hot spot activity in the *pcr1* mutant was significantly reduced, but less than threefold relative to the corresponding *pcr1*⁺ strains, and was 10- to 20-fold greater than that of the non-hot spot control alleles (Table 3). The substantial hot spot activity of these sites in the absence of Pcr1 is consistent with the Pcr1-independent breakage ob-

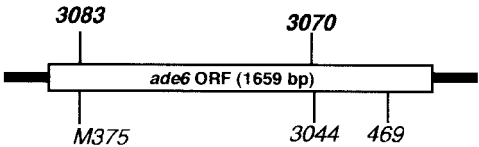


FIG. 3. Positions of *ade6* alleles used in crosses (Tables 3 and 4). The two *M26*_{CS} hot spot alleles, *ade6-3083* and *-3070* (32), are shown in boldface type above the gene; non-hot spot alleles appear below. ORF, open reading frame.

TABLE 3. The *ade6-M26_{CS}* hot spot is only partially Pcr1 dependent

Cross	<i>ade6</i> alleles ^a	<i>pcr1</i>	No. Ade ⁺ /10 ^{6b}
GP4505 × GP3667	3083 × 3044	+	16,900 ± 1,600
GP4938 × GP3700	3083 × 3044	Δ ^c	6,140 ± 560
GP3316 × GP3307	<i>M375</i> × 3044	+	530 ± 50
GP3316 × GP4457	3070 × <i>M375</i>	+	23,500 ± 1,100
GP3698 × GP4937	3070 × <i>M375</i>	Δ	10,600 ± 1,300

^a See Fig. 3 for allele descriptions. *M26_{CS}* hot spot alleles are in bold.^b Mean frequency ± the standard error of the mean of Ade⁺ recombinants per million viable spores from three crosses.^c Δ, *pcr1::his7⁺*.

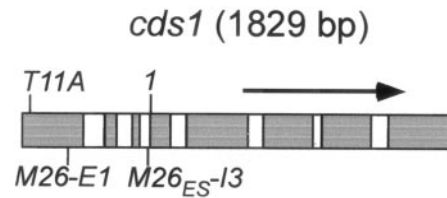
served at or near several of the *M26/CRE* sites in the genome, as noted above.

Given these results, we also tested whether the *M26_{CS}* sites in *ade6* showed *atf1*-dependent hot spot activity. In these experiments, the *atf1* mutations decreased recombination >12-fold relative to the corresponding *atf1⁺* strains for both hot spot alleles (Table 4). For one of the alleles (*ade6-3070*), recombination was reduced to the same level as the non-hot spot control (*ade6-3044*), suggesting that its activity is completely dependent on Atf1; for the other hot spot allele (*ade6-3083*), recombination remained slightly, but significantly, elevated relative to the non-hot spot control ($P < 0.02$, one-tailed *t* test). The residual hot spot activity observed in the absence of either *atf1* or *pcr1* may reflect the ability of these polypeptides to bind weakly as homodimers to *M26* in the absence of its partner protein (36). Homodimer binding to *M26_{CS}*, particularly the Atf1 homodimer, may be substantially greater than to the original *ade6-M26* heptamer, accounting for its activity in the absence of Pcr1.

As mentioned above, five of the sites tested for meiotic DSBs showed breaks >1 kb from the *M26/CRE* motif without an accompanying prominent break at the predicted location (sites 1-1, 2-2, 2-4, 2-7, and 3-3, Table 2; Fig. 2; Fig. S1 of the supplemental material). In one case (site 3-3), a cluster of breaks was observed >6 kb from the predicted site, but at none of the five sites was a second *M26* or *CRE* sequence found near the actual break position. Since most meiotic recombination in *S. pombe* apparently does not require the Atf1 or Pcr1 protein (22; our unpublished observations), the simplest explanation for these breaks is that they are unrelated to the presence of

TABLE 4. The *ade6-M26_{CS}* hot spot is strongly Atf1 dependent

Diploid ^a	<i>ade6</i> alleles ^b	<i>atf1</i>	No. Ade ⁺ /10 ^{6c}
WS102	3083 × 469	+	12,300 ± 1,800
WS111	<i>M375</i> × 469	+	300 ± 40
WS119	3083 × 469	Δ ^d	1,000 ± 90
WS101	3070 × <i>M375</i>	+	12,100 ± 870
WS118	<i>3044</i> × <i>M375</i>	+	320 ± 30
WS103	3070 × <i>M375</i>	Δ	310 ± 10

