A Family of cAMP-Response-Element-Related DNA Sequences With Meiotic Recombination Hotspot Activity in *Schizosaccharomyces pombe*

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ABSTRACT

The heptamer sequence ATGACGT is essential for activity of the *M26* meiotic recombination hotspot in the *ade6* gene of *Schizosaccharomyces pombe*. Hotspot activity is associated with binding of the heterodimeric transcription factor Atf1·Pcr1 to *M26*. We have found that the sequences (C/T/G) TGACGT also bound Atf1·Pcr1 and acted as meiotic hotspots, but unlike *M26* they must be followed by A or C for Atf1·Pcr1 binding and hotspot activity. The basis of the hotspot activity of CTGACGTA (*ade6-3013*) appears to be identical to that of *M26*: hotspot activity of both sequences was abolished in cells mutant for *atf1*, *pcr1*, *spc1*, or *wis1* and was undetectable in mitotic recombination and in meiotic recombination when located on a plasmid. Both hotspot sequences were sites of micrococcal nuclease hypersensitivity in meiotic chromatin, suggesting that they create an open chromatin structure during meiosis at the site of the hotspots. The newly identified hotspot sequences (C/T/G)TGACGT(A/C) and *M26* are closely related to the cAMP response element (CRE) consensus sequence for binding of cAMP-responsive transcription factors such as Atf1·Pcr1, suggesting a link between transcription and meiotic recombination. These results significantly expand the list of identified sequences with meiotic recombination hotspot activity in *S. pombe* from a single sequence to a family of CRE-related sequences.

RECOMBINATION between homologous DNA duplexes plays important roles during mitosis in the repair of DNA damage and during meiosis in the generation of genetic diversity and in the proper segregation of chromosomes. Meiotic recombination does not occur evenly throughout the genome but is elevated in certain regions, called hotspots. Meiotic recombination hotspots have been identified in many organisms from bacteria to mammals (Smith 1994; Lichten and Goldman 1995).

The *M26* hotspot of the fission yeast *Schizosaccharomyces pombe* has been particularly well characterized (reviewed by Fox and Smith 1998). This hotspot results from a G → T transversion in the coding region of the *ade6* gene and elevates intragenic recombination up to 15-fold relative to the nearby *M375* mutation, an identical G → T transversion in the preceding codon (Gutz 1971; Szankasi *et al.* 1988). *M26* also undergoes gene conversion ~10 times more frequently than *M375* and demonstrates disparity of conversion, with *M26* being preferentially converted to wild type.

The hotspot activity of *M26* depends on a specific nucleotide sequence, the heptamer ATGACGT (the site of the *M26* mutation is underlined; Schuchert *et al.* 1991). Prior to the work described here, the *M26* heptamer was the only sequence known to have hotspot activity in *S. pombe*. Extensive mutational analysis demonstrated that hotspot activity required all seven of these nucleotides; however, mutations outside the heptamer had no significant effect on recombinant frequency. The apparently unique nucleotide sequence of *M26* is unusual for meiotic recombination hotspots and greatly facilitates study of the mechanism by which recombination is elevated at this hotspot.

The *M26* heptamer sequence is specifically bound by a heterodimeric complex of the proteins Atf1 and Pcr1 (Kon *et al.* 1997), transcription factors that bind the cAMP response element (Takeda *et al.* 1995; Kanoh *et al.* 1996; Shiozaki and Russell 1996; Watanabe and Yamamoto 1996). Hotspot activity correlates with the ability of mutant sequences to bind the Atf1·Pcr1 heterodimer (Wahls and Smith 1994; Kon *et al.* 1997). Transcriptional activity of Atf1·Pcr1 requires the product of the *wis1* gene, a protein kinase that phosphorylates Spc1, which in turn phosphorylates Atf1 following activation of a stress-induced protein kinase cascade (Shiozaki and Russell 1995, 1996; Degols *et al.* 1996; Stettler *et al.* 1996; Wilkinson *et al.* 1996; Shieh *et al.* 1997). The product of the *spc1* gene is also required for *M26* hotspot function (Kon *et al.* 1998); however, the relationship between transcription and recombination is not clear (Kon *et al.* 1997; see discussion).

Several lines of evidence support a role for chromatin structure in hotspot activity. The *M26* heptamer is inactive on a plasmid and in several transplacements in

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which 3- to 6-kb regions of DNA containing the *ade6-M26* gene were transplaced to other genomic locations (Ponticelli and Smith 1992; Virgin *et al.* 1995). In contrast, the heptamer is active in either orientation at several other sites, when made by site-directed mutagenesis of 1–3 bp without accompanying disruption of gross chromosomal structure (Fox *et al.* 1997). Certain large chromosomal integrations may disrupt a chromatin structure required for hotspot function. The site of the *M26* mutation is also associated with chromatin remodeling, as revealed by changes in patterns of micrococcal nuclease-sensitive sites in chromatin. In cells bearing the *ade6-M375* mutation, MNase-sensitive sites appear at ~150-bp intervals, reflecting nucleosome phasing, along the entire *ade6* coding region. In contrast, the nuclease-sensitivity pattern in cells bearing the *M26* heptamer sequence is similar to that in naked DNA, suggesting that nucleosome positioning is rearranged. In addition, a new hypersensitive site appears near the *M26* heptamer sequence. This hypersensitive site, together with a hypersensitive site in the 5′ noncoding region of the *ade6-M26* locus, is present in chromatin isolated from premeiotic cells and becomes pronounced as cells enter meiosis or become stressed (Mizuno *et al.* 1997). Thus, the *M26* heptamer sequence may promote hotspot activity through the local opening of chromatin, allowing recombination-specific proteins access to the DNA.

