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In Vivo Analysis of the *Saccharomyces cerevisiae* HO Nuclease Recognition Site by Site-Directed Mutagenesis

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HO nuclease introduces a specific double-strand break in the mating-type locus (*MAT*) of *Saccharomyces cerevisiae*, initiating mating-type interconversion. To define the sequence recognized by HO nuclease, random mutations were produced in a 30-base-pair region homologous to either *MAT*α or *MAT*a by a chemical synthesis procedure. The mutant sites were introduced into *S. cerevisiae* on a shuttle vector and tested for the ability to stimulate recombination in an assay that mimics mating-type interconversion. The results suggest that a core of 8 noncontiguous bases near the Y-Z junction of *MAT* is essential for HO nuclease to bind and cleave its recognition site. Other contacts must be required because substrates that contain several mutations outside an intact core reduce or eliminate cleavage in vivo. The results show that HO site recognition is a complex phenomenon, similar to promoter-polymerase interactions.

The mating type of *Saccharomyces cerevisiae* is determined by a genetic locus called *MAT*. One of two alleles, *MAT***a** or *MAT*α, can occur at this locus. Besides *MAT*, there are two more unexpressed copies of mating-type information on the same chromosome (*HML* and *HMR*). Cells replace the allele at *MAT* with the opposite allele from one of the silent loci during an efficient recombination event. Cells can undergo mating-type interconversion as often as once per generation (7, 8, 17; for a recent review, see reference 5). HO nuclease, a site-specific double-strand endonuclease present in haploid *S. cerevisiae*, introduces a double-strand break in the *MAT* locus, initiating mating-type interconversion. The HO nuclease and mating-type interconversion system may belong to a family of recombination systems initiated by site-specific double-strand breaks. For example, double-strand breaks, introduced at specific sites by yeast mitochondrial intron-encoded endonucleases, initiate gene conversion events that transmit introns to other genes (2, 22). The mitochondrial enzyme recognizes a sequence similar in length to that recognized by HO nuclease (about 20 base pairs [bp]). Alt et al. (1) proposed that double-strand breaks may initiate immunoglobulin gene rearrangements in mammals.

HO nuclease recognizes and cleaves a sequence at the border of allele-specific sequences (called Y) and sequences to the right (called Z) present in *MAT*, *HML*, and *HMR* (Fig. 1; 7). Several mutations in *MAT* stop high-frequency mating-type switching and cleavage of *MAT* by HO (4, 10, 18, 19, 21). These inconvertible or *MAT-inc* mutations were found near the junction of allele-specific sequences Y**a** or Yα and the homologous sequences in Z and were healed during rare switching events. Later it was shown that HO nuclease cleaves between bases Z3 and Z4 on one strand and between Z7 and Z8 on the other, leaving a 4-base 3′ extension (8). Bases are numbered to the left and right from the Y-Z junction (Fig. 1).

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We earlier defined the length of the HO recognition sequence in vitro by deletion analysis of *MAT* and by synthesizing a limited set of oligonucleotide substrates homologous to *MAT*, but containing point mutations (11). The shortest sequence cleaved in vitro was 18 bp, including 12 bp of Z and 6 bp of Y, but cleavage of this substrate was not detected in vivo, and it did not stimulate recombination when HO nuclease was expressed. A 24-bp sequence containing 11 bp of Y, and 13 bp of Z inserted into a *ura3* allele stimulated recombination between *ura3* heteroalleles more than 100-fold in the presence of HO and was cleaved in vitro as well (11).

In this study, we have extended the analysis of the in vivo site recognition properties of HO nuclease. We have taken advantage of the increased levels of recombination in the vicinity of the double-strand break made by HO nuclease in developing an assay in which large numbers of mutant HO sites could be rapidly screened in vivo. HO sites complementary to either *MAT***a** or *MAT*α, but containing point or multiple mutations, were synthesized by using cassette mutagenesis (13). These sites were inserted into a yeast shuttle vector and tested for induction of recombination in a yeast strain containing a regulated source of HO nuclease. Mutations in the HO sites were identified by DNA sequence analysis. Many single mutations within the 30-bp region examined do not affect cleavage by HO. Several bases in Z and one base in Y (the core sequence) appear to play critical roles in recognition or cleavage by HO nuclease, although some HO sites containing multiple mutations outside an intact core sequence are not recognized or cleaved by HO nuclease.

MATERIALS AND METHODS

Bacterial transformations, DNA manipulations, and DNA sequence analysis. Bacterial transformations and molecular cloning were performed as described by Maniatis et al. (9). Sequence analysis was performed as described by Zaug et al. (23).

