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Optimizing the Nucleotide Sequence of a Meiotic Recombination Hotspot in *Schizosaccharomyces pombe*

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ABSTRACT

The *ade6-M26* mutation of *Schizosaccharomyces pombe* created a meiotic recombination hotspot. Previous analyses indicated that the heptamer 5′-ATGACGT-3′ was necessary and sufficient for hotspot activity; the Atf1-Pcr1 transcription factor binds to this sequence and activates *M26*. After finding cases in which the *M26* heptamer in *ade6* was, surprisingly, not active as a hotspot, we used an *in vitro* selection method (SELEX) that revealed an 18-bp consensus sequence for Atf1-Pcr1 binding, 5′-GNVTATGACGTCATNBNC-3′, containing the *M26* heptamer at its core. Using this consensus sequence as a guide, we made mutations on each side of the heptamer at two separate sites in *ade6*. These mutations increased the intracellular hotspot activity of the heptamer, in some cases by >15-fold. These results show that *M26*, the eukaryotic recombination hotspot with the most precisely defined nucleotide sequence, is larger than previously thought, and they provide valuable information for clarifying the role of *M26*, and perhaps other hotspots, in meiotic recombination.

MEIOSIS is a special form of cell division in sexually reproducing organisms in which one round of DNA replication is followed by two successive cell divisions, resulting in four haploid products (gametes or spores). The first division of meiosis is termed a reductional division, because homologous chromosomes (*i.e.*, maternal and paternal homologs) are segregated to opposite poles, halving the number of chromosomes in each daughter cell. Prior to the first division, homologous chromosomes recombine with each other at a greatly elevated frequency compared to that during mitosis (Esposito and Klapholtz 1981). This recombination frequently results in crossovers, or chiasmata, which are important in most organisms for the proper segregation of homologs at the first meiotic division (Baker *et al.* 1976). Crossovers also result in the shuffling of genes between homologs and are thereby an important mechanism of increasing genetic diversity within a species.

Meiotic recombination events are not distributed evenly throughout the genome of most organisms. Rather, there are hotspots of recombination, "short segment[s] of chromosome with a conspicuously higher than average rate of recombination" (Stahl 2002, p. 976). In the two distantly related yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, hotspots of recombination coincide with sites of programmed double-strand DNA breaks (DSBs) (Sun *et al.* 1989; Fan *et al.* 1995; Petes 2001; Steiner *et al.* 2002). The broken ends of the DNA initiate recombination by invading intact homologous DNA to form joint molecules, which can then be resolved to produce gene conversions (nonreciprocal exchanges) and crossovers (reciprocal exchanges) (for a review see Pâques and Haber 1999).

The nucleotide sequences determining meiotic recombination hotspots are incompletely understood, but two classes of hotspots have been recognized (Petes 2001). α-Hotspots require the binding of transcription factors, whereas β-hotspots do not require the binding of known transcription factors. Most or all β-hotspots are generated by insertions of foreign DNA (Kirkpatrick *et al.* 1999a,b), which are likely to disrupt the native chromatin structure. Gerton *et al.* (2000) showed that meiotic DNA break sites in *S. cerevisiae* are associated with regions of elevated G + C content—at least 3% above the genomic average when viewed in moving 5-kb windows. Viewing smaller 1-kb windows, Blumental-Perry *et al.* (2000) reported a correlation of DNA break sites with a 50- to 250-bp degenerate motif [termed the *co*mmon *h*omology *r*egion (CoHR)] that is low in G + C content and contains a central poly(A) tract. Current evidence suggests, however, that the CoHR motif does not accurately predict the location or occurrence of DSBs (Haring *et al.* 2004).

The *M26* hotspot of *S. pombe* is the meiotic recombination hotspot whose sequence requirements have been most precisely defined at the nucleotide level. This hotspot was first identified as an allele of the *ade6* gene that produced an unusually high frequency of adenine prototrophs in meiotic crosses with other *ade6* alleles

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(Gutz 1971). This hotspot results from a single G → T nonsense mutation near the 5′-end of *ade6* (Szankasi *et al.* 1988 and Figure 1). A systematic mutational analysis of nucleotides neighboring this mutation indicated a 7-bp sequence, 5′-ATGACGT-3′ (*M26* mutation underlined), to be necessary for hotspot activity (Schuchert *et al.* 1991). The *ade6-M26* mutation creates a binding site for a heterodimeric transcription factor, Atf1-Pcr1, that is also essential for its hotspot activity; in *atf1*Δ or *pcr1*Δ mutants the *M26* hotspot is inactive (Wahls and Smith 1994; Kon *et al.* 1997).

To test if the *M26* heptamer sequence is sufficient for activity, site-directed mutagenesis of 1–4 bp was used to create the heptamer at five locations within the *ade6* and *ura4* genes (Fox *et al.* 1997). A hotspot was created in each case, suggesting that the *M26* heptamer sequence was sufficient for hotspot activity. Importantly, this study also showed that hotspot activity was independent of the orientation of the *M26* heptamer, since sequence inversions at two of the sites were also hotspots.

Although Schuchert *et al.* (1991) found that any single-base-pair change within the *M26* heptamer abolished activity of the hotspot, Fox *et al.* (2000) showed that a partially overlapping sequence, 5′-TGACGT$^{A}/_{C}$-3′, termed *c*yclic AMP *r*esponse *e*lement (*CRE*), at the same position in *ade6* (Figure 1, site 1) is also an Atf1-Pcr1-dependent hotspot. Thus, the first A of the *M26* heptamer is not essential provided that the heptamer is followed by an A or C (normally a G in the wild-type sequence at site 1). These data showed that the Atf1-Pcr1 recognition sequence is more flexible than previously thought and that base pairs outside the *M26* heptamer can influence hotspot activity. Here, we identify an 18-bp consensus sequence for binding of the Atf1-Pcr1 transcription factor to purified DNA. This sequence contains the *M26* heptamer at its core plus other significantly overrepresented bases to either side of the heptamer. Using the consensus sequence as a guide, we made mutations that showed that some of these additional bases are necessary for intracellular hotspot activity at a site within the *ade6* gene (Figure 1, site 2), where the *M26* heptamer alone is essentially inactive. The more complete definition of the *M26* hotspot nucleotide sequence reported here allows a more accurate assessment of its role in meiotic recombination.

[For clarity, the terms *M26* and *CRE* refer here to the heptamer sequences 5′-ATGACGT-3′ and 5′-TGAC GT$^{C}/_{A}$-3′, respectively, while *ade6-M26* refers only to the *ade6* allele in which the *M26* heptamer was originally identified (Gutz 1971).]

MATERIALS AND METHODS

S. *pombe* strains, growth media, and meiotic crosses: The *ade6* alleles used in this study are listed in Table 1; the strain numbers and genotypes are available on request. Solid and liquid growth media were made as described by Gutz *et al.* (1974). Yeast extract agar (YEA) was supplemented with 100 μg/ml each of adenine, uracil, leucine, and lysine and 50 μg/ml of histidine (YEA + 5S). Yeast extract liquid medium (YEL) was supplemented with 100 μg/ml of adenine and uracil (YEL + 2S). For selection of Ade^{+} recombinants, guanine (80 μg/ml) was substituted for adenine in YEA + 5S (YEA + 4SG) (Grimm *et al.* 1991).