Work on the mammalian transcription factor ATF1 has defined a binding consensus sequence, cAMP response element (CRE), which is present in genes that are transcriptionally regulated by ATF1 in response to intracellular cAMP levels (Montminy *et al.* 1986). This consensus, TGACGTC, bears striking similarity to the *M26* heptamer sequence ATGACGT: both have in common the hexamer TGACGT. *S. pombe* protein extracts contain a factor that specifically binds to a DNA probe containing the sequence CTGACGTAAC with this hexamer (Jones and Jones 1989). *S. pombe atf1* was also cloned as a gene called *gad7*, required for the starvation-induced arrest of cells in G_1 (Kanoh *et al.* 1996). The Gad7 (Atf1) protein binds to a DNA probe containing the CRE sequence CTGACGTCAG. These studies suggested that *S. pombe* Atf1·Pcr1 can bind to sequences other than the *M26* heptamer. From the work cited above, one such sequence appeared to be CTGACGT, and it seemed plausible that other *M26*-like sequences may also be binding sites for Atf1·Pcr1. Since binding to *M26* correlates with hotspot activity, these sequences may also be novel meiotic recombination hotspots. To test this hypothesis, we constructed *S. pombe* strains in which the *ade6* gene was altered at the site of the *M26* heptamer to make the following sequences: CTG ACGTN, TTGACGTN, and GTGACGTN. Atf1·Pcr1 bound to a subset of these sequences, but binding depended on the nucleotide following the heptamer. Sequences that bound Atf1·Pcr1 *in vitro* had meiotic recombination hotspot activity *in vivo*. These studies have thus identified a new family of sequences that can act as meiotic recombination hotspots in *S. pombe*.

MATERIALS AND METHODS

***S. pombe* strains and crosses:** The wild-type strains used for mutagenesis and preparation of protein extracts were GP18 (*h⁻ leu1-32*) and GP20 (*h⁺ leu1-32*). Strains containing the mutant *ade6* alleles studied here are listed in Table 1. Strains GP14 (*h⁺ ade6-52*) and GP5 (*h⁺ ade6-M216*) were used for the crosses in Figure 2. The homothallic strain used in Table 4 was GP341 (*h^90 ade6-469 ura4-294*). Strains mutant for *atf1*, *pcr1*, *spc1*, or *wis1*, provided by K. Shiozaki and P. Russell (Shiozaki and Russell 1996), have the following genotypes: JM544 (GP2204, *h⁺ wis1::ura4⁺ leu1-32 ura4-D18*); KS1517 (GP2206, *h⁺ spc1::ura4⁺ leu1-32 ura4-D18*); KS1512 (GP2208, *h⁺ atf1::ura4⁺ leu1-32 ura4-D18*); KS1648 (GP2435, *h⁻ pcr1::his7⁺ his7-366 ura4-D18 leu1-32*). The unstable diploids used for preparation of chromatin were GP2611 (*h⁺/h⁻ ade6-3013/ade6-3013 leu1-32/leu1-32 ura1-61/+ pro1-1/+*) and GP2612 (*h⁺/h⁻ ade6-3015/ade6-3015 leu1-32/leu1-32 ura1-61/+ lys3-37/+*). Genealogies and sources of strains are available on request.

All strains were grown on rich yeast extract agar (YEA) solid or yeast extract liquid (YEL) liquid media (Gutz *et al.* 1974) or on modified Edinburgh minimal medium 2 (EMM2; Nurse 1975) supplemented as appropriate. Sporulation was at 25° on sporulation agar (SPA) solid medium supplemented with adenine and leucine (Gutz *et al.* 1974). The frequency of recombination between *ade6* alleles in heterothallic crosses was determined by random spore analysis (Ponticelli *et al.* 1988). Spores were plated on supplemented EMM2 with or without adenine to determine total spores or Ade^+ recombinants, respectively. Each cross was repeated from independent cultures at least four times, and more than 50 colonies were counted for each determination. The frequency of Ade^+ revertants, as determined by meiotic selfings, was $<0.1/10^6$ viable spores (data not shown; DeVeaux *et al.* 1992).

***ade6* alleles:** *ade6* alleles used in recombination studies were *ade6-M26* (G1010T), *ade6-M375* (G1007T), *ade6-469* (C2342T), *ade6-M216* (G921A) (Szankasi *et al.* 1988), and *ade6-52* (G1670A) (M. E. Fox, unpublished data). Nucleotides are numbered according to Szankasi *et al.* (1988); the *ade6* coding region extends from 875 to 2530. It should be noted that the mutation corresponding to the *ade6-52* allele differs from that previously reported (Fox *et al.* 1997). The G1670A mutation was identified by sequencing of genomic DNA isolated from three different *ade6-52* strains from our laboratory and a different *ade6-52* strain provided by J. Kohli. *ade6* alleles created in this study are described in Table 1.

Mutagenesis of the chromosomal *ade6* locus: Mutations were created in the chromosomal *ade6* locus by site-directed mutagenesis of the *ade6⁺* gene on plasmid pMF1 (1.45-kb *Bam*HI-*Xho*I fragment of *ade6⁺* subcloned into pKS^+; Stratagene, La Jolla, CA), followed by transformation of strain GP18 to adenine auxotrophy with a linear DNA fragment bearing the desired mutation, as follows. Site-directed mutagenesis of plasmid pMF1 was by polymerase chain reaction (PCR)-based mutagenesis (Fox *et al.* 1997) or by the Morph mutagenesis system (5Prime → 3Prime, Boulder, CO) and was confirmed by sequencing. Mutations were transferred to the chromosomal *ade6* locus by transformation (Ito *et al.* 1983). Strain GP18 (*h⁻ leu1-32*) was cotransformed with at least 1 μg of a 1.45-kb *Bam*HI-*Xho*I fragment of *ade6* bearing the desired mutation and 1 μg of circular plasmid pDB248′ (Beach *et al.* 1982) carrying the *Saccharomyces cerevisiae LEU2*

TABLE 1

***ade6* alleles and nucleotide sequences**

ade6 allele	Sequence (nt 1009–1018)[a]	Strain[b]
+, *M375*[c]	AGGACGTGAG/CTCACGTCCT	GP43
M26	**AT**GACGT**G**AG/CTCACGTC**A**T	GP203
3024	CTTACGTCAG/**CTGACGTA**AG	GP2749
3017	**CTGACGTC**AG/**CTGACGTC**AG	GP2716
3016	CTGACGTGAG/CTCACGTCAG	GP2714
3020	CTGACGTTAG/CTAACGTCAG	GP2722
3012	GTTACGTGAG/CTCACGTAAC	GP2555
3013	GTTACGTCAG/**CTGACGTA**AC	GP2556
3014	ATTGCGTGAG/CTCACGCAAT	GP2557
3015	ATTGCGTCAG/CTGACGCAAT	GP2558
3030	**GTGACGTA**AG/CTTACGTCAC	GP2756
3032	**GTGACGTC**AG/CTGACGTCAC	GP2816
3025	GTGACGTGAG/CTCACGTCTC	GP2751
3026	GTGACGTTAG/CTAACGTCAC	GP2752
3029	**TTGACGTA**AG/CTTACGTCAA	GP2755
3033	**TTGACGTC**AG/CTGACGTCAA	GP2756
3028	TTGACGTGAG/CTCACGTCAA	GP2754
3027	TTGACGTTAG/CTAAACTGAA	GP2753

[a] The first sequence is that of the coding strand, nucleotides (nt) 1009–1018, inclusive, as numbered by Szankasi *et al.* (1988); the second is its complement. Both are written 5′ → 3′. The nucleotides in boldface are the sequences deduced to be responsible for hotspot activity (Schuchert *et al.* 1991; see Table 5 and discussion). Underlined nucleotides differ from those in *ade6*$^+$. Alleles *3012–3033* also contain a C → T mutation at nucleotide 998 to create a UAA stop codon to ensure an Ade$^-$ phenotype.

