Identification of a Protein That Binds to the Ho Endonuclease Recognition Sequence at the Yeast Mating Type Locus

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Mating type switching in *Saccharomyces cerevisiae* initiates when Ho endonuclease makes a site-specific double-stranded break at *MAT*, the yeast mating type locus. To identify other proteins involved in this process, we examined whether extracts prepared from *ho*[−] mutants contain additional factors that bind near the recognition sequence for Ho. Using an electrophoretic mobility shift assay, we isolated a chromatographic fraction that contains an activity, named YZbp, which binds to two sequences flanking the recognition sequence at *MAT* α and to one sequence overlapping it at *MATa*. *MAT* plasmids carrying mutations in the YZbp recognition sequence are cleaved by purified Ho at wild-type efficiencies in an in vitro assay. These same plasmids, however, are not cleaved by Ho inside cells, demonstrating that YZbp acts as a positive activator of in vivo cleavage. YZbp is present in all cell types, even those not undergoing mating type switching, suggesting that it has additional cellular functions.**

Genetic recombination is the intracellular process that moves DNA sequence information from one genomic location to another. In the yeast *Saccharomyces cerevisiae*, several lines of evidence indicate that recombination initiates with a double-stranded break (DSB) in DNA. First, DSBs are repaired by using gene products required for recombination (reviewed in reference 22). Second, certain meiotic segregation patterns of yeast genes are consistent with molecular models of recombination that posit a DSB initiation event (28). Third, DSBs occur at regions of the genome defined genetically as initiators of recombination (2, 4, 6, 8, 21, 26, 27, 33, 34). Fourth, DSBs introduced into DNA during meiosis stimulate nearby meiotic recombination (13). Fifth, meiotic DSBs precede and are independent of pairing between homologs, implying that DSBs initiate chromosomal pairing (3, 7). Finally, mating type switching, a specialized form of mitotic gene conversion, has been shown unambiguously to initiate with a DSB (14, 15, 25). While these results clearly show that DSBs play a major role in the initiation of recombination, it is important to state that other types of intermediates, such as single-stranded nicks in DNA, might perform similar functions.

Given the important role of DSBs in the initiation of recombination, it seems likely that their formation is a highly regulated process that depends on multiple gene products. The goal of this study was to identify factors involved in this regulation, particularly those that function by direct interaction with DNA at the site of the break. To carry out this analysis, we have focused on the well-characterized DSB that occurs at the *MAT* locus during mating type switching.

The *MAT* locus is the central element in the yeast mating type system (reviewed in reference 10). It exists in two allelic states, known as *MAT**a* and *MAT* α , which determine whether a haploid cell has an *a* or α mating type, respectively. *MAT* contains five sequence blocks: W, X, Y, Z1, and Z2 (1) (Fig. 1). The W, X, Z1, and Z2 blocks are identical in *a* and α cells, but each mating type contains a unique Y sequence—Y α in *a* cells

and Y α in α cells. Wild-type haploids continuously interconvert between the *a* and α mating types, a process mediated at the molecular level by the interconversion between Y α and Y α . The switching reaction initiates when an endogenous endonuclease known as Ho makes a DSB at the border between Y and Z1 (14, 15, 25). This DSB triggers a mitotic recombination reaction that replaces Y information at *MAT* with Y information from one of two unlinked and transcriptionally inactive loci known as *HML* α and *HMR**a* (31) (Fig. 1).

The cleavage reaction catalyzed by Ho has been analyzed in detail by Heffron and colleagues (14, 15, 18, 19). Ho cleaves *MAT* DNA between Z7 and Z8 on one strand and Z3 and Z4 on the other (by convention, base pairs are numbered in relation to the YZ border, e.g., Z8 and Y8 are each 8 bp away from the YZ border [see Fig. 7]). This generates a DSB with two 3' overhangs that are 4 nucleotides long. The recognition sequence for Ho was determined by an in vitro assay which examined how bacterially expressed protein cleaved mutant derivatives of the *MAT* loci. These studies showed that the recognition sequence for Ho was located in a 16-bp region from Y6 to Z10. Eight noncontiguous base pairs were necessary for cleavage: Y4, an AT base pair conserved in both Y α and Y α , and Z1, Z2, Z3, Z4, Z6, Z7, and Z9 (see Fig. 7). The recognition sequence for Ho was also determined by an in vivo assay, which monitored cleavage of yeast plasmids carrying mutant *MAT* loci. Surprisingly, the minimal recognition sequence determined from this assay was longer than the one determined in the in vitro assay. Thus, the 16 bp required for cleavage in vitro (Y6 to Z10) were found to be insufficient to support cleavage in vivo. The same was true of a 20-bp fragment from Y8 to Z12. Instead, a 24-bp sequence from Y11 to Z13 was required for cleavage to be observed inside cells (see Fig. 7).