^a *atf1* mutants show severe defects in mating and meiosis (23, 29, 38). Thus, in order to obtain adequate numbers of viable spores, unstable diploids were used for these experiments.^b See Fig. 3 for allele descriptions. *M26_{CS}* hot spot alleles are in bold.^c Mean frequency ± the standard error of the mean of Ade⁺ recombinants per million viable spores from three crosses.^d Δ, *atf1::ura4⁺*.FIG. 4. The *cds1* gene. The positions of two Cds1-inactivating mutations, *T11A* and *1* (see Materials and Methods) and the two *M26* motifs, *M26-E1* in exon 1 and *M26_{ES}-I3* in intron 3, are shown. Exons and introns are indicated by gray and white boxes, respectively.

the nearby *M26* sequence. However, most of these breaks appeared to be strongly dependent on the Pcr1 protein, for example, sites 1-1, 2-2, and 3-3 (Fig. 2; Fig. S1 of the supplemental material). Since many other meiosis-specific DSBs were seen in the same *pcr1* mutant meiosis (for example, at sites 1-3, 1-4, and 3-5, Fig. 2), the lack of DSBs at some sites must, therefore, reflect Pcr1 dependence.

Might *M26* stimulate DNA breaks distant from it? Previously, we showed that one *M26* hot spot (*ade6-3011*) is able to stimulate visible breaks up to 1.3 kb distant from it (31). In that case, there were also breaks much closer to *M26*. Nevertheless, this result demonstrated that *M26* can stimulate breaks a considerable distance from it. A similar phenomenon might explain breaks distant from *M26* at some of the sites mentioned above, but it does not explain the absence of breakage proximal to *M26* at those same sites. Perhaps the DNA near *M26* at these sites is protected from cleavage, which occurs instead at the nearest available position. This admittedly unorthodox model would be one means of explaining how hot spots of recombination are preserved through evolutionary time (27): if hot spots of recombination are determined by the DNA sequence at or near the site of breakage, and break repair involves copying from a homolog without the hot spot, they should be lost at high frequency. If, however, the break determinant is located distant from the actual site of breakage, it would be lost at a much lower frequency.

***M26* is a recombination hot spot in *cds1*.** Meiotic DSBs are generally assumed to be hot spots of recombination. In the two organisms in which meiotic DSBs have been observed (*S. cerevisiae* and *S. pombe*), strong hot spots of recombination are sites of DNA breakage and vice versa, where tested (e.g., 8, 11, 26, 31, 33). Thus, the DSBs we found in this study are very likely also hot spots of recombination. In order to confirm this assumption, we analyzed one site (3-4, Fig. 1 and 2) more thoroughly because it lies within a well-characterized gene, *cds1*, encoding a checkpoint protein kinase (14, 25, 35). The *cds1* gene contains two *M26* motifs; *M26_{ES}*, which we identified originally in our genome survey, occurs in the third intron (*M26_{ES}-I3*), and a second site containing all but the last nucleotide of the *M26_{ES}* motif occurs 343 bp upstream, in the first exon of the gene (*M26-E1*; Fig. 4). Since either or both of these sites could contribute to the DSBs observed in Fig. 2, we mapped the break sites more precisely in order to determine their positions relative to the two *M26* motifs. Figure 5 shows a 6-kb DNA fragment containing *cds1* probed from each side for meiotic DSBs. In both analyses, *M26_{ES}-I3* fell between two prominent break sites ~200 bp apart, making it the likely