[b] These strains are also *h*$^-$ *leu1-32*.

[c] *ade6-M375* is a G → T mutation two nucleotides to the left of the first sequence and served as the nonhotspot control for *M26*. The sequence shown is present in both *ade6*$^+$ and *ade6-M375*.

marker, which complements the *S. pombe leu1-32* mutation. Leu$^+$ transformants were selected on EMM2 medium with limiting adenine (10 μg/ml) on which Ade$^-$ cells produce red colonies (Gutz *et al.* 1974). Ade$^-$ strains were purified on media containing leucine, and Leu$^-$ segregants were identified. Chromosomal mutagenesis was confirmed by PCR amplification and sequencing and by Southern blot analysis (data not shown).

Binding assays: The DNA probes used were 215-bp dsDNA fragments containing the mutated *ade6* sequences, prepared by *Sty*I digestion of plasmid DNA and gel purification. Probes were end-labeled using [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA polymerase I (New England Biolabs, Beverly, MA).

Crude whole-cell protein extracts were prepared from exponentially growing cultures of strains GP20, GP2204, GP2206, GP2208, and GP2435 as follows. Cell pellets (~2 g wet weight) were lysed in an equal volume of 50 mm HEPES-NaOH (pH 7.9), 500 mm NaCl, 20% glycerol, 1 mm EDTA, 1 mm dithiothreitol (DTT), 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 10 μg/ml bestatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin by the addition of acid-washed glass beads and 10 rounds of vortexing for 30 sec each with cooling on ice between each round. Following centrifugation at 15,000 × *g* for 30 min at 4°, the supernatant was removed and protein concentration determined using a modified Bradford assay (Bio-Rad, Richmond, CA). Extracts were diluted with the buffer above to 10 mg/ml, stored in aliquots at −70°, and thawed on ice immediately before use.

Binding reactions were performed in a final volume of 10 μl containing 0.2 ng labeled probe DNA, 10 μg protein extract, 1 μg poly(dI-dC) (Sigma, St. Louis), 12% glycerol, 12 mm HEPES-NaOH (pH 7.9), 5 mm $MgCl_2$, 4 mm Tris-HCl (pH 7.9), 0.6 mm EDTA, and 0.6 mm DTT. Reactions were incubated at room temperature for 30 min, and the products were separated on 5% nondenaturing polyacrylamide gels at 16 V/cm in 1× TGE buffer [50 mm Tris-HCl (pH 7.9), 380 mm glycine, 2 mm EDTA]. Gels were dried onto Whatman 3MM paper and autoradiographed or analyzed using a Molecular Dynamics (Sunnyvale, CA) PhosphorImager and ImageQuant software.

***In vitro* binding site selection:** DNA sequences bound by Atf1·Pcr1 were selected using a modified Selex procedure as described (Tuerk and Gold 1990; Cui *et al.* 1995). The probe used was a 65-bp dsDNA molecule containing a central core of 11 randomized nucleotides centered over the site of the *M26* heptamer and flanked on the left by GG and 24 bp of *ade6* DNA and on the right by 20 bp of the *ade6* DNA and GGATCCGG to provide restriction sites for convenient cloning (5′ GGAATTCTCCTGCCAAACAAATTGATNNNNNNNN NNNGCACATTGATGCATCATTTAGGATCCGG). This probe was end-labeled using T4 polynucleotide kinase (New England Biolabs) and incubated with protein extract from strain GP18. The products were analyzed as above using an equivalent probe containing the *M26* heptamer sequence as a control marker for migration of bound probe. The region of the gel corresponding to the position of bound probe was excised and crushed in 100 μl of H_2O. After elution from the gel, nucleic acids were precipitated with ethanol and used as template in a PCR amplification with primers hybridizing to the ends of the 65-bp probe (5′ GGAATTCTCCTGCCAAAC and 5′ CCGGATCCTAAATGATGC). The PCR product was end-labeled and used as a probe in a subsequent round of binding and fractionation. After three rounds of binding and PCR amplification, bound probe was readily detectable as a shifted band migrating at the position of bound probe containing the *M26* heptamer. PCR products from rounds three and four were subcloned into pCRII (Invitrogen, San Diego), and the inserts were sequenced. Since binding to the desired DNA targets should depend on Atf1 protein, specificity of binding to candidate sequences was determined in binding reactions containing *Bam*HI-digested plasmid DNA and protein extract from *atf1*$^+$ and *atf1*Δ cells.

Micrococcal nuclease sensitivity of chromatin: Meiotic chromatin structure of *ade6* was analyzed as described (Mizuno *et al.* 1997). Chromatin was isolated from diploid strains GP2611 and GP2612, homozygous for *ade6-3013* and *ade6-3015*, respectively, at 0 and 3 hr after induction of sporulation. Chromatin was digested with 0, 20, and 30 units/ml of micrococcal nuclease (Amersham Pharmacia). MNase-digested DNA was cleaved with *Xho*I and the resulting restriction fragments were analyzed by Southern hybridization using a probe for the 3′ terminus of the *ade6* coding region (a 238-bp *Xho*I-*Eco*RI fragment). Digestion of naked genomic DNA was as described (Mizuno *et al.* 1997).