For crosses, single colonies were picked to YEL + 2S and grown for 2 days at 30° to saturation. Cells from 0.5 ml of culture of each parent strain were mixed in microfuge tubes, washed twice in 1 ml of H_2O, resuspended in 0.1 ml of H_2O, spread on synthetic sporulation agar (Gutz *et al.* 1974) supplemented with 100 μg/ml each of adenine and leucine and 50 μg/ml of uracil, and incubated for 2 days at 25°. The resulting spores were harvested and treated with glusulase to kill re-

TABLE 1

***ade6* alleles**

ade6 allele	Mutation(s) in *ade6*[a]
M26	G1010T
M216	G921A
M375	G1007T
469	C2342T
3043	C2011T G2024T
3044	G2024T
3047	T2004A G2024T
3049	C2088A
3059	Δ1005-1020::*kanMX6-ura4*$^{+}$[b]
3060	Δ2002-2017::*kanMX6-ura4*$^{+}$[b]
3070	C2000G T2002C T2004A A2013T T2017C G2024T
3071	A1005G G1010T
3072	G1007T G1010T
3073	G1008T G1010T
3074	G1010T G1016C
3075	G1010T A1017G
3076	G1010T G1018T
3077	G1010T A1020G
3078	G1010T A1022C
3079	G1010T G1016C G1018T
3080	G1010T G1016C G1018T A1022C
3081	A1005G G1010T G1016C G1018T A1022C
3082	A1005G G1008T G1010T G1016C G1018T A1022C
3083	A1005G G1008T G1010T G1016C G1018T A1020G A1022C
3084	T2004A A2013T G2024T
3086	A2013T G2024T
3087	C2003T T2004A G2024T
3088	T2002C T2004A G2024T
3089	C2000A T2004A G2024T
3090	T2002C C2003T T2004A G2024T
3091	C2000A T2002C C2003T T2004A G2024T
3093	C2000A T2002C C2003T A2013T A2014G G2015A T2017G G2024T
3094	A2013T A2014G G2015A T2017G G2024T

[a] Numbering is as described by Szankasi *et al.* (1988; GenBank accession no. X14488).

[b] The indicated nucleotides of *ade6* are substituted with a 3.2-kb DNA fragment containing *kanMX6-ura4*$^{+}$ from pura4-Sph-kanMX6 (see materials and methods).

maining vegetative cells as previously described (Ponticelli and Smith 1989) with the following exception: after glusulase treatment, 0.45 ml of 100% ethanol was added to the 1 ml of spore suspension, mixed, and incubated for 20 min at room temperature. Spores were washed twice and resuspended in 1 ml of H_2O. Total and *ade6*$^+$ spore yields were determined by plating appropriate dilutions on YEA + 5S and YEA + 4SG, respectively. For the experiments shown in Figures 2 and 8, repeat crosses of the same strains were performed on different days using different batches of growth and sporulation media. For the experiments shown in Figures 7 and 9, all crosses with a given test strain were performed in parallel under identical growth and sporulation conditions to minimize day-to-day fluctuations in recombinant frequencies.

Generation of new *ade6* alleles: Generation of *ade6* alleles for this study was by site-directed mutagenesis using an overlap extension polymerase chain reaction (PCR; Vallejo *et al.* 1995). These PCR products were used directly for lithium acetate-mediated transformation (Bähler *et al.* 1998) of strains GP4258 (*h*$^-$ *ade6-3059 leu1-32 ura4-D18*) or GP4261 (*h*$^-$ *ade6-3060 leu1-32 ura4-D18*), each containing a 3.2-kb insertion of a *ura4*$^+$*-kanMX6* construct at sites 1 and 2 of the *ade6* gene, respectively (Figure 1). Following transformation, cells were allowed to grow for 2 days at 32° in 100 ml of YEL + 2S to dilute the remaining *ura4*$^+$ gene product in transformed cells. Samples (0.1 ml) of these cultures were plated on NBA (0.67% Difco yeast nitrogen base without amino acids, 1% glucose, 2% agar) supplemented with required nutrients at 100 μg/ml and 1 mg/ml of 5-fluoroorotic acid (5-FOA) to select for Ura$^-$ transformants. Alternatively, some *ade6* mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) on pJF63 [pBluescript KS+ (Stratagene) containing a 2.9-kb *Pvu*II-*Spe*I *ade6* insert; Farah *et al.* 2002)]. The mutagenized plasmid was digested with *Stu*I and *Spe*I and used for linear transformation of strain GP3162 (*h*$^-$ *ade6-3037 leu1-32 ura4-D18*) or GP3163 (*h*$^+$ *ade6-3037 leu1-32 ura4-D18*). All new *ade6* alleles were verified by nucleotide sequencing and Southern blot hybridizations.

SELEX: The *s*ystematic *e*volution of *l*igands by *ex*ponential enrichment (SELEX) procedure selects for nucleic acid sequences among a random pool of sequences capable of binding to a particular target (Tuerk and Gold 1990; Ausubel *et al.* 2003). In this procedure, sequences that bind the target are purified and amplified by a PCR, followed by additional cycles of binding, purification, and amplification. For the experiments described here, the starting library contained a randomized 30-bp central region. A 70-base oligonucleotide, 5′-CAAGAATCTAGACGTAGGTG-$(N)_{30}$-CGAATCACCTAA GCTTGGTA-3′ (Integrated DNA Technologies), was made double stranded by annealing it with a 3′-complementary primer and incubation at 37° with the Klenow fragment of DNA polymerase I and dNTPs in Klenow reaction buffer (Fisher, Pittsburgh). One hundred micrograms of this double-stranded (ds) oligonucleotide ($\sim 10^{15}$ molecules) was incubated with gentle rotation overnight at 4° with 25 μl of protein A-conjugated Dynabeads (Dynal, Great Neck, NY) and 4 μg of anti-HA antibody (clone 12CA5; Roche, Indianapolis) in 0.8 ml of binding solution [12% glycerol, 12 mm HEPES (pH 7.9), 4 mm Tris-HCl (pH 7.9), 60 mm KCl, 1 mm EDTA, and 1 mm DTT]. This "preclearing" step was used to eliminate oligonucleotides that bound directly to protein A or anti-HA antibody.

Extracts of strain GP2436 (*h*$^+$ *ura4-D18 leu1-32 atf1*::*HA6His*; Shiozaki and Russell 1996) were prepared from frozen cell pellets by grinding with a mortar and pestle under liquid N_2. A solution (0.5 ml) containing 50 mm HEPES (pH 7.9), 500 mm NaCl, 20% glycerol, 1 mm DTT, 1 mm EDTA, and 2× complete protease inhibitors (Roche) was added to ~2 ml of loosely packed frozen cell powder and gently rotated at 4° for 10 min. The resulting slurry was adjusted to 500 mm NaCl by addition of 5 m NaCl, and rotation was continued for an additional 10 min. The slurry was centrifuged twice (16,000 × *g*, 4°, 30 min) to remove insoluble cell debris. The protein concentration of the clarified cell extract was measured (Bio-Rad protein assay, Richmond, CA) and adjusted to 10 mg/ml. The extracts were frozen in 100-μl aliquots in liquid N_2 and stored at −80°.