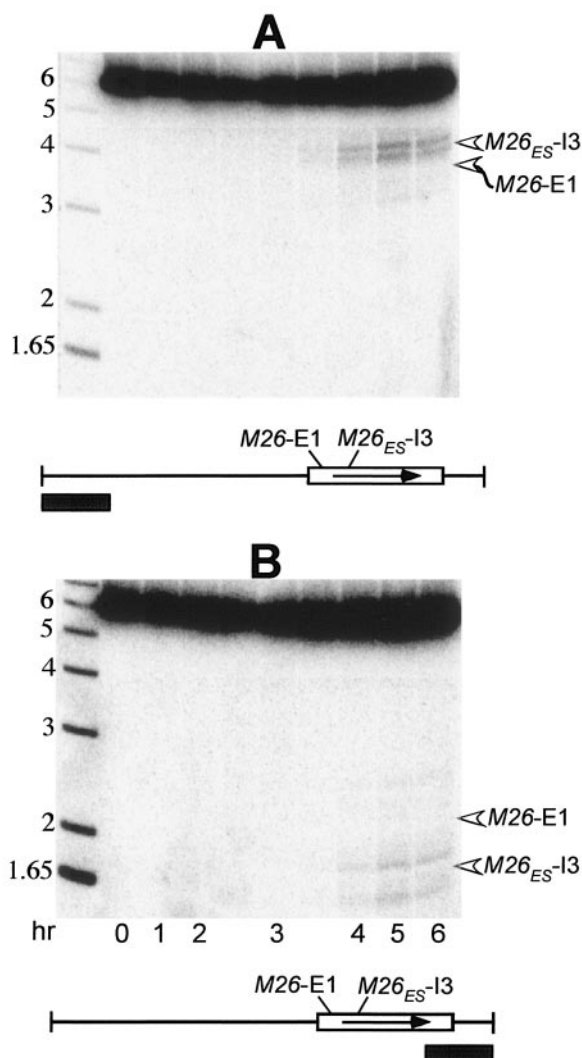


FIG. 5. $M26_{ES}$ lies between two prominent meiotic break sites in *cds1*. Genomic DNA from meiotically induced cells was digested with *Hae*II and probed from each end of the resulting 6.0-kb *cds1* fragment, as indicated in the schematic diagram below each blot. The inferred positions of both *M26* motifs are shown to the right of the blots. The values on the left are molecular sizes in kilobase pairs.

feature responsible for most, if not all, of the observed breakage. Twin break sites bracketing the *M26* sequence were also observed at the *ade6-M26* hot spot (31). This pattern of breakage may reflect exclusion of Rec12 over the narrow region of DNA to which Atf1-Pcr1 is actually bound. Thus, while stimulating the cleavage of neighboring DNA, the hot spot sequence itself is protected.

In order to test our prediction that $M26_{ES-I3}$ was responsible for the DSBs in *cds1*, we also analyzed it genetically for hot spot activity. Mutations in *cds1* result in sensitivity to the DNA synthesis inhibitor HU (25, 35), so *cds1*⁺ recombinants can be readily selected. Figure 6 shows results from crosses between *cds1-T11A* (35) and *cds1-1*, a nonsense mutation 512 bp downstream of the *T11A* mutation (Fig. 4). When no other mutations were present, we observed approximately 3,700 *cds1*⁺ recombinants per million viable spores. This frequency of re-