RESULTS

***In vitro* binding of Atf1·Pcr1 to a probe containing CTGACGTAAC:** To test the hypothesis that multiple sequences can bind Atf1·Pcr1, we constructed plasmids carrying mutated sequences at the site of the *M26* heptamer in the *ade6* gene. Initial experiments involved the

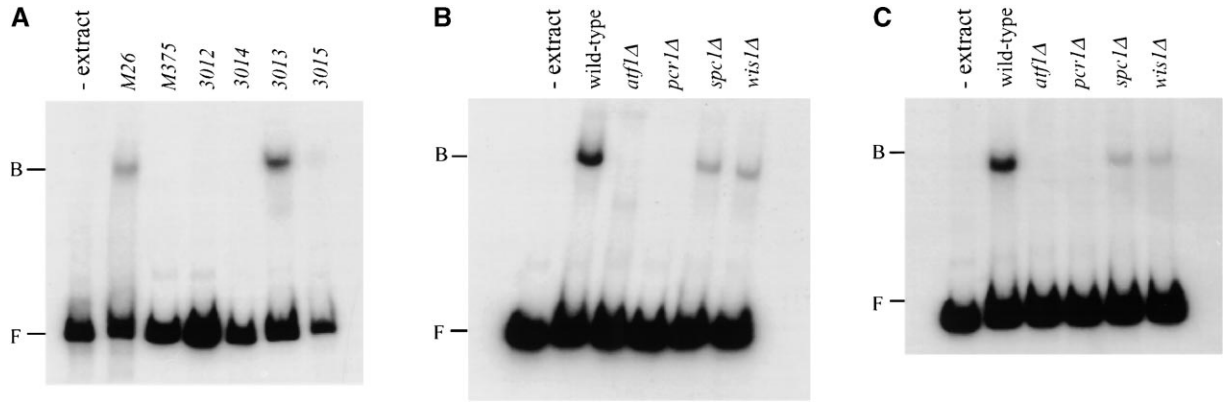

FIGURE 1.—*In vitro* binding to DNA probes containing CRE-like sequences. – extract, control reactions containing *M26* probe and lacking protein extract; F, free probe; B, bound probe. (A) Binding reactions containing wild-type extract and probes as indicated. The CRE-like sequences in these probes were *ade6-M26* (ATGACGTGAG), *ade6-M375* (AGGACGTGAG), *ade6-3012* (CTCACGTAAC), *ade6-3014* (CTCACGTAAT), *ade6-3013* (CTGACGTAAC), and *ade6-3015* (CTGACGCAAT). (B) Binding of extracts from wild-type and mutant strains to probe containing *ade6-3013* (CTGACGTAAC). (C) Binding of extracts from wild-type and mutant strains to probe containing *ade6-M26* (ATGACGTGAG).

candidate sequence CTGACGTAAC (JONES and JONES 1989; KANOH *et al.* 1996). Gel-shift assays using wild-type *S. pombe* extract showed binding to 215-bp dsDNA fragments containing CTGACGTAAC (*ade6-3013*), but not to probes containing the closely related sequences CTCACGTAAC (*ade6-3012*), CTCACGCAAT (*ade6-3014*), or CTGACGCAAT (*ade6-3015*) (Figure 1A; these sequences differ from *ade6-3013* at the underlined positions). Binding to the probe containing CTGACGTAAC (*ade6-3013*) was abolished in the absence of Atf1 or Pcr1 and was reduced in the absence of Spc1 or Wis1 (Figure 1B). Similar requirements for binding were observed with a probe containing the *M26* heptamer (Figure 1C). These probes were therefore bound by a factor whose synthesis or activity is under the control of *atf1*, *pcr1*, *spc1*, and *wis1*. The similar electrophoretic mobilities of the numerous complexes reported here (Figure 1 and additional data not shown) suggest that this factor is Atf1·Pcr1 itself, as shown for *M26* with purified Atf1·Pcr1 protein (WAHLS and SMITH 1994; KON *et al.* 1997). These binding results suggested that CTGACG TAAC (*ade6-3013*) might be a recombination hotspot similar to *M26*.

CTGACGTAAC (*ade6-3013*) is a meiotic recombination hotspot: To test this hypothesis, the sequence CTGACGTAAC (*ade6-3013*) and the control sequence CTGACGCAAT (*ade6-3015*) were introduced into the *ade6* gene on the chromosome and assayed for recombination hotspot activity. The *ade6-3015* control was selected because it involves the same number and type of base-pair changes as *ade6-3013* and thus reduces potential complications due to differential mismatch correction. Meiotic crosses were performed between strains carrying these alleles and strains carrying either the *ade6-52* or the *ade6-M216* allele, which map to either side of *M26*. For comparison, crosses were also performed with strains carrying the *ade6-M26* hotspot mutation or its nonhotspot control *ade6-M375*. In crosses with *ade6-52* the presence of the CTGACGTAAC sequence (*ade6-3013)* increased the Ade^+ recombinant frequency about 13-fold relative to the control CTGACGCAAT (*ade6-3015*); the hotspot activity of *3013* was indistinguishable from the 13-fold enhancement of *M26* relative to *M375* (Figure 2A; Table 2). In crosses with *ade6-M216*, the *3013* mutation increased the recombinant frequency 6-fold relative to its control (Figure 2B); this also is comparable to the 5-fold hotspot activity of *M26* relative to *M375* in crosses with *ade6-M216* (Figure 2B). *3013* is thus active as a hotspot in crosses with markers to either side of itself, as previously shown for *M26* (GUTZ 1971). As further controls, strains with the sequences CTCACGTAAC (*ade6-3012*) and CTCACG CAAT (*ade6-3014*), which did not bind Atf1·Pcr1 (Figure 1A), were constructed and tested for hotspot activity in crosses with *ade6-52* and *ade6-M216*. For both sequences recombinant frequencies were comparable to those obtained with *M375* (Figure 2). Thus, *in vitro* binding of protein correlated with hotspot activity *in vivo*. These results established that *ade6-3013* creates a meiotic recombination hotspot with properties similar to those of *M26* (see additional results below).

Other CRE-related sequences that bind Atf1·Pcr1 and act as recombination hotspots: The sequence previously shown to bind Gad7 (Atf1), CTGACGTCAG (*ade6-3017*) (KANOH *et al.* 1996), was similarly tested and found to bind Atf1·Pcr1 *in vitro* and to have recombination hotspot activity *in vivo* (Table 3; Figure 2). This result suggested that multiple related sequences might bind Atf1·Pcr1 and have hotspot activity.