The SELEX was performed by mixing 0.1 ml of cell extract, 0.1 ml of poly(dI-dC) (1 mg/ml; Sigma, St. Louis), 40 μl of 25× complete protease inhibitors (Roche), and 0.8 ml of the precleared oligonucleotide library (above); the mixture was rotated gently at 4° for 30 min. Protein A-conjugated Dynabeads (25 μl) and 4 μg of anti-HA antibody were added, and rotation continued for 3 hr to immunoprecipitate Atf1::HA6-His-Pcr1 and associated oligonucleotides. The Dynabeads were washed once in binding solution (1 min, 4° with rotation) and boiled 2 min in 0.1 ml of H_2O. The supernatant was distributed equally into eight 100-μl PCR mixtures using primers to the constant region of the starting 70-bp oligonucleotide (5′ sense and 3′ complementary). Thirty cycles of PCR were performed using Platinum *Taq* DNA polymerase (Invitrogen, San Diego) and the manufacturer's recommended conditions with 4 mm $MgCl_2$. PCR products were purified on a 4% Metaphor agarose (Cambrex) gel, and the above procedures were repeated five times with the following exceptions: (1) the enriched oligonucleotide libraries were precleared with only 10 μl of Dynabeads and 2 μg of anti-HA antibody, and (2) after immunoprecipitation, one additional wash was added per cycle to a maximum of four washes.

After six cycles of SELEX, the remaining oligonucleotides were digested with *Xba*I and *Hin*dIII, restriction enzymes that cut at sites in the constant region, and cloned into pBluescript KS(+) (Stratagene).

Gel-mobility shift assays: These assays were performed as described by Fox *et al.* (2000) with the following modifications. For the experiment shown in Figure 3, 215-bp DNA fragments were generated by a PCR using genomic DNA as template and primers with sequences 5′-TGCATCTTTATGGTAAAGCTG-3′ and 5′-CACCTTGAATTCATCTAAAATGACGG-3′. PCR products were gel purified and end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Extracts were made from strains GP2429 (*h*$^+$ *atf1::ura4*$^+$ *leu1-32 his7-366 ura4-D18 ade6-M210*) and GP2435 (*h*$^-$ *pcr1::his7*$^+$ *leu1-32 his7-366 ura4-D18*), described by Shiozaki and Russell (1996) and Fox *et al.* (2000). Complete protease inhibitor mixture (Roche) was used in place of individual protease inhibitors and was also included in binding reactions. For the experiment shown in Figure 5, 70-bp probes were generated by PCR using the pBluescript clones described in the preceding paragraph as template and 5′-sense and 3′-complementary primers to the constant regions of the 70-bp oligonucleotide used for SELEX.

RESULTS

Apparent orientation dependence of an *M26* hotspot: Fox *et al.* (2000) showed that the *CRE* sequence (5′-TGACGT$^C/_A$-3′), which partially overlaps the *M26* sequence (5′-ATGACGT-3′), has significant Atf1-Pcr1-dependent hotspot activity when located at the same position as the original *ade6-M26* mutation (Figure 1, site 1). This sequence, unlike *M26*, is found in the wild-type *ade6* gene, at nucleotides 1131–1138 of the *ade6* ORF (Figure 1, site 2). Given that both the *M26* and

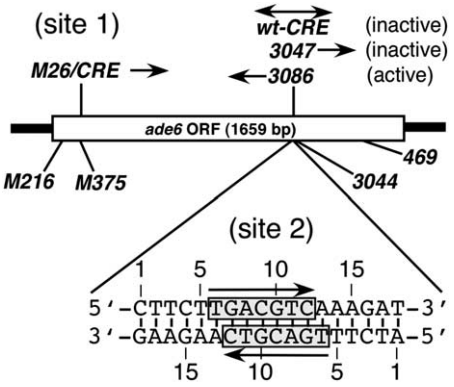

FIGURE 1.—Allele positions within *ade6*. Arrows indicate the orientation of the *M26* and *CRE* sequences when applicable. The expanded portion shows the nucleotide sequence of the palindromic *wt-CRE* site (site 2; *CRE* sequences boxed). Each strand of the DNA at this site is numbered equivalently (5′ → 3′) relative to the *CRE* sequence in the strand.

CRE sequences are Atf1-Pcr1-dependent hotspots, and given that *M26* generated a hotspot at every site where it was created within *ade6* (Fox *et al.* 1997), it seemed probable that this wild-type *CRE* sequence (*wt-CRE*) would act as a natural hotspot within the gene.

To test this hypothesis, a translational stop mutation (*ade6-3044*, G1150T in the ORF) was introduced next to *wt-CRE* to serve as a marker for gene conversion at that site. In crosses with a strain containing the *ade6-M375* allele, the mean frequency of recombinants was 400 ± 40 (SEM) *ade*$^+$ per million viable spores (Figure 2). We then abolished the *wt-CRE* sequence by a C → T substitution at nucleotide position 12 (*ade6-3043*) in both strains used in the cross. [A comparable C → T mutation abolishes the *CRE* hotspot at site 1 (Fox *et al.* 2000)]. However, the *ade6-3043* mutation resulted in no significant decrease in recombination (Figure 2), indicating that the *wt-CRE* sequence is not a hotspot.

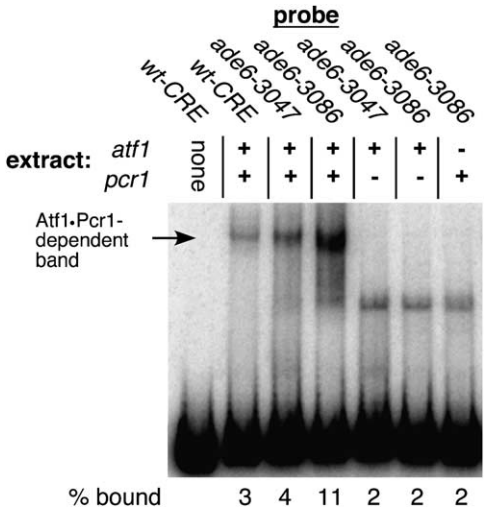

FIGURE 3.—The inactive *wt-CRE* and *M26* heptamers bind the Atf1-Pcr1 transcription factor less strongly than an active heptamer at the same site. Gel mobility shift assays were conducted with 215-bp DNA fragments containing the indicated *ade6* allele. DNA was end labeled and mixed with *S. pombe* protein extracts of cells with the indicated genotype. The percentage of Atf1-Pcr1-dependent shift is shown below each lane. Similar results were obtained in repeat experiments. The *atf1*Δ and *pcr1*Δ mutant extracts also produced a distinct mobility shift in these experiments that was 2% of the total probe in each case; the basis of this band is not clear.