Plasmids. Plasmid pFH800 is a derivative of pUC19 containing *TRP1*, *CEN4*, *ARS1*, and HO nuclease under *GAL10*

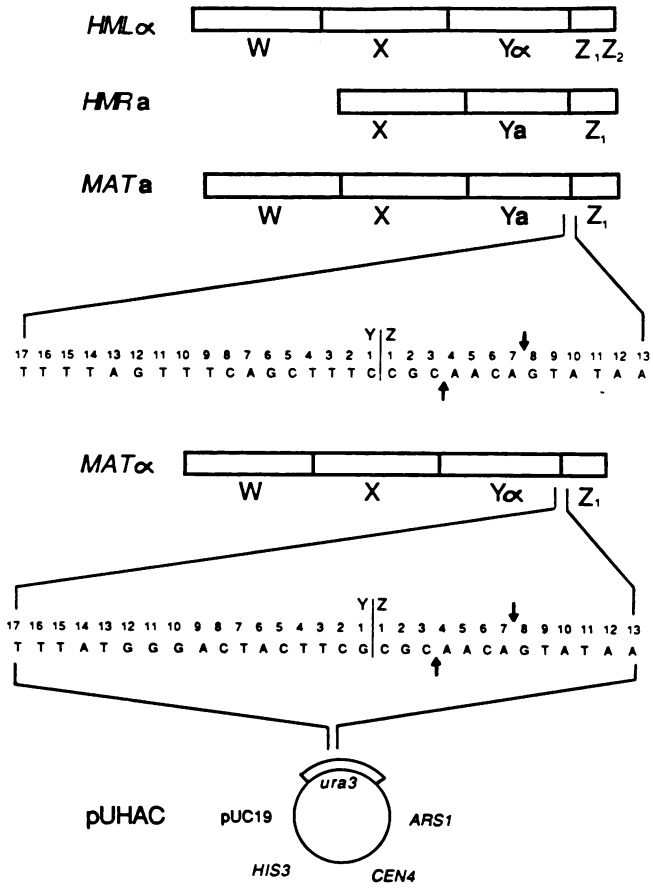

FIG. 1. Structure of mating-type loci, sequence of *MAT***a** and *MAT*α HO sites and structure of pUHAC derivatives. The top two lines show the structures of the silent mating-type loci, *HML*α and *HMR***a**. The boxes indicate regions of homology (W, X, and Z). The next line shows the structure of *MAT***a**, and below is the DNA sequence of the *MAT***a** Y-Z junction. Bases are numbered from the junction, leftward into Y and rightward into Z. The HO cleavage site is shown by arrows. Similar diagrams are shown for *MAT*α. Below is a diagram of pUHAC; synthetic 30-bp *MAT***a** and *MAT*α HO sites are present in the *ura*3 gene.

promoter control (11). Plasmid pUHAC is a pUC19 derivative with a 1.1-kbp *Hin*dIII fragment containing *ura3*, a 1.7-kbp *HIS3* *Bam*HI fragment, and a 2.5-kbp *Xba*I fragment with *ARS1* and *CEN4* (Fig. 1). An *Eco*RI linker was inserted into the unique *Nco*I site in the coding region of *ura3*.

Mutagenesis of the HO nuclease recognition sequence. Both strands of the 30-bp *MAT***a** or *MAT*α HO sites (Fig. 1) were synthesized. Point mutations were created by introducing mutant bases during synthesis at a rate of 0.9 mutations per 30 bases (13) (low-level mutagenesis). A second set of oligonucleotides was synthesized to generate sites with multiple mutations. In these oligonucleotides, mutant bases were introduced at a rate of 4 mutations per 30 bases (high-level mutagenesis). In this case, C was added at twice the concentration of the other bases to further increase the level of mutagenesis. Each oligonucleotide chain had four 5′-terminal bases cohesive to *Eco*RI termini. Complementary oligonucleotides were heated to 95°C for 3 min and then cooled to 15°C during a 3-h period. Slow cooling allowed chains carrying complementary mutations to anneal. The duplex DNAs were inserted into the *Eco*RI site of pUHAC. Since insertion of these oligonucleotides destroyed the *Eco*RI sites, ligated DNAs were digested with *Eco*RI before transformation of *Escherichia coli*. Plasmids without HO sites were linearized by this procedure and transformed the *E. coli* host infrequently. HO sites did not ligate to each other since they did not carry 5′ phosphate groups. Sequence analysis of more than 370 pUHAC derivatives confirmed that nearly all *E. coli* transformants contained pUHAC derivatives with single HO site insertions (see below).