Why does Ho require a larger recognition sequence in vivo than in vitro? While it is possible to advance several hypotheses, perhaps the most economical one proposes that other proteins bind to the additional sequences required in vivo. In this study, we tested this model and found that yeast does have a protein with the predicted binding characteristics. The protein, which was named YZbp for YZ binding protein, is present in all cell types and binds to *MAT**a* and *MAT* α YZ

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FIG. 1. The mating loci in *S. cerevisiae*. During mating type switching, Y information from HML α (black bar) or from HMRa (stippled bar) is transposed into the expressed MAT locus. Switching initiates when Ho endonuclease makes a DSB at the MAT YZ border as shown.

borders. We further show that the binding sites for YZbp are required for cleavage *in vivo*, but not *in vitro*, strongly arguing that the protein is a positive activator of DSB formation at MAT. The identification of YZbp provides experimental support for the idea that DSB formation at MAT depends upon multiple proteins, including at least Ho and YZbp.

MATERIALS AND METHODS

Yeast strains. The diploid strain used in this study was commercial yeast cake (Imperial Yeast Distributors, Inc., Parlin, N.J.). The haploid strains were BJ5462 (MAT α *ho ura3-52 trp1 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1Δ1.6R can1 Gal⁺*), kindly supplied by Elizabeth Jones (11); W303-1A (MATa *ho ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*), kindly supplied by Andrew Vershon; and DN1005 (MATa *ho thr4*).

Plasmids. Plasmid pGALHOT, kindly supplied by Mike Fasullo, is a yeast centromere plasmid containing TRP1 and the HO gene driven by a GAL1 promoter. Plasmid pRW17 contains the following sequence inserted between the HindIII and BamHI sites of YCp50 (24): AGC TCA CGG AAT ATG GGA CTA CTT CGC GCA ACA GTA TAA TTT TAT AAA TCT AGA. This sequence contains a wild-type, MAT α YZ border from Y22 to Z22. Plasmid pRW3 is the same as pRW17, with the exception that Y7 to Y12 were changed to TTCGAC. Plasmid pRW24 is also the same as pRW17, with the exception that Z11 to Z14 were changed to GCCG.

Isolation of oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems 392 synthesizer according to the manufacturer's instructions and subsequently purified by electrophoresis through 12% denaturing polyacrylamide gels (5). Quantitation was carried out by absorbance spectroscopy (260 nm) using the following extinction coefficients: dATP = 15,200 M⁻¹; dCTP = 9,300 M⁻¹; dGTP = 13,700 M⁻¹; and dTTP = 9,600 M⁻¹.

Isolation of end-labeled, double-stranded oligonucleotides. Single-stranded oligonucleotides (500 pmol) were end labeled with T4 polynucleotide kinase (New England BioLabs) according to the manufacturer's instructions. Unlabeled complementary oligonucleotides (500 pmol) were added to the terminated kinase reaction mixture, and the mixture was heated for 5 min at 95°C and then slowly cooled to room temperature to permit renaturation. The resulting double-stranded oligonucleotides were separated from unincorporated radionucleotides by size exclusion chromatography using Sephadex G-50 (Pharmacia). For each gel shift, 20 fmol of labeled, double-stranded oligonucleotide was used (2,000 to 8,000 cpm/reaction mixture).

Isolation of the active YZbp fraction. All purification steps were carried out in M buffer (20 mM morpholinepropanesulfonic acid [MOPS] [pH 7.5], 1 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% [wt/vol] glycerol, 40 mM NaHSO₃, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM benzimidazole). Two hundred fifty grams of yeast cell pellet was resuspended in an equal volume of M₂₅₀ (M buffer containing 250 mM NaCl). The cells in the slurry were disrupted by mechanical lysis in a Biospec Beadbeater (six 1-min bursts, separated by 1.5-min cooling periods) using acid-washed, 0.45-μm-diameter glass beads. The lysate was centrifuged for 30 min in a Sorvall SS34 rotor (8,000 rpm, 4°C) to remove broken cell debris, and the supernatant was removed and centrifuged for a further 2 h in a Beckman 45 Ti rotor (40,000 rpm, 4°C). The resulting supernatant was dialyzed against M₅₀ to generate fraction I (220 ml, 33.7 mg/ml). Fraction I was loaded at 75 ml/h on a 2.5- by 20-cm DEAE-Sephacel column (Pharmacia) equilibrated in M₅₀. After being loaded, the column was washed with M₅₀ until the A₂₅₄ of the effluent reached 0. The column was then developed with an 800-ml linear gradient from M₅₀ to M₄₀₀ (75 ml/h). YZbp eluted at 230 to 280 mM NaCl. The protein fraction used in the gel shift assays eluted at 240 mM NaCl and had a protein concentration of 2 to 2.5 mg/ml.