Since all previous *M26* creations within *ade6* were hotspots, we next tested if the *wt-CRE* sequence (site 2) could be activated by mutation to an *M26* sequence. In contrast to previous observations, this change produced no significant increase in recombination (Figure 2, *ade6-3047*). However, since the *CRE* sequence at site 2 is palindromic, it was also possible to create an *M26* heptamer by a comparable mutation on the complementary strand. Remarkably, this mutation created a strong hotspot (Fig-

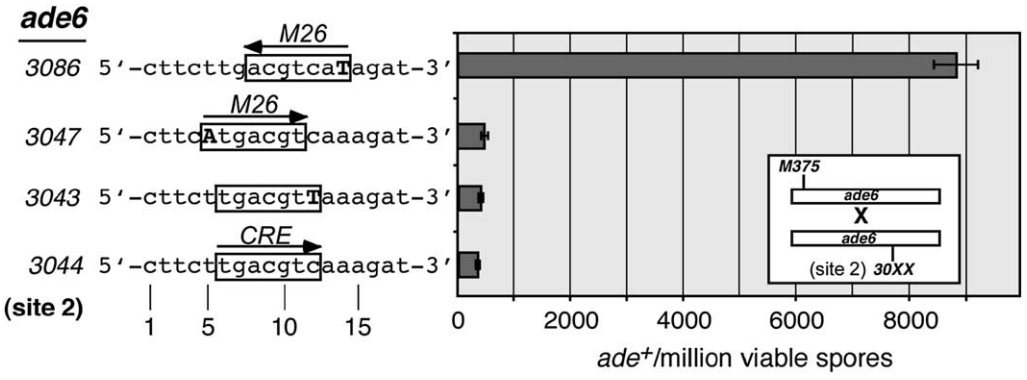

FIGURE 2.—Apparent orientation dependence of the *M26* heptamer. The frequency of recombination was measured in crosses between *ade6-M375* and the indicated alleles of *ade6*. The frequency of recombination in these crosses was not significantly affected by mutations abolishing *CRE* (*ade6-3043*) or creating *M26* in the forward orientation (*ade6-3047*). However, mutation to *M26* in the opposite orientation (*ade6-3086*) produced a large increase in recombination. Bars show the frequency of *ade*$^+$ recombinants from each pair of alleles (mean ± SEM; n = 3–13).

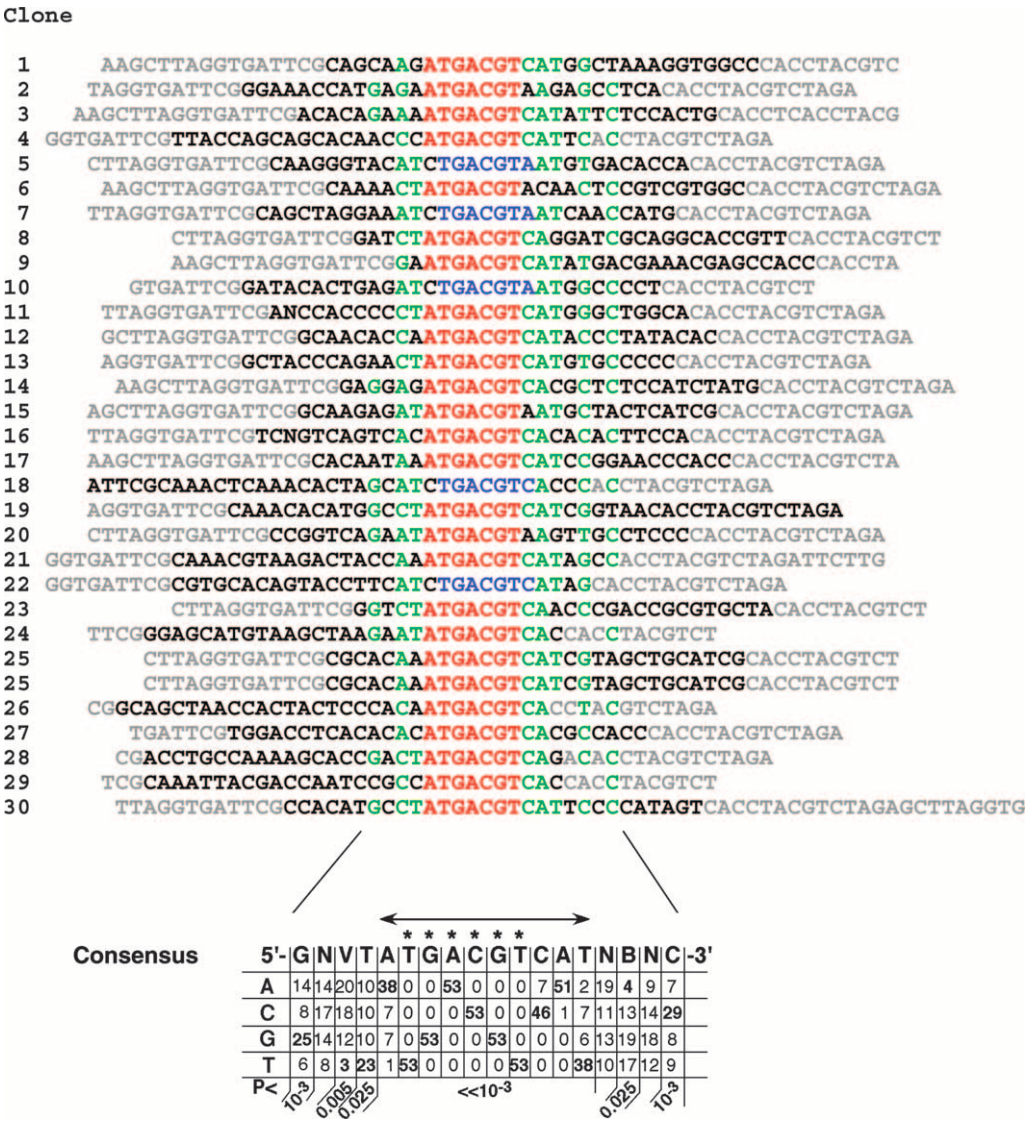

FIGURE 4.—The sequence of 30 clones derived from SELEX and the deduced consensus sequence. Sequences are aligned relative to the common region, 5′-TGACGT-3′ (asterisks at bottom). Color code is as follows: gray, nucleotides from constant (nonrandom) region of the 70-bp oligonucleotide library; black or color, 30-bp randomized region; red, *M26* heptamers; blue, *CRE* heptamers; green, matches to the consensus sequence for Atf1-Pcr1 binding outside the *M26* or *CRE* heptamer (a few of these matches are in the constant region). Clones 18 and 19 were isolated as two inserts in a single plasmid but were counted as separate clones. Both strands of a clone were included when the common region 5′-TGACGT-3′ occurred within a palindrome. The frequency of a given nucleotide at each position is indicated in the box at the bottom, and the most frequent (or significantly underrepresented) nucleotide is shown in boldface type. The probability that the observed distribution derives from a random distribution (25% of each nucleotide) for relevant columns is shown in the bottom row (χ^2-test, d.f. = 3). V represents A, C, or G and B represents C, G, or T. N represents any nucleotide. The double-headed arrow spans the 10-bp *M26* palindrome.

ure 2, *ade6-3086*), >10 times hotter than *ade6-3047* with the *M26* heptamer in the opposite orientation. Given that these two *M26* heptamers are at essentially the same position within the *ade6* gene (overlapping at 4 of 7 bp), it seemed unlikely that the difference in activity could be due to an effect of local chromatin structure as had been previously observed with transplacements of the entire *ade6-M26* allele (PONTICELLI and SMITH 1992; VIRGIN *et al.* 1995). *A priori*, these data imply that hotspot activity is dependent on the orientation of the *M26* heptamer at site 2, in contrast to the previously observed orientation independence (FOX *et al.* 1997).