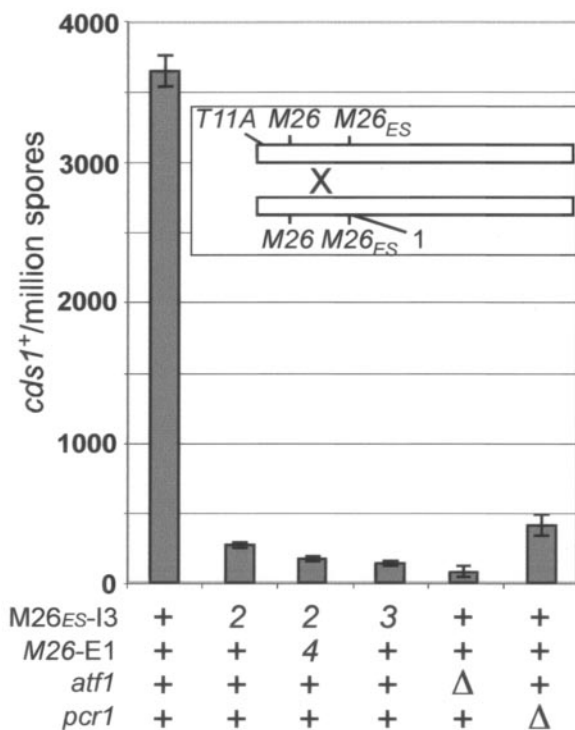


FIG. 6. *M26* is a hot spot of recombination in *cds1*. Bars show the mean frequency of *cds1*⁺ recombinants ($\pm$ the standard error of the mean) obtained from crosses between strains with the *cds1-T11A* and *cds1-1* mutations (inset) and *M26*, *atf1*, and *pcr1* mutations as indicated at the bottom. Each cross was performed three times, except for the *atf1* mutant, which was performed twice; error bars for that cross show the range of values obtained. $M26_{ES-I3}$ and $M26-E1$ are the *M26* sites in the third intron and first exon of *cds1*, respectively (Fig. 4). Mutations in these sites are indicated by allele numbers (2, 3, and 4) at the bottom (see Materials and Methods for allele descriptions).

combination is comparable to that observed for the *ade6-M26* hot spot (12, 17), which shows about the same frequency of meiotic DSBs (31). When $M26_{ES-I3}$ was also mutated in both parent strains by a single base pair change (*cds1-2*), the frequency of recombinants dropped 13-fold. When the second *M26* motif ($M26-E1$) was also abolished (*cds1-4*), recombination was reduced further, compared to mutation of only the first site ($P < 0.004$, one-tailed *t* test). Thus, while the majority of recombination can be attributed to only one site ($M26_{ES-I3}$), there may be a small contribution from the second site as well. (The *cds1-2* single mutant is wild type with respect to HU sensitivity [unpublished observations], and the *cds1-4* mutation is translationally silent.) This observation supports our earlier conclusion that bases outside of the originally defined 7-bp *M26* sequence can dramatically affect hot spot activity (32). It also suggests that some of the >800 *M26*/CRE sites scattered throughout the genome, which may be only weakly recognized by Atf1 or the Atf1-Pcr1 heterodimer, could contribute to low-level (non-hot spot) recombination in regions of the genome lacking prominent break sites (40). Consistent with this view, we noted that the single-base-pair mutation in the active hot spot of *cds1* (*cds1-2*) may not have completely eliminated its activity, since a double-base-pair change at that hot spot (*cds1-3*) also decreased recombination significantly, albeit

modestly, compared to the single-base-pair change ($P < 0.0007$, one-tailed t test; Fig. 6).

Since DSBs in *cds1*⁺ were largely, but not completely, eliminated by mutation of *pcr1* (Fig. 2), we also tested how *cds1* hot spot activity was affected by mutation of *pcr1* or its partner *atf1*. Recombination was reduced 9-fold by a mutation in *pcr1* but ~40-fold by mutation of *atf1* (Fig. 6). This large differential, along with the results in Tables 3 and 4, provides additional evidence that Atf1 can act independently of Pcr1 to promote recombination at M26 or related sites.

Conclusions. Here we have shown that the M26/CRE sequence motif defines the locations of multiple meiotic DNA break sites in the wild-type *S. pombe* genome. At the one break site occurring within a gene, *cds1*, we also confirmed genetically that M26 acts as a recombination hot spot. Given this result and our current understanding of how recombination is initiated, it is very likely that M26/CRE is also a recombination hot spot at the other break sites we observed. To our knowledge, this is the first example in any eukaryote of a simple sequence motif accurately predicting the locations of recombination hot spots. However, since breaks were not observed at all of the sites examined and since there were large variations in break frequency at the different sites, there are clearly other factors involved in break site selection. These factors could include an influence of nucleotides adjacent to the M26_{ES} sequence (32) or some broader aspect of the local chromatin structure.

Based on the results of our previous analysis of the *ade6* gene (32), we restricted our present investigation to genomic sites containing the 9- to 10-bp M26_{ES} and 10-bp CRE sequences, which we hypothesized were most likely to be sites of meiotic DSBs. The majority of these sites showed meiotic DNA breaks. However, this result does not imply that those longer sequences are essential for M26/CRE hot spot activity, and many of the hundreds of shorter versions of the motif could also be hot spots. M26/CRE may be just the first example among a diverse group of simple sequences determining the locations of recombination hot spots. Knowledge of such sequences would allow further predictions about meiotic recombination hot spots, similar to those presented here. This knowledge would facilitate the comparison of genetic and physical maps and aid, for example, the location of human disease genes by linkage studies.

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