Electrophoretic mobility shift assay. Five microliters of the DEAE fraction containing YZbp was added to a mixture containing 1 μl of 20 nM end-labeled oligonucleotide and 15 μl of binding cocktail (4 mM Tris-HCl [pH 8.0], 4 mM MgCl₂, 40 mM NaCl, 10 μg of double-stranded salmon sperm DNA per ml, and 6% glycerol). After incubation at room temperature for 30 to 120 min, the binding reaction mixtures were loaded onto a 6% nondenaturing polyacrylamide gel in 0.5× TBE (0.045 M Tris base, 0.045 M boric acid, 0.001 M EDTA) and electrophoresed at 17 V/cm. The resulting gels were subsequently processed for autoradiography or analysis on a Molecular Dynamics PhosphorImager. Dilution of protein fractions was done in protein dilution buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM 2-mercaptoethanol, 50 mM NaCl, 1 mg of bovine serum albumin per ml).

Isolation of Ho endonuclease. Biochemically homogeneous Ho endonuclease was purified by using the Kostriken and Heffron bacterial overexpression system (14). This system utilizes a plasmid containing the yeast HO gene driven by the P_R promoter of bacteriophage λ. Also carried on the plasmid is the gene for the thermolabile repressor cI857, which represses the P_R promoter at low but not high temperatures. Ho endonuclease was induced by heat shock, and the enzyme was purified by the method of Kostriken and Heffron (14) with slight modifications (10a).

RESULTS

Identification of YZbp. To test whether yeast has a factor that binds to MAT Y-Z1 borders, extracts were prepared from haploid MAT α cells and subjected to anion-exchange chromatography through DEAE-Sephacel (see Materials and Methods). The resulting fractions were analyzed by an electrophoretic mobility shift assay as described in Materials and Methods. The substrate DNA was a 44-bp double-stranded oligonucleotide called *yaz* (Table 1), which contained the last 22 bp of Y α DNA and the first 22 bp of Z1 DNA (Table 1).

A fraction eluting from DEAE between 230 and 280 mM NaCl contained an activity that bound to oligonucleotide *yaz* (Fig. 2A) (see Materials and Methods). This activity was named YZbp. YZbp activity was competed away in the presence of excess unlabeled oligonucleotide *yaz* (Fig. 2A, lanes *yaz*) but not in the presence of excess nonspecific oligonucleotide (Fig. 2A, lanes RW8). These and subsequent data demonstrated that YZbp bound in a sequence-specific fashion. We also noted a less intense band shift migrating faster than the true YZbp band shift in many of the lanes in Fig. 2. As shown below (see "At least two polypeptides are responsible for the YZbp band shift"), YZbp is a multimeric protein, and this second band reflects the breakdown of the YZbp complex.

YZbp binds to two separate half-sites flanking the Y α -Z1 border. Two complementary approaches were used to map the binding site for YZbp. The first approach examined whether mutant Y α -Z1 oligonucleotides could compete with oligonucleotide *yaz* in a standard band shift assay. Four mutant 44-bp oligonucleotides were constructed, each of which contained the same 11-bp substitution block located at a different position (Table 1). Oligonucleotide *yaz*-y1222 contained the block from positions Y12 to Y22, *yaz*-y0111 contained the block from positions Y1 to Y11, *yaz*-z0111 contained the block from positions Z1 to Z11, and *yaz*-z1222 contained the block from positions Z12 to Z22. (The sequence of the substitution block was designed by comparing the four consecutive 11-bp blocks that comprise the wild-type oligonucleotide [Y12 to Y22, Y1 to Y11, Z1 to Z11, and Z12 to Z22] and choosing the base pair at each position of the substitution block which was least represented at that same position in the wild-type blocks.) Each reaction mixture contained buffer, radioactively labeled oligonucleotide *yaz*, and one of the mutant oligonucleotides. The mutant was unlabeled and present in 2-, 20-, 250-, or 500-fold excess over the labeled wild-type oligonucleotide. The results of this experiment are shown in Fig. 2A.