Yeast strains and transformations. The parent yeast strain is a derivative of strain YP52 (11). Its genotype is *matΔ his3-200 trp1Δ901 lys2-101 ade2-101 ura3*::*Xho*I(*Stu*I) and contains pFH800. This strain was transformed with derivatives of pUHAC, using a modification of the lithium acetate method of Ito et al. (6). Ninety-six transformations with individual pUHAC derivatives were done simultaneously in microdilution dishes as follows: 50 μl of lithium acetate-treated cells, 0.2 to 0.3 μg of a pUHAC derivative, and 40 μg of calf thymus carrier DNA were mixed and incubated at 30°C for 30 min; 250 μl of 40% polyethylene glycol (Sigma Chemical Co.) (average M_w, 4,000) was added, and the solution was incubated for 1 h at 30°C. The cells were heat shocked at 42°C for 5 min and pelleted in the microdilution dish. The supernatant was removed, and the cells were suspended in 200 μl of a solution containing 10% YPD (1% bacto-yeast extract [Difco Laboratories], 2% Bacto-Peptone, 2% glucose), 0.9 M sorbitol, and 0.1 M ethylenedinitrilo-tetraacetic acid (pH 8.0). The contents of one well were spread on one quarter of a synthetic defined medium plate lacking tryptophan (to maintain pFH800) and histidine (to select for the pUHAC derivative).

RESULTS

Screening HO site mutations with an in vivo recombination assay. There were several reasons for examining the recognition sequence of HO nuclease in vivo instead of in vitro. First, HO recognizes and cleaves non-*MAT* yeast DNA in vitro (R. Kostricken and F.H., unpublished results; J.D.S. and F.H., unpublished results), but single point mutations in *MAT* can relieve lethality in *HO rad52-1* strains, indicating that these sites are probably not cleaved in vivo. HO does not appear to stimulate generalized recombination (12, 20), supplying additional support for the notion that few non-*MAT* sites are cleaved by HO in vivo. Lastly, an 18-bp sequence from *MAT* and a full-length *MAT* fragment were cleaved in vitro, but not in vivo (11). The recognition sequence for HO nuclease must therefore be determined in vivo, because HO has a lower specificity in vitro.

The following assay, which mimics mating-type interconversion, was used to determine whether HO nuclease cleaves mutant HO sites in vivo. Derivatives of the yeast shuttle vector pUHAC (pUC19, *ura3*, *HIS3*, *ARS1*, *CEN4*; Fig. 1), a high proportion of which contained a mutant HO site, were transformed into *E. coli* DH5α. Plasmid DNA was isolated from *E. coli* transformants, and each DNA was independently transformed into the yeast strain containing a heteroallelic *ura3* gene and plasmid pFH800. The *ura3*::*Xho*I(*Stu*I) allele present at the normal *ura3* locus on chromosome V recombines spontaneously with the plasmid-borne *ura3* heteroallele to yield Ura$^+$ colonies at a frequency of 3.4×10^{-6}. If a wild-type HO site is present in the *ura3* allele in pUHAC and HO nuclease expression is induced with galactose, cleavage of the HO site increases the recombination frequency nearly 3 orders of magnitude, to 2.6×10^{-3} (J.A.N. and F.H., unpublished observations).

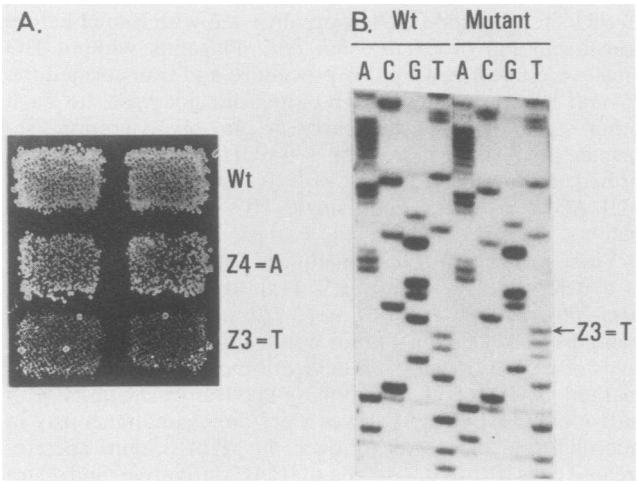

FIG. 2. Sample results of in vivo recombination assay and DNA sequence analysis. (A) Transformants of the parent yeast strain containing pUHAC derivatives with wild-type (top) or mutant (middle and bottom) HO sites were plated in duplicate to rich medium containing glycerol and replica plated to rich medium containing galactose and then to plates lacking uracil, pictured. Ura^+ papillae are indicative of HO-induced recombination. Shown are examples of high, reduced, and no stimulation of recombination. These patches are about four times larger than patches used for screening strains containing pUHAC derivatives. (B) Sample autoradiograph of sequencing gel with wild-type (Wt) or mutant HO sites. Designations of mutations in this figure correspond to the bottom strand and are therefore opposite to those shown in Fig. 3 (e.g., the mutation Z3 = T is shown in Fig. 3 as Z3 = A).