In parallel studies, a search for DNA sequences that are bound by Atf1·Pcr1 was performed using a modification of the Selex approach (TUERK and GOLD 1990; CUI *et al.* 1995). This search was not carried to exhaustion but did yield several sequences that demonstrated

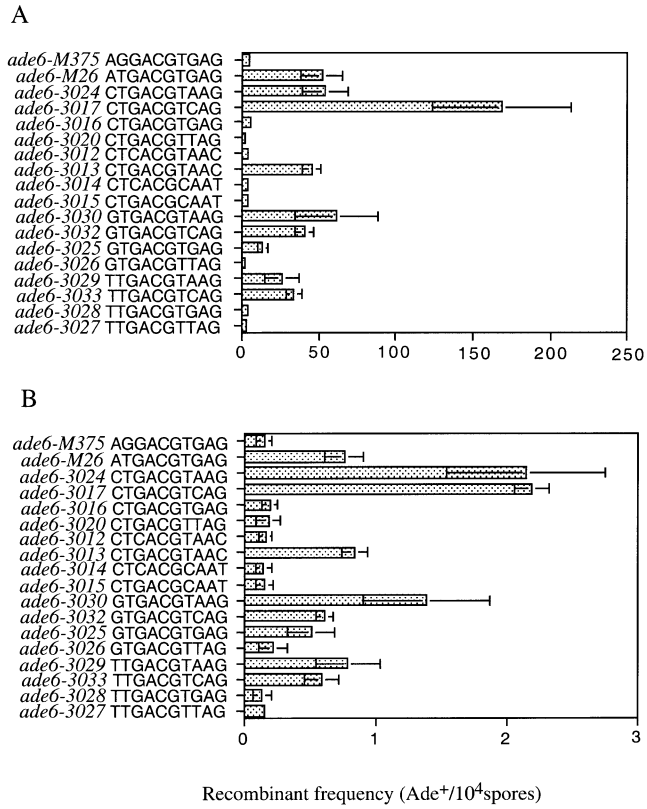

FIGURE 2.—Hotspot activity of CRE-like sequences. Strains carrying the sequences indicated were crossed with strains carrying either *ade6-52* (A) or *ade6-M216* (B). Recombinant frequencies are arithmetic means of 4–8 independent crosses, with standard error of the mean indicated by bars. Subsequent crosses with the *ade6-3017* strains, recovered from frozen stocks, produced recombinant frequencies only slightly greater than those with *ade6-M26* strains (data not shown); the basis of this difference is unclear.

Atf1-dependent binding (data not shown). These included the *M26* heptamer and the sequence GTGACG TAAG. The similarity of this sequence to those shown above to have hotspot activity suggested that other sequences related to the CRE consensus also act as hotspots. To test this suggestion by more rigorous genetic means, a series of mutant sequences, (C/T/G) TGACGTN, was generated and introduced into the *ade6* gene; the four sequences ATGACGTN had previously been tested during identification of the *M26* heptamer and found to be active as meiotic recombination hotspots (SCHUCHERT *et al.* 1991). This series of sequences and their corresponding *ade6* allele designations are shown in Table 1.

In vitro binding assays were performed using probes carrying these mutated sequences with extract from wild-type cells or from *atf1*Δ or *pcr1*Δ mutant cells (Table 3). The following sequences bound significantly more protein than the others: ATGACGTGAG, CTGACG TAAG, CTGACGTCAG, GTGACGTAAG, GTGACGT CAG, TTGACGTAAG, and TTGACGTCAG (only the underlined nucleotides differ among these sequences). In all cases significant binding was dependent on the presence of Atf1 and Pcr1 (Table 3 and additional data not shown).

To test whether these Atf1·Pcr1-binding sequences have recombination hotspot activity, strains bearing the sequences in Table 1 on their chromosomes were crossed with strains bearing either the *ade6-52* allele or the *ade6-M216* allele, and recombinant frequencies were determined (Figure 2). All sequences that displayed *in vitro* binding above background were also meiotic recombination hotspots, while those that had no significant binding displayed recombinant frequencies comparable to those of *M375*. Hotspot activity thus correlated with *in vitro* binding, although the variability in the binding assay precludes a quantitative comparison.

These results indicate that the (C/G/T)TGACGT heptamers are recombination hotspots, but only if followed by either A or C. This contrasts with *M26* for which changing the position following ATGACGT from G to A, C, or T has no significant effect on hotspot activity (SCHUCHERT *et al.* 1991). Limited studies suggest that hotspot activity is independent of the next two nucleotides as well: CTGACGTAAC (*ade6-3013*),

TABLE 2

The *M26* and *3013* hotspots require the Atf1·Pcr1 transcription factor and the Spc1 and Wis1 protein kinases

	Ade+ recombinant frequency[b]			
Strain[a]	*ade6-M26*	*ade6-M375*	*ade6-3013*	*ade6-3015*
+	48.0 ± 2.6 (8)	3.8 ± 0.39 (8)	43.0 ± 1.9 (6)	3.4 ± 0.81 (4)
*atf1*Δ	1.6 ± 0.29 (7)	2.6 ± 0.16 (8)	2.6 ± 0.24 (4)	2.4 ± 0.39 (4)
*pcr1*Δ	2.4 ± 0.28 (4)	3.8 ± 0.51 (4)	2.7 ± 0.41 (4)	3.0 ± 0.48 (4)
*spc1*Δ	3.7 ± 0.34 (8)	3.1 ± 0.60 (11)	3.3 ± 0.39 (4)	2.7 ± 0.30 (4)
*wis1*Δ	4.3 ± 0.33 (11)	2.8 ± 0.56 (7)	2.4 ± 0.54 (4)	3.1 ± 0.45 (4)

[a] Both parents in each cross carried the indicated mutation.
[b] Number of Ade+ recombinants per 10^4 viable spores from crosses between the indicated *ade6* allele and *ade6-52*. Data are the means ± the standard error of the mean from the number of crosses in parentheses.