The first conclusion of these studies is that each of the mutant oligonucleotides exhibited higher levels of competition than oligonucleotide RW8, the nonspecific oligonucleotide

TABLE 1. *MAT* oligonucleotides used in this study

Oligonucleotide	Sequence ^a									
MAT α										
y α z.....	cacgg	aatat	gggac	tactt	cgCGC	AACAG	TAAAA	TTTTA	TAAA	
y α z-y1222.....	tcgac	ggccg	tggac	tactt	cgCGC	AACAG	TAAAA	TTTTA	TAAA	
y α z-y0111.....	cacgg	aatat	<u>gtcga</u>	<u>cggcc</u>	<u>gtCGC</u>	AACAG	TAAAA	TTTTA	TAAA	
y α z-z0111.....	cacgg	aatat	gggac	tactt	<u>cgTCG</u>	ACGGC	CGTAA	TTTTA	TAAA	
y α z-z1222.....	cacgg	aatat	gggac	tactt	cgCGC	AACAG	<u>TAATC</u>	<u>GACGG</u>	<u>CCGT</u>	
y α z-y0612.....	cacgg	aatat	<u>ttcga</u>	<u>cgttt</u>	cgCGC	AACAG	TAAAA	TTTTA	TAAA	
y α z-y05z06.....	cacgg	aatat	gggac	<u>tatcg</u>	<u>acGGC</u>	CGTAG	TAAAA	TTTTA	TAAA	
y α z-z0713.....	cacgg	aatat	gggac	tactt	cgCGC	<u>AACGC</u>	<u>CGTTC</u>	TTTTA	TAAA	
MAT α										
y α z.....	ttttc	tttta	gtttc	agctt	tcCGC	AACAG	TATAA	TTTTA	TAAA	
y α z-y0612.....	ttttc	tttta	<u>tggga</u>	ctctt	tcCGC	AACAG	TATAA	TTTTA	TAAA	
y α z-y05z06.....	ttttc	tttta	gtttc	<u>agagg</u>	<u>gaATA</u>	CCAAG	TATAA	TTTTA	TAAA	
y α z-z0713.....	ttttc	tttta	gtttc	agctt	tcCGC	<u>AACCT</u>	<u>GCGCC</u>	TTTTA	TAAA	
y α z-y0612z0713.....	ttttc	tttta	<u>tggga</u>	<u>ctctt</u>	tcCGC	<u>AACCT</u>	<u>GCGCC</u>	TTTTA	TAAA	
Nonspecific										
RW8.....	GCTAG	TACTC	TTTAG	ACCCC	TCGGC	GGCTA	CCGCG	TGCGG	CAGGA	GCGCG CTAGA A

^a Only one strand of each double-stranded oligonucleotide is shown. Nucleotides in Y DNA (lowercase), nucleotides in Z1 DNA (uppercase), and substitution mutations (underlined) are indicated.

(Table 1). This demonstrates again the sequence specificity of YZbp and shows that none of the mutant yz oligonucleotides is completely deficient in binding. Nonetheless, among the yz oligonucleotides, yaz-y0111 and yaz-z0111 showed the lowest level of competition, as more-pronounced band shifts were seen when these mutants were present in 250- and 500-fold excesses (Fig. 2A, lanes yaz-y0111 and yaz-z0111). For instance, when oligonucleotides yaz-y0111 and yaz-z0111 were present in 250-fold excess, the resulting band shifts contained 23.3 and 24.3%, respectively, of the radioactive counts present in the control band shift that contained no competitor (Fig. 2A, lane 2). By comparison, when oligonucleotides yaz-y1222 and yaz-z1222 were present in 250-fold excess, the resulting band shifts contained 7.5 and 7.4%, respectively, of the counts present in the control. These results suggested that the binding site for YZbp resided in the middle 22 bp of yaz.

The second approach to localize the binding site for YZbp used a direct binding experiment. In this approach, the four mutant oligonucleotides were labeled and directly examined in the band shift assay. (All of the oligonucleotides had specific activities which were within 1.1-fold of each other [data not shown].) The results were in agreement with those seen in the preceding competition experiments (Fig. 2B). Oligonucleotides yaz-y1222 and yaz-z1222 had band shift patterns nearly identical with those for oligonucleotide yaz, although yaz-z1222 consistently exhibited a slightly lower level of shifting. Oligonucleotides yaz-y0111 and yaz-z0111, however, produced a significantly smaller amount of the specific band shift; quantitation indicated approximately five- to sixfold-lower binding to the latter mutant oligonucleotides.

To further localize the binding site for YZbp in the 22 nucleotides flanking the YZ border, another oligonucleotide which contained a substitution block from positions Y5 to Z6 was constructed. This oligonucleotide, called yaz-y05z06 (Table 1), was used in the same competition and direct binding experiments previously described. The competition (Fig. 3, lanes yaz-y05z06) and direct binding (data not shown) experiments indicated that YZbp bound normally to yaz-y05z06, suggesting that the binding site for YZbp consisted of two separate half-sites at positions Y6 to Y11 and Z7 to Z11.