***In vitro* selection for Atf1-Pcr1-binding sequences:** An alternative explanation to a strict orientation dependence of *M26* hotspot activity at site 2 is that nucleotides adjacent to the heptamer are necessary for hotspot activity. If so, this might be reflected in differential binding of purified DNA with these sequences to the Atf1-Pcr1 transcription factor. Thus, we tested the *wt-CRE* sequence and both the active and the inactive orientations of *M26* for their ability to bind Atf1-Pcr1 *in vitro* by gel mobility shift assays. Although all of these sequences bound Atf1-Pcr1, the active allele, *ade6-3086*, showed greater binding than either of the two inactive alleles (Figure 3), supporting the hypothesis that nucleotides adjacent to the *M26* (or *CRE*) sequence influence binding of the transcription factor. Binding of Atf1-Pcr1 to purified *ade6-3086* DNA was 3–4 times greater than to *wt-CRE* or *ade6-3047* DNA, but intracellular hotspot activity of *ade6-3086* was more than 10 times greater than

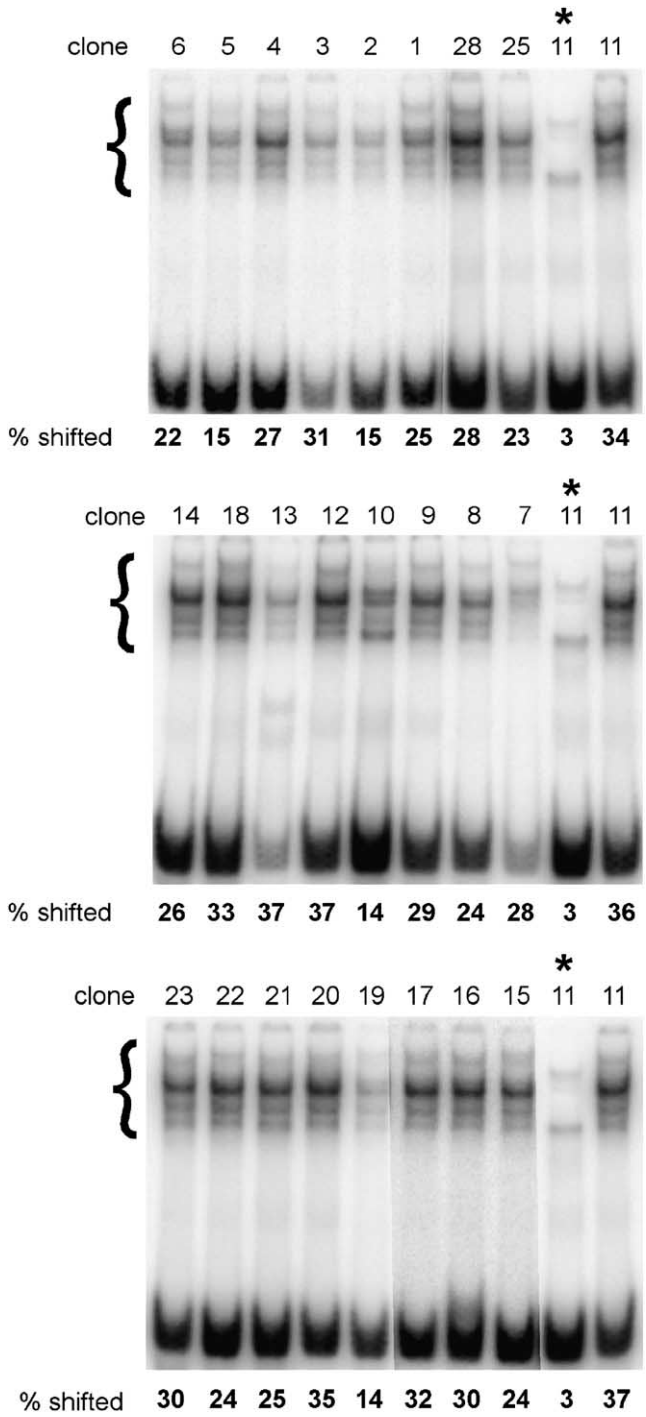

FIGURE 5.—*In vitro* binding of the SELEX clones to Atf1-Pcr1. The 70-bp clones indicated were end labeled, incubated with protein extracts, and assayed by gel mobility shift, as described in MATERIALS AND METHODS. Clone 11 was used as a standard for comparison in all such gels. All bands within the bracketed region were used for quantitation, and the percentage of bound probe is indicated above each lane. Lanes marked with an asterisk contained cell extracts from a *pcr1*Δ mutant strain.

that of *wt-CRE* (*ade6-3044*) or *ade6-3047* (Figure 2). This quantitative difference may reflect the influence of intracellular chromatin structure on hotspot activity (see DISCUSSION).

To determine which nucleotides in addition to the *M26* heptamer are needed for binding to purified DNA, we sought a wide range of sequences capable of strongly binding the Atf1-Pcr1 transcription factor. To this end we utilized the SELEX procedure (TUERK and GOLD 1990; AUSUBEL *et al.* 2003) on a ds-oligonucleotide library containing a 30-bp randomized central region. This technique selects for nucleic acid sequences among a random pool of sequences capable of binding to a particular target. After six cycles of SELEX, we sequenced 30 randomly chosen clones. All 30 clones were unique, but a 6-bp sequence, 5′-TGACGT-3′, common to both the *M26* and *CRE* hotspots, was found in all 30 (Figure 4). To derive a consensus Atf1-Pcr1-binding sequence, we aligned the 30 clones relative to this 6-bp common region. For those clones in which the common region occurred as a palindrome (5′-TGACGTCA-3′), we included both strands in the alignment. The *M26* heptamer was apparently the preferred recognition sequence for Atf1-Pcr1 in these experiments, as it was found on at least one strand in 27 of the 30 clones analyzed, and in 12 clones *M26* occurred as a perfect 10-bp palindrome (5′-ATGACGTCAT-3′). In addition, there were nonrandom distributions of bases extending four nucleotides to the left and four nucleotides to the right of the central 10-bp *M26* palindrome. The distribution of bases at positions outside of this region was not significantly different from random ($P > 0.05$, χ^2-test, 3 d.f.).

We confirmed the ability of the SELEX-derived sequences to bind Atf1-Pcr1 *in vitro* by gel mobility shift assays as above. All of the sequences showed substantial mobility shifts when mixed with wild-type (but not *atf1*Δ or *pcr1*Δ mutant) cell extracts (Figure 5). In general, those sequences most closely resembling the consensus sequence showed the strongest mobility shifts (Table 2), with one curious exception, clone 7, which contained a single *CRE* sequence.

As described below, we used the consensus sequence for Atf1-Pcr1 binding to purified DNA as a guide for mutagenesis to determine more precisely the nucleotide sequences necessary and sufficient for intracellular *M26* hotspot activity.

Apparent orientation dependence of the *M26* heptamer is due to adjacent nucleotides: We noted earlier the large difference in hotspot activity between the active and inactive orientations of the *M26* heptamer at site 2 (*ade6-3086* and *ade6-3047*, respectively; Figure 2). Could this difference be explained by their flanking nucleotides? Comparison of each of these sequences with the consensus sequence revealed two bases adjacent to the first A of each heptamer that could explain the difference in activities (Figure 6, positions 3 and 4). These bases matched the consensus sequence in the active, but not the inactive, allele. In addition, the data from Figure 4 suggested that the A at position 1 of the active sequence is preferred over the C found at the same position of the inactive sequence (Figure 6). Therefore, we tested whether the inactive orientation