TABLE 3

***In vitro* binding of CRE-containing DNA probes by protein extracts**

ade6 allele	Probe DNA sequence[a]	Hotspot[b] activity	% probe bound[c]		
			wt	*atf1*Δ	*pcr1*Δ
+	AGGACGTGAG	−	0.5, 0.9	0.06	1.2
M26	**ATGACGT**GAG	+	12.2, 3.9	1.2	1.6
3024	**CTGACGTA**AG	+	8.4, 15.0	0.7	2.3
3017	**CTGACGTC**AG	+	5.8, 4.5	0.09	0.8
3016	CTGACGTGAG	−	0.4, 1.2	1.0	1.7
3020	CTGACGTTAG	−	1.8, 1.0	0.6	1.3
3030	**GTGACGTA**AG	+	7.7, 7.8	0.4	0.7
3032	**GTGACGTC**AG	+	8.4, 12.3	1.7	1.4
3025	GTGACGTGAG	±	0.8, 0.8	0.05	2.0
3026	GTGACGTTAG	−	0.6, 0.1	0.9	1.4
3029	**TTGACGTA**AG	+	4.8, 4.6	0.2	1.3
3033	**TTGACGTC**AG	+	5.8, 10.3	0.6	0.5
3028	TTGACGTGAG	−	0.6, 0.04	0.2	1.6
3027	TTGACGTTAG	−	0.1, 0.3	0.08	0.9

[a] DNA probes were the 215-bp *Sty*I fragments extending from bp 902 to bp 1116 of *ade6* (Szankasi *et al.* 1988) containing the indicated *ade6* alleles and sequences. Boldface indicates sequences deduced to have hotspot activity. Data for *ade6* alleles *3012–3015* are shown in Figure 1A.

[b] Hotspot activity relative to that with *M26*: +, >65%; ±, 40%; −, <10%. Hotspot activity was calculated from the data in Figure 2 after subtracting the recombinant frequency with *ade6-M375* and averaging the hotspot values from crosses with *ade6-M216* and *ade6-52* (Figure 2; Schuchert *et al.* 1991).

[c] The fraction (%) of the probe bound by extracts of cells with the indicated genotypes. Results of two experiments are shown for wild-type (wt) extracts. The wt, *atf1*Δ, and *pcr1*Δ strains are GP20, GP2208, and GP2435, respectively.

CTGACGTAAT (*ade6-3019*), and CTGACGTAGG (*ade6-3018*) all demonstrated Atf1-dependent protein binding and had comparable hotspot activity in crosses with both *ade6-52* and *ade6-M216* (Figures 1 and 2 and data not shown).

The sequence GTGACGTGAG (*ade6-3025*) had an intermediate recombinant frequency in crosses with both *ade6-52* and *ade6-M216*; recombinant frequencies were increased approximately threefold relative to equivalent crosses involving *ade6-M375* but decreased relative to *M26* (Figure 2). The original mutational analysis of the *M26* heptamer also found recombinant frequencies for this sequence reduced approximately threefold relative to *M26* but above that with *ade6-M375* (Schuchert *et al.* 1991); this was a significant reduction, although not as great as that observed for other single base changes at the first position of the *M26* heptamer (ATGACGT). The *ade6-3025* sequence did not bind Atf1·Pcr1 significantly above background levels (Table 3), but a low level might not have been detected in this assay.

In summary, *M26* is not the only sequence that displays meiotic hotspot activity. Rather, there is a family of related sequences, ATGACGT and (C/G/T)TGA CGT(C/A), that bind Atf1·Pcr1 and function as meiotic recombination hotspots.

The CTGACGTAAC sequence (*ade6-3013*) forms a meiosis-specific hotspot with properties similar to those of the *M26* hotspot: The sequences shown above to have hotspot activity are closely related to the *M26* heptamer sequence and, like *M26*, bind Atf1·Pcr1, suggesting that the mechanism of the new hotspots may be similar to that of *M26*. The following experiments were performed to determine whether one of the novel hotspots, CTGACGTAAC (*ade6-3013*), behaves in a manner similar to *M26*. The control used was CTGACGCAAT (*ade6-3015*), a sequence with the same number and type of nucleotide changes but with a recombinant frequency similar to that of *M375* (Figure 2; Table 2).

In vitro binding and hotspot activity require Atf1 and Pcr1: To test the genetic requirements for binding and hotspot activity, null alleles of *atf1*, *pcr1*, *wis1*, or *spc1* were introduced into strains carrying either *ade6-M26*, *ade6-M375*, *ade6-3013*, or *ade6-3015*. As shown previously, hotspot activity of *M26* requires Atf1 and Pcr1 (Table 2; Kon *et al.* 1997). Furthermore, hotspot activity of *M26* appears to require activation of the protein kinase cascade involving Wis1 and Spc1, since *M26* was inactive in mutants lacking Wis1 (Table 2) or Spc1 (Table 2; Kon *et al.* 1998). The *3013* hotspot behaved like the *M26* hotspot. As noted previously, the *3013* sequence increased recombinant frequency 13-fold relative to its control (*3015*), a hotspot activity comparable to that of *M26* relative to *M375*, and the *3013* hotspot activity was also dependent on Atf1, Pcr1, Wis1, and Spc1 (Table 2). *In vitro* binding to probe containing *3013* or *M26* was abolished in the absence of Atf1 and Pcr1 and re-

TABLE 4

Parallel inactivity of the *M26* and *3013* hotspots

Chromosomal allele[a]	Recombinant frequency[b]
A. Mitotic chromosome × plasmid recombination: *ade6-469* on plasmid	
ade6-M26	1.6 (1.2–2.2)
ade6-M375	3.2 (1.5–4.4)
ade6-3013	2.0 (1.6–3.5)
ade6-3015	1.9 (1.4–3.3)
Plasmid allele[a]	**Recombinant frequency[c]**
B. Meiotic chromosome × plasmid recombination: *ade6-469* on chromosome	
ade6-M26, M375[d]	4.2 ± 0.77
ade6-M375	4.5 ± 0.90
ade6-3013	4.0 ± 0.24
ade6-3015	3.3 ± 0.88

[a] Nucleotide sequences of these alleles are in Table 1.

[b] Data are the median number of Ade$^+$ recombinants per 10^4 viable plasmid-containing cells in five independent cultures (range in parentheses). Each culture contained 1.4–2.6 × 10^7 cells.

[c] Number of Ade$^+$ recombinants per 10^4 viable spores. Data are the mean ± the standard error of the mean of four crosses.

[d] Since high-copy-number plasmids with *ade6-M26* complement *ade6-469*, the *ade6-M26, M375* hotspot allele was used (Ponticelli and Smith 1992).