Two more mutant oligonucleotides were therefore constructed. The first, called yaz-y0612 (Table 1), had the wild-

type sequence with the exception of a single substitution block from positions Y6 to Y12. The second, yaz-z0713 (Table 1), also had the wild-type sequence with the exception of a single substitution block, from positions Z7 to Z13. In this oligonu-

FIG. 2. Preliminary mapping of the YZbp binding sites at the MAT α YZ border. (A) Band shift assays were carried out in the presence of increasing quantities of unlabeled competitor DNAs with labeled oligonucleotide yaz used as a substrate (sequences of the competitors are in Table 1). MAT α competitor DNAs were present in each experiment in 2-, 20-, 250-, and 500-fold excesses over yaz. RW8 competitor DNA was present in 20-, 250-, and 1,000-fold excesses. Lane 1, oligonucleotide yaz in the absence of the YZbp fraction; lane 2, the YZbp band shift in the absence of competitor DNA. (B) Band shift assays were carried out in the presence of decreasing quantities of the YZbp fraction using the indicated oligonucleotides as substrates (Table 1). In each experiment, 5, 2.5, 0.5, and 0.05 μ l of the YZbp fraction were used. Lanes 1, 2, 3, 4, and 5, oligonucleotides yaz, yaz-y1222, yaz-y0111, yaz-z0111, and yaz-z1222, respectively, in the absence of the YZbp fraction.

FIG. 3. YZbp binds to two separate half-sites at the Y α -Z1 border. Band shift assays were carried out in the presence of increasing quantities of unlabeled competitor DNAs using labeled oligonucleotide y α z as a substrate (Table 1). *MAT* α competitor DNAs were present in each experiment in 2-, 20-, 250-, and 500-fold excesses over y α z. Lane 1, oligonucleotide y α z in the absence of the YZbp fraction (see Materials and Methods); lane 2, the YZbp band shift in the absence of competitor DNA.

cleotide, mutations were introduced into positions Z12 and Z13 because y α z-z1222 showed lower levels of band shifting in direct binding experiments (Fig. 2B), suggesting that distal parts of the YZbp binding site might extend beyond position Z11. Both y α z-y0612 and y α z-z0713 exhibited significantly lower levels of competition than y α z-y05z06 (Fig. 3). For instance, when y α z-y0612 and y α z-z0713 were each present in 250-fold excess, the resulting band shifts contained 100 and 32%, respectively, of the radioactive counts present in the control band shift that contained no competitor (Fig. 3, lane 2). By comparison, when oligonucleotide y α z-y05z06 was present in 250-fold excess, the resulting band shift contained 9.7% of the counts present in the control. These results confirm that the binding site for YZbp consists of two separate half-sites, one at positions Y6 to Y11 and the other at positions Z7 to Z13. Moreover, the efficient binding of YZbp required both sites, since the ability to compete with wild-type oligonucleotide was eliminated by the removal of either one.

At least two polypeptides are responsible for the YZbp band shift. In Fig. 2B, the intensity of the y α z band shift decreased with decreasing amounts of YZbp. This experiment was carried out by adding progressively decreasing volumes of the YZbp fraction to the band shift reaction mixtures. However, this experiment can be carried out in a different way: first, the YZbp fraction can be serially diluted in a standard protein dilution buffer (see Materials and Methods), and second, fixed amounts of the diluted fractions can then be added to the reaction mixtures. Under the latter conditions, the results are different, i.e., the y α z band shift breaks down into a higher-mobility form upon predilution (Fig. 4). The reason for this difference remains unclear, but control experiments show that it is not due to any proteolytic activities in the dilution buffer (data not shown). Instead, we interpret the results to mean that the original band shift is a complex between the oligonucleotide and at least two proteins and that this complex is less stable when the YZbp fraction is prediluted. Whether YZbp consists of independent polypeptides that bind separately to the half-sites or is a multimer that binds to both has yet to be determined.

YZbp binds to both *MAT* loci and is present in all cell types. One of the YZbp binding sites in *MAT* α maps to Z1 and is

therefore present in both *MAT* alleles (Fig. 1). The other binding site, however, maps to Y α and is not present in *MAT* α . Since the latter binding site plays an essential role in YZbp binding (Fig. 3), and since the last 22 bp in Y α show little sequence homology with the same sequences in Y α (1), we predicted that YZbp would have little affinity for the Y α -Z1 border of *MAT*. To test this prediction, a double-stranded oligonucleotide containing the last 22 bp of Y α DNA and the first 22 nucleotides of Z1 was constructed. This oligonucleotide, which was called y α z (Table 1), was used as a competitor in a reaction mixture containing radioactively labeled oligonucleotide y α z. Surprisingly, oligonucleotide y α z competed well in the standard band shift assay (Fig. 5, lanes y α z). YZbp therefore binds to Y α -Z1 borders.