Two independent yeast transformants of each of 1,010 pUHAC derivatives were plated to medium lacking tryptophan and histidine. Cells were replica plated to rich medium containing glycerol as the carbon source and grown for 1 day to eliminate intracellular glucose, an inhibitor of the *GAL10* promoter. The cells were replica plated to rich medium containing glucose, to repress HO nuclease expression, and to rich medium containing galactose, to induce HO expression. These plates were incubated for 24 h and then replica plated to synthetic defined medium lacking uracil. Ura^+ papillae were scored after 3 days. Control strains containing wild-type HO sites in pUHAC produced many Ura^+ papillae after growth on galactose, but not glucose (Fig. 2). Cells grown on glucose typically had few or no Ura^+ papillae, as did strains containing pUHAC without an HO site or strains lacking pFH800 (data not shown). Strains containing plasmids with mutant HO sites exhibited one of three phenotypes in this assay. The HO-induced recombination level was either similar to that observed when a wild-type HO site was present, was eliminated (i.e. was similar to spontaneous levels), or was reduced to an intermediate level.

A total of 677 pUHAC derivatives with HO sites produced with a low level of mutagenesis were tested, including 353 with *MAT***a** sites and 324 with *MAT*α sites. Of these *MAT***a** HO sites, 80% stimulated recombination at levels comparable with that seen with a wild-type HO site, 3% showed reduced levels of recombination, and 10% showed no stimulation (the remaining 7% gave mixed results in the assay and were not analyzed further). Similar results were found with *MAT*α sites: 82% had wild-type recombination levels, 3% had low levels, and 11% showed no HO-induced recombination.

pUHAC derivatives containing heavily mutated HO sites were analyzed in the in vivo assay as described above. Of 210 *MAT***a** derivatives analyzed, 77% showed no stimulation, 20% had reduced recombination levels, and 3% had wild-type levels. Similar results were obtained with 117 *MAT*α derivatives: 82% showed no stimulation, 13% showed reduced levels, and the remainder were wild type.

Sequence analysis of mutant HO sites. The HO sites in pUHAC derivatives characterized in the recombination assay were sequenced to identify mutations (Fig. 2). A total of 178 *MAT***a** derivatives produced with low-level mutagenesis were sequenced, including 145 exhibiting wild-type phenotypes, 8 with low-level stimulation, and 19 with no HO-induced recombination. Of the sequenced plasmids, 52% carried wild-type HO sites and 26% had point mutations. The remainder contained multiple mutations or were mixtures of plasmids with mutant and wild-type sequences. Of those with point mutations, 64% had wild-type phenotypes, 15% showed low-level stimulation, and 21% showed no stimulation by HO in vivo. We isolated 36 derivatives containing different point mutations. No HO sites with mutations at *MAT***a** position Y1 were isolated; HO sites with Y1 mutations were synthesized separately. A compilation of recombination assay and sequencing results for *MAT***a** sites with point mutations are shown in Fig. 3.

The results for *MAT*α derivatives produced with a low level of mutagenesis paralleled the *MAT***a** results. A total of 130 *MAT*α derivatives were sequenced, including 110 with wild-type, 5 with low-level, and 15 showing no HO-induced recombination. Of these, 47% had wild-type sequences and 35% had point mutations. Of the 45 sites with point mutations, 30 had wild-type phenotypes, 5 had low-level, and 10 showed no HO-induced recombination. *MAT*α point mutant results are shown in Fig. 3. Point mutations at four positions in Y in *MAT***a** (Y9, -10, -12, and -17) and at three positions in Y in *MAT*α (Y14, -16, and -17) were not isolated. However, derivatives with mutations at these positions were found among plasmids with multiple mutations (see below). Seven bases in Z and one base in Y appear to be critical for HO nuclease recognition. This consensus sequence is shown in Fig. 3.