TABLE 2

Relative Atf1-Pcr1-binding efficiencies of SELEX-derived clones

Clone	Relative binding (%)	Sequence (5′ → 3′)
12	170 ± 32	acCaATGACGTCATaCcC
13	164 ± 30	aaCTATGACGTCATgTgC
7	132 ± 28	aaATcTGACGTaATcaaC
9	110 ± 18	tcATATGACGTCATtCcg
11	[100]	ccCTATGACGTCATgGgC
4	100 ± 13	acCcATGACGTCATtCaC
3	95 ± 14	GaATATGACGTCATtTtC
20	91 ± 4	ccAaATGACGTCATaGcC
1	86 ± 8	agCcATGACGTCATcTtg
17	80 ± 5	ccGgATGACGTCATtTat
23	79 ± 3	GaATATGACGTCAccacC
28	79 ± 5	cgCcATGACGTCAccacC
16	77 ± 4	tcAcATGACGTCAcaCaC
25	69 ± 5	caCaATGACGTCAccTaC
14	68 ± 8	GgAgATGACGTCAcgCtC
6	65 ± 3	aaCTATGACGTacaaCtC
8	64 ± 2	atCTATGACGTCAggatC
15	60 ± 6	agATATGACGTaATgCta
21	57 ± 8	tgCTATGACGTCAgaTga
22	51 ± 7	GtCTATGACGTCAacCcg
10	42 ± 2	agATcTGACGTaATgGcC
5	42 + 6	acATcTGACGTaATgTga
2	39 ± 6	GaGaATGACGTaAgaGcC
19	34 ± 5	GaATATGACGTaAgtTgC
Consensus		*GNV*TATGACGTCAT*NBN*C

Binding of the indicated 70-bp SELEX clone to Atf1-Pcr1 was determined by gel mobility shift assays in four independent experiments (see Figure 5 for examples). Numbers in the center column show the mean percentage (±SEM) of probe shifted relative to clone 11, which was used as a standard in each gel. Matches to the consensus sequence (Figure 4) are in uppercase and underlined. *V* represents A, C, or G; *B* represents C, G, or T. *N* represents any nucleotide. Only clones yielding a single 70-bp PCR product were analyzed.

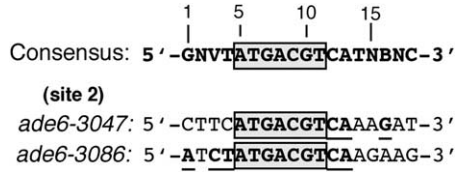

FIGURE 6.—Comparison of the active (*ade6-3086*) and inactive (*ade6-3047*) *M26* sequences with the consensus sequence. Matches or close matches to the consensus sequence are underlined. *M26* heptamers are boxed, and numbering is as in Figure 1.

of *M26* (*ade6-3047*) could be activated by mutating each of the nucleotides at positions 1, 3, and 4 to match those found in the active orientation. Figure 7 (bottom set of sequences) shows that mutation of any one of these bases resulted in a 2- to 3-fold increase in the frequency of ade^+ recombinants observed in test crosses (*t*-test, $P < 0.05$). The double mutant T3 → C, C4 → T (*ade6-3090*) produced an ~6-fold increase, and the triple mutant (*ade6-3091*) produced an ~15-fold increase or about the same activity as *ade6-3086* (the active orientation).

The data above imply that at least three additional nucleotides to the 5′-side of the *M26* heptamer (positions 1, 3, and 4) at site 2 are required for optimal hotspot activity. If so, it should also be possible to inactivate the active *M26* orientation, *ade6-3086*, by mutating the equivalent bases to match those of the inactive heptamer, *ade6-3047*. As predicted, this allele (*ade6-3094*) showed a significant reduction in recombination (Figure 7, *t*-test, $P < 0.001$). However, recombination with this allele was not reduced to the level of *ade6-3047* (the inactive orientation allele). Thus, we made additional mutations to the other side of the active *M26* orientation (*ade6-3093*, equivalent to an 18-bp inversion of the entire inactive sequence), but these changes failed to eliminate the residual activity. Hence, there may be additional nucleotides outside of the inverted segment affecting hotspot activity, although some degree of orientation or chromatin structure dependence cannot be completely excluded.

The consensus sequence (Figure 4) and the data from Table 2 suggested that the 10-bp *M26* palindrome was the preferred target for Atf1-Pcr1 binding. Therefore, we also tested this sequence (*ade6-3084*) as well as the full consensus sequence (*ade6-3070*; shown in Figure 7 as the complement of the consensus sequence in Figure 4) to see whether recombination at site 2 could be further increased over that already observed. *ade6-3070* showed a marginally significant increase of hotspot activity relative to *ade6-3086* in crosses with one, but not the other test allele (Figure 7). Thus, the single-base-pair change of *ade6-3086* may be sufficient (or nearly so) for optimal hotspot activity at site 2.

Nucleotides outside the *M26* heptamer at a second site also affect hotspot activity: The preceding results suggested that *M26* hotspot activity is not an all-or-nothing phenomenon. Rather, there is a continuum between inactivity and full activity. Consistent with this view, we observed an ~10-fold range in activities among previous *M26* creations in the *ade6* gene (FOX *et al.* 1997; STEINER *et al.* 2002; and our unpublished results). Thus, we wished to know whether the activity of other *M26* heptamers was also affected by their flanking sequences. SCHUCHERT *et al.* (1991) had previously tested the sequence requirements for hotspot activity in the original *ade6-M26* allele (site 1) and concluded that only the heptamer 5′-ATGACGT-3′ was necessary for hotspot activity. We conducted a similar analysis at that site by testing every nucleotide outside of the *ade6-M26* heptamer that showed a nonrandom distribution in the SELEX consensus sequence (Figure 4). A total of 13 new *ade6* alleles containing mutations at site 1 were generated and crossed with 2 test alleles: *ade6-M216* and *ade6-469*. Substantial differences in recombinant frequencies were observed among these alleles (Figure

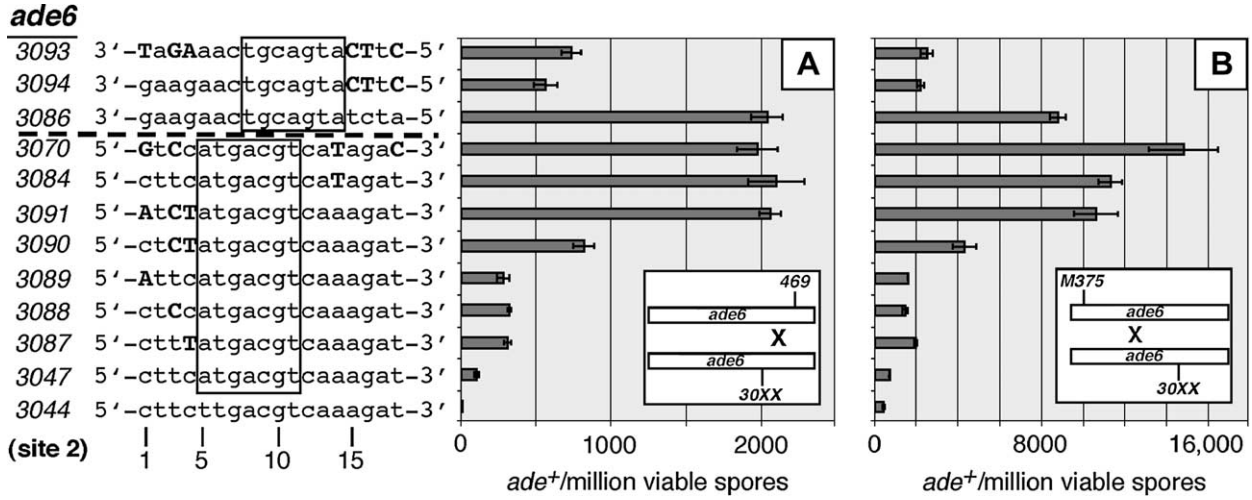

FIGURE 7.—Control of hotspot activity by nucleotides flanking the *M26* heptamer. Multiple alleles of *ade6* were constructed containing one or more base pair changes at the *wt-CRE* site of *ade6* (site 2). The frequency of recombinants (mean ± SEM; $n = 3$) was measured in crosses between these alleles and *ade6-469* (A) or *ade6-M375* (B). The *M26* heptamer, *ade6-3047*, has little hotspot activity in these crosses, but activity is increased by sequence changes (boldface type) to the left or right of the heptamer (boxed). See RESULTS and DISCUSSION for further details. The dashed line demarcates sequences written 5′ → 3′ from those written 3′ → 5′, the "top" and "bottom" strands of *ade6*, respectively.