These unexpected results suggested that YZbp might be expressed in other cell types. Extracts from *MAT* α haploid and *MAT* α /*MAT* α diploid cells were therefore analyzed by the standard band shift assay. Both extracts generated band shifts that had electrophoretic mobilities identical to the one present in *MAT* α extracts (data not shown). Moreover, the binding activities present in the *MAT* α and *MAT* α /*MAT* α extracts could be efficiently competed by oligonucleotides y α z and y α z (data not shown). (The sequence specificity and sensitivity to dilution of YZbp in these extracts have not yet been examined.) From these studies, we conclude that YZbp is expressed in all cell types, even those normally incompetent to undergo mating type interconversion. This suggests that YZbp may have other functions inside the cell in addition to its role in Ho cleavage.

YZbp has a different binding specificity at the Y α -Z1 border. YZbp binds to both *MAT* alleles, even though a critical recognition sequence in *MAT* α mapped to Y α and was therefore absent from *MAT* α . This implied that YZbp recognized different sequences in the two *MAT* alleles. To test this possibility, the YZbp binding site(s) in the *MAT* α allele was mapped by using the competition approach described above. Thus, electrophoretic mobility shift assays using radioactively labeled oligonucleotide y α z and unlabeled competitor DNAs were conducted. Four y α z-based competitor oligonucleotides were used: y α z-y0612, which contained a substitution block from positions Y6 to Y12; y α z-y05z06, which contained a substitution block from positions Y5 to Z6; y α z-z0713, which contained a substitution block from positions Z7 to Z13; and y α z-y0612z0713, which contained two substitution blocks from positions Y6 to Y12 and Z7 to Z13. The mutations in these

FIG. 4. YZbp is not monomeric. Band shift assays were carried out in the presence of decreasing quantities of the YZbp fraction with oligonucleotide y α z used as a substrate (Table 1). The assays used 5 (lane 2), 2.5 (lane 3), 1 (lane 4), and 0.2 (lane 5) of the YZbp fraction. Lane 1, oligonucleotide y α z in the absence of YZbp.

FIG. 5. Mapping of the YZbp binding sites at the *MATa* YZ border. Band shift assays were carried out in the presence of increasing quantities of unlabeled competitor DNAs with labeled oligonucleotide *yaz* used as a substrate (Table 1). *MATa* competitor DNAs were present in each experiment in 2-, 20-, 250-, and 500-fold excesses over *yaz*. RW8 competitor DNA was present in 20-, 250-, and 1,000-fold excesses. Lane 1, *yaz* in the absence of the YZbp fraction; lane 2, the YZbp band shift in the absence of competitor DNA.

oligonucleotides were in positions identical to those used to map the YZbp binding sites in oligonucleotide *yaz*. The results of this analysis are shown in Fig. 5. Quantitation showed that the addition of unlabeled oligonucleotide *yaz* in 250-fold excess decreased the band shift to 11.4% of that of the control reaction mixture containing no competitor (Fig. 5, lane 2). The addition of *yaz*-y0612, *yaz*-z0713, *yaz*-y0612z0713, and *yaz*-y05z06 in 250-fold excesses decreased the band shifts to 11.3, 33.2, 50.5, and 34.6%, respectively, of that of the control reaction.

These results show that YZbp recognizes both different and similar sequences at the *MATa* and *MATα* YZ borders. Thus, an oligonucleotide containing a substitution block from positions Z7 to Z13 in *yaz* exhibited a lower level of competition than wild-type *yaz*. The *MATa* allele, however, did not appear to have a sequence in *Ya* analogous to the half-site in *Yα* required for YZbp binding. This was shown by oligonucleotide *yaz*-y0612, which exhibited wild-type levels of competition in the standard band shift assay. The base pairs from positions Y6 to Y11 in *MATa* may have a subtle role in YZbp binding, however, as oligonucleotide *yaz*-y0612z0713 consistently exhibited lower levels of competition than oligonucleotide *yaz*-z0713, indicating that mutations from Y6 to Y12 in *Ya* further decrease the binding of YZbp to an oligonucleotide that already contained mutations from Z7 to Z13. Finally, oligonucleotide *yaz*-y05z06, which contained a mutation that spanned the *Ya*-Z1 border from Y5 to Z6, exhibited a lower level of competition than wild-type *yaz*. By comparison, a competitor oligonucleotide containing mutations in the same positions as in *yaz* competed as well as wild-type *yaz*. A comparison of the binding sites at *MATa* and *MATα* will be presented below (Discussion).