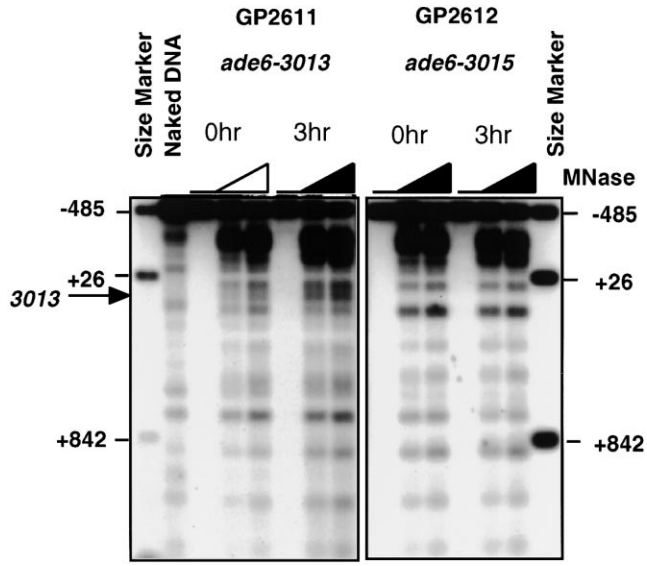

Figure 3.—Chromatin structural change at the *ade6-3013* hotspot. Before (0 hr) and after (3 hr) meiotic induction, chromatin was isolated from strain GP2611 (*ade6-3013*) and from the negative control strain GP2612 (*ade6-3015*). Samples were analyzed by partial digestion of chromatin with MNase (0, 20, and 30 units/ml) followed by *Xho*I digestion and indirect end-labeling of Southern blots as described (Mizuno *et al.* 1997). One microgram of genomic DNA was analyzed in each lane. An arrow indicates the position of the *3013* mutation. The nucleotide positions of restriction sites for the size markers are indicated relative to the first A of the *ade6* coding sequence (Szankasi *et al.* 1988).

duced, but not abolished, in the absence of Wis1 or Spc1 (Figure 1, B and C). Thus, the genetic and biochemical properties of the *3013* hotspot resemble those of *M26*.

The 3013 sequence is inactive in mitosis and when on a plasmid: Activity of the *M26* hotspot is meiosis specific: no enhancement of recombinant frequency relative to that with *M375* is seen during mitotic growth (Ponticelli *et al.* 1988; Schuchert and Kohli 1988). *M26* is also inactive in plasmid-by-chromosome crosses when on the plasmid but active when on the chromosome (Ponticelli and Smith 1992). The *3013* hotspot was tested to determine whether its activity is meiosis- and context-dependent in a similar manner. The control used was *ade6-3015*, which was inactive for both *in vitro* binding and *in vivo* hotspot activity (Figure 1A and Table 2). Strains bearing this hotspot or the control sequence were transformed with a plasmid carrying the *ade6-469* allele [a C → T mutation located 1.33 kb from *3013* (Szankasi *et al.* 1988)] and grown mitotically to allow plasmid-by-chromosome recombination to generate Ade$^+$ products. The presence of the *3013* hotspot had no significant effect on mitotic recombinant frequency (Table 4A). The *3013* hotspot was active, however, when meiosis was induced in these transformed cultures (data not shown). Hotspot activity of the *3013* sequence, like that of *M26*, is thus meiosis specific.

To test for meiotic activity of *3013* when on a plasmid, we constructed homothallic strains bearing *ade6-469* on the chromosome and the hotspot alleles or their controls on a 3-kb fragment of *ade6* in the plasmid pade6 (Szankasi *et al.* 1988). The frequency of meiotic Ade$^+$ recombinants was nearly the same for all four alleles (Table 4B), demonstrating that neither the *M26* nor the *3013* hotspot was active when on a plasmid. These results suggest that hotspot activity of the *3013* hotspot, like that of *M26*, is dependent on its genomic location and may require a specific chromatin structure.

Chromatin structure is altered at the 3013 sequence: To determine whether chromatin structure of the *ade6* gene is altered in strains carrying the *ade6-3013* hotspot, chromatin isolated from diploids homozygous for *ade6-3013* or the control *ade6-3015* was digested with MNase and analyzed by Southern blotting (Figure 3). MNase sensitivity of the *ade6* region in the *3015* control had a pattern similar to that observed previously for both wild-type and *ade6-M375* (Mizuno *et al.* 1997). In contrast, the pattern of MNase-sensitive sites around the *3013* sequence (*ca.* +50 to +800) was different and was more similar to that observed in the naked DNA digestion (Figure 3; additional data not shown). In addition, a new hypersensitive site was observed in *3013* at the site of the new sequence, which was absent in *3015* chromatin. This hypersensitive site was detectable at low levels in premeiotic cultures and increased in intensity following induction of meiosis. Such modified patterns in the MNase sensitivity are very similar to those at the *M26*

TABLE 5

Nucleotide sequences of the *M26* and CRE hotspots

	Nucleotide position							
Element[a]	1	2	3	4	5	6	7	8
M26 hotspot	A	T	G	A	C	G	T	N[b]
CRE hotspots	C, G, T	T	G	A	C	G	T	C, A[c]
CRE consensus	N	T	G	A	C	G	T	C, A

[a] The nucleotide sequence of the *M26* hotspot is from Schuchert *et al.* (1991); those of the CRE hotspots are from this study (see Figure 2 and Table 3); that of the CRE consensus for transcriptional activation is from Montminy *et al.* (1986).

[b] N, any nucleotide.

[c] G at position 8 supports partial activity, provided 1 is G.

site (Mizuno *et al.* 1997). Thus, both the *M26* and *3013* hotspots are associated with specific remodeling of chromatin in the region of *ade6* as cells enter meiosis.

DISCUSSION

We show here that *M26*, previously considered a unique sequence (Schuchert *et al.* 1991; Lichten and Goldman 1995; Fox and Smith 1998), is not the only meiotic recombination hotspot in *S. pombe* and describe a family of sequences related to the *M26* heptamer that act as hotspots in *S. pombe*. Since an exhaustive search for sequences that bind the hotspot-activating transcription factor Atf1·Pcr1 was not performed, there may well be additional sequences that function as hotspots. In the currently sequenced 11.3 Mb of *S. pombe* DNA there are 286 copies of the *M26* hotspot sequence and 587 copies of the new hotspot sequences (Cre hotspots, Table 5). If these naturally occurring sequences act similarly to enhance recombination, our findings suggest that recombination hotspots contribute more to meiotic recombination than previously calculated (Kon *et al.* 1997).