8), showing that nucleotides near, but outside of, the *M26* heptamer also affected hotspot activity at site 1.

To quantitate this effect more rigorously, three of the most active alleles in these experiments (*ade6-3074*, *ade6-3079*, and *ade6-3083*) and *ade6-M26* were analyzed side-by-side in crosses with two test alelles (Figure 9). Each of these very active alleles showed comparable frequencies of recombination, and each was significantly more active than *ade6-M26* (*t*-test, $P < 0.05$; Figure 9). Surprisingly, addition of the single G → C mutation at position 12 (*ade6-3074*) to the *ade6-M26* sequence was enough to stimulate recombination three- to fourfold in these crosses. Additional mutations, including mutation to the full consensus sequence, showed little if any further stimulation, suggesting that only the additional C is necessary for optimal hotspot activity at site 1. SCHUCHERT *et al.* (1991) also observed increased levels of recombination caused by the G → C mutation at position 12 (referred to as 16C in their article) but concluded that this level was not significantly higher than that of *ade6-M26* itself. However, in those experiments the strains used for comparison were derived in different ways. The *ade6-M26* allele was created in a strain exposed to X-ray mutagenesis (GUTZ 1971) and, hence, could have been associated with other mutations affecting recombination frequencies. In fact, in the experiments of SCHUCHERT *et al.* (1991), when the *ade6-M26* allele was recreated by site-directed mutagenesis in the same strain background as that of the 16C mutant, recombination frequencies were significantly lower compared to those of 16C (*t*-test, $P < 0.05$). Thus, their results are compatible with our results showing that nucleotides outside of the *M26* heptamer are necessary for optimal hotspot activity.

DISCUSSION

The *ade6-M26* hotspot was previously characterized as a unique 7-bp sequence, 5′-ATGACGT-3′, thought to be necessary and sufficient for the elevated frequency of recombination observed with that allele (SCHUCHERT *et al.* 1991; FOX *et al.* 1997). Here, we report that the *M26* heptamer is not sufficient for hotspot activity, at least at one site and in one orientation within *ade6*. The inactivity of *M26* at that site is probably due, at least in part, to its relatively weak binding of the Atf1-Pcr1 transcription factor (Figure 3). Hence, we used an *in vitro* selection and amplification procedure (SELEX) to identify the nucleotide sequences of purified DNA that bound strongly to Atf1-Pcr1 and found an 18-bp consensus sequence containing *M26* as a 10-bp palindrome at the center. Positions on each side of the central palindrome also showed significant over- or underrepresentation of particular bases (Figure 4). We used this consensus sequence as a guide to test nucleotides flanking the *M26* heptamer for a role in intracellular hotspot activity. Many mutations altering these nucleotides were capable of either increasing or decreasing recombination by as much as 15-fold relative to the *M26* heptamer without the additional mutations (Figures 7–9). Thus, more than the *M26* heptamer is necessary for optimal hotspot activity.

The most striking feature of the consensus sequence shown in Figure 4 is the *M26* palindrome at the central 10 bp. This is not necessarily a remarkable result—if one *M26* heptamer is sufficient for some binding of Atf1-Pcr1, then two overlapping heptamers could bind even more strongly and apparently do, since 9 of the 10 strongest binding sequences shown in Table 2 con-