The YZbp binding sites are required for cleavage in vivo but not in vitro. We initially hypothesized that yeast contains a protein that (i) binds near the Ho recognition sequence and (ii) acts in vivo to stimulate Ho cleavage. Since the preceding studies showed that YZbp has the requisite binding characteristics, we next examined how YZ borders lacking the YZbp recognition sequences are cleaved in vivo. To do so, we constructed a set of YCp50-based shuttle vectors carrying different versions of the *MATα* border. The first vector, pRW17, contained the wild-type *MATα* sequence from Y22 to Z22. The second vector, pRW3, was identical to pRW17 except for six substitution mutations from Y7 to Y12; these mutations were in the YZbp sequence located in the *Yα* block. The third vector, pRW24, was also identical to pRW17 except for four substitution mutations from Z11 to Z14; these mutations were in the YZbp sequence located in the Z1 block.

The three plasmids were then individually transformed into a strain containing the pGALHOT vector, which carries the *HO* gene under the transcriptional control of the *GAL1* promoter. The resulting transformants were grown under noninducing conditions to mid-logarithmic phase, at which point transcription of the *HO* gene was induced by adding galactose. At various times after induction (0, 20, 40, and 60 min), cells were collected and DNAs were prepared. The resulting DNAs were digested with restriction enzyme *XhoI* and subjected to Southern analyses using a probe that hybridized to both plasmids within each strain. If cleavage failed, the resulting linearized fragment would be approximately 7,900 bp long. If cleavage occurred, then two fragments would be generated, one 4,450 bp long and the other 3,450 bp long. Plasmid pRW17, the wild-type vector, was cleaved efficiently in vivo (Fig. 6A). By comparison, pRW3 and pRW24, which contain mutations in the YZbp binding sites, were not cleaved (Fig. 6A). These data demonstrate that the YZbp binding sites are required in vivo for cleavage.

Given the unusual characteristics of the Ho recognition sequence, we wondered whether the preceding mutations might prevent in vivo cleavage through a direct effect on Ho. To rule this out, we examined how the vectors acted as substrates in an in vitro cleavage assay. This was accomplished by linearizing them with *XhoI* and treating the resulting DNAs with purified Ho endonuclease (see Materials and Methods). Ho cleaved all three DNAs efficiently in vitro (Fig. 6B). Therefore, removal of the YZbp recognition sequences eliminates cleavage in vivo but not in vitro, strongly arguing that YZbp acts as a positive activator of Ho inside cells.

DISCUSSION

In yeast, mating type switching initiates when Ho endonuclease makes a DSB at *MAT*, the yeast mating type locus. The recognition site for Ho consists of a sequence spanning the YZ border sufficient to direct cleavage in vitro and two short flanking sequences required solely for cleavage in vivo. Hypothesizing that the extra in vivo sequences were binding sites for non-Ho proteins, we examined whether yeast extracts prepared from *ho⁻* mutants contain factors that bind to the *MAT* YZ border. We identified an activity with the predicted binding characteristics by using an electrophoretic mobility shift assay. This activity, which was named YZbp, consists of at least two polypeptides, binds to both *MAT* loci, and is present in all cell types.

The binding sites for YZbp in relation to the in vivo and in vitro sequences required for Ho cleavage are shown in Fig. 7.

FIG. 6. The YZbp recognition sequences are required for in vivo, but not in vitro, cleavage. (A) In vivo cleavage was monitored as described in the text. The substrates were pRW17, which contains a wild-type YZ border; pRW24, which lacks the YZbp recognition sequence in *MAT* Z; and pRW3, which lacks the YZbp recognition sequence in *MAT* Y. The strain used in these studies was W303-1A (*MATa ade2-1 trp1-1 his3-11,15 can1-100 ura3-1 leu2-3,112*). Lane C, DNA prepared from a strain carrying only pGALHOT; the top band in the preceding lanes therefore comes from this plasmid. DNA was prepared 0, 20, 40, and 60 min after galactose induction, represented by the increasing slope. The cleavage products (arrows) are indicated. (B) In vitro cleavage was monitored as described in the text (see also Materials and Methods). Plasmids were the same as in panel A. – and +, absence and presence of Ho endonuclease, respectively, in the cleavage reaction mixture. Lane λ, λ *BstEII* marker.

At *MATα*, YZbp binds to two noncontiguous half-sites, one in the Yα block and the other in the Z1 block. At *MATa*, YZbp binds to the in vitro Ho recognition sequence and to immediately adjacent base pairs located in the Z1 block. These data make two important points. First, the YZbp binding sites overlap with sequences that are required solely for cleavage in vivo, suggesting that YZbp acts as a positive activator of Ho inside cells. Second, the recognition sequences for YZbp at the two *MAT* alleles are different. This characteristic may explain a previously isolated mutation—an A-to-G transition at Z4, i.e., within the unique YZbp recognition sequence for *MATa*—which decreases Ho cleavage by 75% at *MATa* but has no effect at *MATα* (19). It is important to state that the chromatographic fraction used in these studies is only partially purified, and the *MAT*-specific differences in YZbp binding may therefore result in part from other proteins in the extract. Nonetheless, the ability to cross-compete with *MATa* and *MATα* borders indicates that at least one of the YZbp polypeptides binds to both *MAT* alleles.