The recombination hotspot sequences described here demonstrate an unusual dependence upon surrounding nucleotides. The *M26* heptamer, ATGACGT, is active irrespective of the nucleotide following the heptamer. In contrast, the novel hotspot sequences described here display a strong dependence on the nucleotide following the heptamer: CTGACGT, TTGACGT, and GTGACGT are all active if followed by a C or A, but inactive if followed by a G or T (Figure 2; Table 5). Nucleotides within a consensus are typically considered to be independent: the effects of changing one base to another are generally independent of changes at another position, and interdependence of nucleotides, such as that between nucleotides at positions 1 and 8 in Table 5, is unusual. The mammalian CRE binding protein CREB1 shows a similar interdependence of nucleotides within the CRE consensus sequence for binding and transcriptional activation (Benbrook and Jones 1994). In this case, sequences in which the G at position 6 (Table 5) is mutated to either C or A are active only if the nucleotide at position 3 is a G and that at position 8 is a C. These observations underscore the necessity of testing multiply mutated sequences to determine the nucleotide sequence(s) necessary for activity.

Activity of the *M26* hotspot is associated with binding of the transcription factor Atf1·Pcr1 (Kon *et al.* 1997). It appears that binding of Atf1·Pcr1 is also required for activity of the novel hotspots identified here. All sequences associated with hotspot function bound a factor whose synthesis or activity is controlled by *atf1* and *pcr1* (Figure 1; Table 3); we infer that this factor is Atf1·Pcr1 itself (see results). Binding of this factor was not significantly above background with sequences that did not enhance recombination. The correlation of binding and hotspot activity suggests that Atf1·Pcr1 directly activates the *M26* hotspot (Wahls and Smith 1994) as well as the family of hotspots studied here.

The Atf1 transcription factor is activated through a stress-induced protein kinase cascade in which Wis1 phosphorylates Spc1, which in turn phosphorylates Atf1 (Shiozaki and Russell 1995, 1996; Degols *et al.* 1996; Stettler *et al.* 1996; Wilkinson *et al.* 1996; Shieh *et al.* 1997). Spc1 and Wis1 are required for transcriptional activity of Atf1, although it is not clear whether phosphorylation itself is required. Recombination hotspot activity of *M26* also requires Spc1 and Wis1 (Kon *et al.* 1998; Table 2), indicating that this protein kinase cascade is also required for the recombination functions of Atf1·Pcr1 bound to the *M26* heptamer. For the one other sequence tested further, *3013*, hotspot activity similarly required Atf1, Pcr1, Spc1, and Wis1 (Table 2). Interestingly, for both *M26* and *3013 in vitro* binding was undetectable in the absence of Atf1 or Pcr1 but was detectable at reduced levels in the absence of Wis1 or Spc1 (Figure 1). The reduced levels of binding in the absence of these protein kinases might reflect reduced transcription of *atf1* in these mutants (Shiozaki and Russell 1996; Kon *et al.* 1998), although Atf1·Pcr1 protein levels are reported to be unaltered in these mutants

(Degols *et al.* 1996; Kon *et al.* 1998). Alternatively, the reduced levels of binding might reflect a weaker affinity of unphosphorylated Atf1 protein relative to phosphorylated, although treatment of purified Atf1·Pcr1 protein with phosphatase is reported not to affect binding (Kon *et al.* 1998). Further studies are required to elucidate the role of the Spc1 and Wis1 protein kinases in meiotic recombination hotspot activity.

The hotspot sequences described here are very similar to the CRE consensus sequence TGACGT(C/A) that is required for transcriptional activation of cAMP-responsive genes (Montminy *et al.* 1986). However, the relationship between recombination and transcription is complex. It has been suggested that hotspot activity of *M26* requires transcription of the *ade6* gene. Deletion of ~500 bp containing the putative *ade6* promoter abolishes *M26* activity (Zahn-Zabal *et al.* 1995), and replacement of the weak *ade6* promoter with the strong *adh1* promoter increases recombination at *ade6* (Grimm *et al.* 1991). However, both of these results could reflect the gross structural changes made to the *ade6* region, rather than effects on transcription of *ade6*. Evidence against a requirement for transcription of *ade6* is the observation that *ade6* transcript levels are equivalent in *M26* and *M375* strains and are not affected by mutations in *atf1* or *pcr1* (Kon *et al.* 1997). In addition, while the Atf1 transcription factor and the Wis1 and Spc1 kinases are required for both transcriptional activation and for meiotic hotspot activity, the stress response and hotspot activity are separable: *pcr1* is required for *M26* hotspot activity but not for the stress response (Kon *et al.* 1997, 1998). It is possible that Atf1 has other, as yet unidentified, binding partners that are required for transcriptional activation in response to stress.

In addition to the genetic requirements for *atf1*, *pcr1*, *wis1*, and *spc1* discussed above, the *3013* hotspot shares other properties with *M26*: both hotspots are inactive during mitosis and when located on a plasmid. It has been proposed that hotspot activity of *M26* requires a particular chromatin structure that is absent on a plasmid and during mitosis (Ponticelli and Smith 1992; Virgin *et al.* 1995; Fox *et al.* 1997). Indeed, the entry of cells into meiosis is associated with an increase in the intensity of a micrococcal nuclease hypersensitive site at *M26* and in the 5′ noncoding region of the *ade6-M26* locus, indicating that chromatin structure becomes more open at a time when the hotspot is active (Mizuno *et al.* 1997). A similar micrococcal nuclease hypersensitive site was observed at the site of the *3013* hotspot; as with *M26*, the chromatin structure at this sequence became more "open" as cells entered meiosis (Figure 3). It is possible that local opening of the chromatin at hotspot sequences increases accessibility for binding of proteins required for recombination, including Atf1·Pcr1. Alternatively, Atf1·Pcr1 constitutively bound to hotspot sequences may promote local chromatin remodeling in response to meiotic induction. Meiosis-specific recombination factors may be recruited to such open chromatin regions. Although it is not clear how sequences such as *M26* and *3013* act to increase recombinant frequency, it is likely that they act in a similar manner, since both require the Atf1·Pcr1 transcription factor and the Wis1 and Spc1 kinases, and they induce similar specific changes to local chromatin structure.

The elucidation of a new family of meiotic recombination hotspots in *S. pombe* will facilitate further studies on the mechanism of meiotic recombination hotspots and the role they play in determining the frequency and distribution of recombination during meiosis.

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