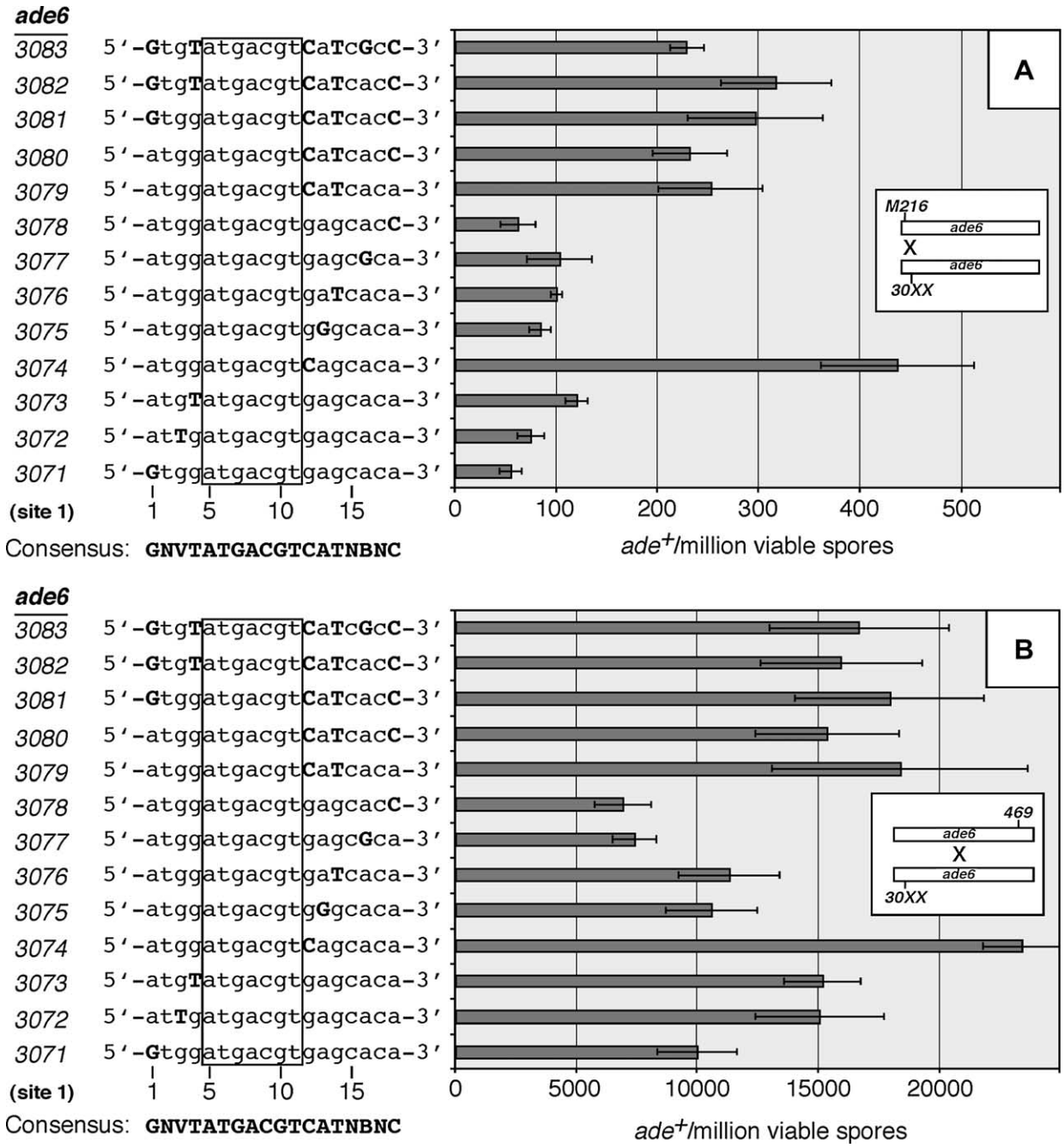

FIGURE 8.—Nucleotides flanking the *M26* heptamer at a second site also affect hotspot activity. Crosses were performed with the indicated *ade6* allele and *ade6-M216* (A) or *ade6-469* (B). Each nucleotide outside the *M26* heptamer (boxed) at site 1 (Figure 1) showing a nonrandom distribution in the consensus sequence (Figure 4) was tested for its effect on hotspot activity. If the nucleotide present in the wild-type sequence was different from the consensus, it was changed to match the consensus; otherwise, it was changed not to match (*ade6-3072* and *ade6-3075*). Bars show the frequency of *ade*$^+$ recombinants (mean ± SEM; $n = 3$ in A and 5 in B).

tain the 10-bp *M26* palindrome, and the remaining 14 do not. Surprisingly, this palindrome, or even the full consensus sequence, was not necessarily more active as an intracellular hotspot than some other sequences at either of the two sites tested within *ade6*. A possible explanation for this apparent difference is that the *in vitro* binding conditions used in these experiments may not accurately reflect the conditions within the cell, where transcription factors must interact with chromatin rather than naked DNA. Indeed, *ade6-M26* is often inactive as a hotspot when transplaced on 3- to 5-kb DNA fragments into novel sites in the genome, presumably due to an alteration in chromatin structure (PONTICELLI and SMITH 1992; VIRGIN *et al.* 1995). Alternatively, factors other than the quality of a protein-binding site may limit recombination even when a stronger binding

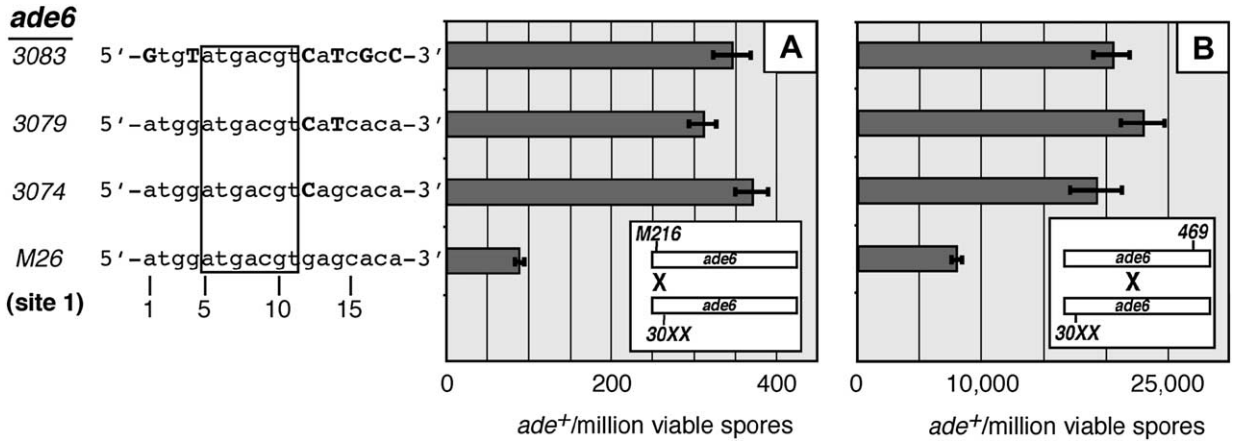

FIGURE 9.—Only a single additional base change is sufficient for optimal hotspot activity at site 1. Hotspot activities of three of the most active *ade6*-alleles (Figure 8) were determined in side-by-side comparisons with each other and *ade6-M26*. Bars show the frequency of *ade6*$^+$ recombinants (mean ± SEM; $n = 3$) in crosses between these alleles and *ade6-M216* (A) or *ade6-469* (B).

site is created. Nevertheless, the most active hotspots we found contained at a minimum overlapping *M26* and *CRE* sequences, *i.e.*, 5′-ATGACGTCA-3′ (Figures 7 and 9, *ade6-3086* and *ade6-3074*, respectively). The most active hotspot we previously found, *ade6-3049*, also contains overlapping *M26* and *CRE* sequences and shows activity comparable to that of *ade6-3074* (our unpublished results). [*ade6-3049* is identical to *ade6-3011* but lacks the extraneous mutation found with *ade6-3011* (FOX *et al.* 1997; STEINER *et al.* 2002).]

Our mutational analysis of *M26* hotspot sequences was based on the consensus sequence derived in Figure 4; we focused only on those bases showing a distribution significantly different from random and at fixed positions relative to the central common region. (None of the full 30-bp sequences found by SELEX were directly tested for intracellular hotspot activity.) We assumed that those nucleotides were the ones most likely to affect binding of Atf1-Pcr1 and, hence, hotspot activity. While this assumption was largely supported by our experiments, we do not purport to have identified every nucleotide affecting *M26* activity. However, nucleotides outside of the consensus sequence are likely to have only small effects on hotspot activity unless they exert those effects from nonfixed positions or by some means other than binding of the Atf1-Pcr1 transcription factor. The sequence requirements for optimal activity could also vary from site to site depending on the influence of local chromatin structure. Meiosis-specific changes in chromatin structure occur at site 1 with either *ade6-M26* or a CRE sequence (*ade6-3013*) and may determine in part the level of hotspot activity (MIZUNO *et al.* 1997; FOX *et al.* 2000; YAMADA *et al* 2004). An influence of chromatin structure may account for the quantitative differences between Atf1-Pcr1 binding to purified DNA (Figure 3) and intracellular hotspot activity (Figure 2) of certain sequences studied here.

The *M26* hotspot was discovered as a single-base-pair mutation that fortuitously created a binding site for a transcription factor (GUTZ 1971; SZANKASI *et al.* 1988; WAHLS and SMITH 1994; KON *et al.* 1997). Identification of this mutation has since allowed a detailed dissection of the nucleotide sequence required for its activity. Although we are aware of no other eukaryotic recombination hotspot whose nucleotide sequence has been defined at this level, *M26* is similar to other hotspots that also require transcription-factor binding (α-hotspots), for example, the *HIS4* hotspot of *S. cerevisiae* (WHITE *et al.* 1993). In fact, most hotspots in *S. cerevisiae* appear to occur in gene promoters (BAUDAT and NICOLAS 1997; GERTON *et al.* 2000), where transcription factors and other proteins often bind. The SELEX procedure we describe here could be utilized to characterize other α-hotspots in similar detail. Simple sequences found by this type of analysis may be active at many sites in the genome.

The *M26* heptamer is found at ~300 sites in the sequenced *S. pombe* genome (http://www.sanger.ac.uk/Projects/S_pombe/; our unpublished observation). Although it seems plausible that this sequence acts as a hotspot in at least some of those locations, any such natural *M26* hotspot has yet to be identified. The results reported here provide a more complete picture of the nucleotide sequence requirements for *M26* activity and, therefore, a tool with which to search for natural *M26* hotspots in the *S. pombe* genome. Identifying such natural hotspots will help to elucidate further the control of meiotic recombination.

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