Although these findings are highly suggestive, one cannot conclude from the potentially fortuitous overlap of their recognition sequences that YZbp acts as a positive activator of Ho. To obtain direct evidence for this, a series of yeast shuttle vectors which carried mutated versions of the *MATα* YZ border were constructed. These plasmids were then used as substrates for in vivo and in vitro cleavage assays. Plasmids lacking the YZbp binding sites were cleaved efficiently in vitro by using purified Ho endonuclease, indicating that these sequences lie outside the Ho recognition sequence. These same plasmids, however, were not cleaved in vivo, strongly arguing that YZbp acts as a positive activator of DSB formation inside cells. Earlier mutational analyses by Heffron and colleagues support this conclusion (14, 18, 19).

Two major classes of *MAT* mutations which reduce the efficiency of mating type switching have been isolated by selection (9, 16, 23, 30). The first class, the *MAT-inc* mutations, map within the in vitro recognition sequence for Ho and reduce

FIG. 7. Recognition sequences for YZbp and Ho at the *MAT* loci. Shown is a closeup of the *MAT* locus near the HO recognition sequence. Ho makes a staggered DSB that centers on Z5-Z6 (base pairs are numbered going away from the YZ border). The sequence required for in vitro cleavage by Ho (Recognition sequence in vitro) and base pairs that contact Ho (20, 21) (black regions), sequences that function or fail to function in an in vivo cleavage assay (20, 21) (Inactive in vivo and Active in vivo, respectively), base pairs that are important for YZbp binding at the *MATα* and *MATa* loci (gray bars), and base pairs at the *MATa* YZ border that play a less important role in YZbp binding (+/- bar) are indicated.

switching to mitotic recombination levels. The second class, the *MAT-stk* and *MAT-survivor* mutations, are identical T-to-A transversions at position Z11 that reduce switching to a much lesser extent. The latter transversion is enigmatic for several reasons. First, a C introduced into the same position has no effect on Ho cleavage in vivo (19). Second, the *MAT* locus originally sequenced by Astell et al. contained the *MAT-stk* mutation, suggesting that the transversion exists naturally in some laboratory strain backgrounds (1). Finally, the *MAT-stk* mutation has different effects in the two mating types (8a). Nonetheless, position Z11 is in the YZbp half-site located in Z1, which suggested to us that *MAT-stk* might reduce switching by affecting the binding of YZbp. We found, however, that YZbp binds to a *MAT-stk* YZ border with an essentially wild-type affinity (data not shown). Thus, the mechanism by which this mutation decreases mating type switching remains unknown.

We have recently found that Ho cleaves DNA extremely inefficiently in an in vitro cleavage assay (10a). This characteristic prompted us to examine whether an extensively purified fraction of YZbp could stimulate the in vitro activity of Ho. Surprisingly, we found that YZbp neither stimulated nor inhibited Ho cutting in vitro (10b). Thus, the in vivo activation by YZbp is unlikely to result from a direct stimulation of Ho. Nonetheless, the interdigitation of the Ho and YZbp recognition sequences, in combination with the fact that no inhibition of in vitro cleavage was observed, suggests that the two proteins must interact productively in some fashion. We currently hypothesize that YZbp is required for the restructuring of chromatin at the *MAT* locus to allow entry of Ho. This model is based on a set of studies by Nasmyth, who showed that the Y-Z1 border of *MAT* is in a DNase I-hypersensitive site, whereas the same sequence present at *HML* or *HMR* exhibits no such hypersensitivity (17). In strains carrying *sir* mutations, however, the Y-Z1 borders of the silent loci become substrates for cleavage and, concomitantly, become hypersensitive to DNase I digestion (12, 17). Thus, the ability to cleave the mating cassettes correlates with the presence of DNase I-hypersensitive sites at their respective Y-Z1 borders.

It remains to be seen whether proteins like YZbp regulate DSB formation during meiotic recombination. It has been shown in *Schizosaccharomyces pombe*, however, that an allele of *ade6* which acts as a hot spot of meiotic recombination binds an unknown heteromeric protein (29). Moreover, since meiotic DSBs in *S. cerevisiae* occur in DNase I-hypersensitive sites, it has been suggested that the responsible endonuclease has a prerequisite for proteins that open chromatin, reminiscent of the function we propose for YZbp (20, 32, 33). Further genetic and biochemical analyses of meiotic DSBs and the *MAT* DSB will be required to address this question.

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