Point mutations at most positions do not affect HO-induced recombination levels. At those positions where mutations have effects, the effects are often similar for *MAT***a** and *MAT*α sites. Except Y4, all point mutations in either Y**a** or Yα are silent; Y4 T-to-C transitions in both **a** and α alleles reduced HO-induced recombination. Since the nucleotide sequences in the Z regions of *MAT***a** and *MAT*α are identical, one might expect that identical Z mutations in the two alleles would have the same phenotype. Usually, this was true. *MAT***a** and *MAT*α mutants with similar phenotypes are seen at positions Z1 (C to T) and Z5 (A to G), which are silent, and at positions Z2 (G to A), Z3 (C to A), and Z6 (C to T), which eliminate HO-induced recombination. However, in three cases, the same mutations in Z do not have the same effect in *MAT***a** and *MAT*α sites (i.e., Z4, Z5, and Z7; see below).

We assumed most of the highly mutated HO sites that did not stimulate recombination were inactivated by mutations in the consensus sequence. We were therefore, most interested in determining the nucleotide sequences of those sites that exhibited at least low-level stimulation, reasoning that these sites would carry mutations outside the core sequence. The nucleotide sequences of 49 *MAT***a** and 14 *MAT*α sites with multiple mutations were determined. Sequences and phenotypes of those sites without mutations in the consensus sequence are shown in Fig. 4. Clearly, HO site recogni-

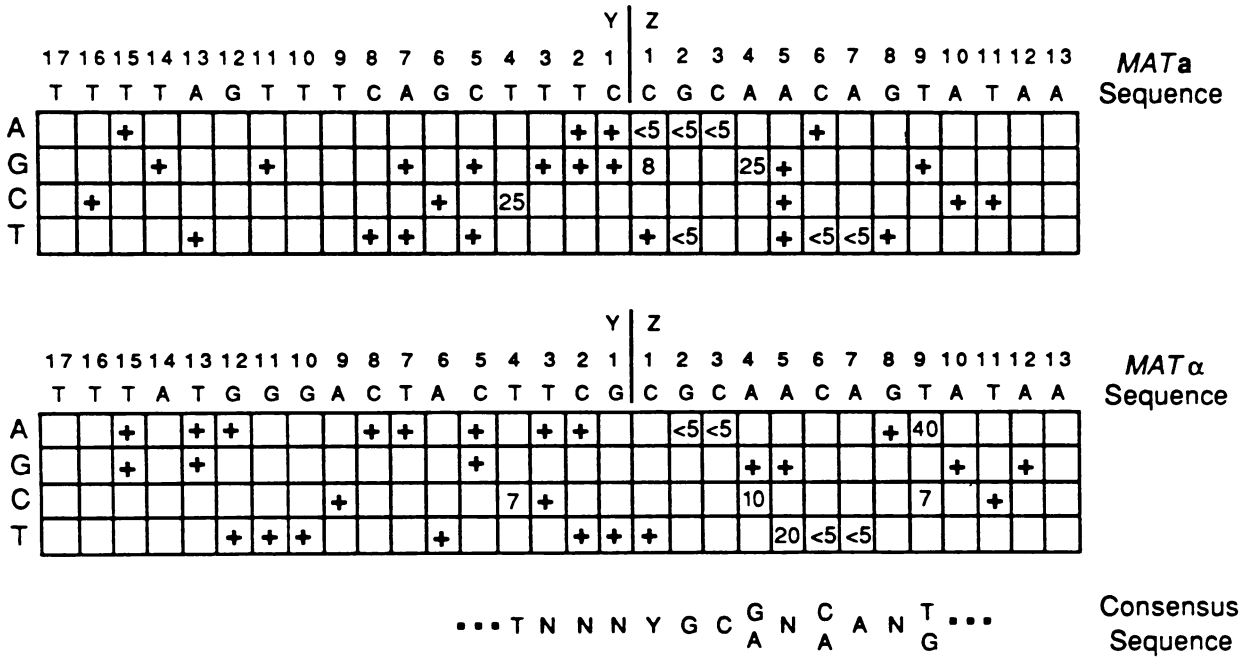

FIG. 3. Compilation of recombination results for HO sites with point mutations. For both *MAT*a and *MAT*α, bases Y1 to Y17 and Z1 to Z13 are shown. HO sites with point mutations and their phenotypes are shown in columns below each base. Phenotypes are as follows: +, wild-type levels (>100 papillae per patch); #, reduced levels of stimulation (<100 papillae per patch); values of <5 papillae per patch are similar to background recombination levels found with no HO site in pUHAC. The approximate error in these determinations is twofold. The consensus HO recognition sequence for both *MAT*a and *MAT*α is shown at the bottom of the figure.

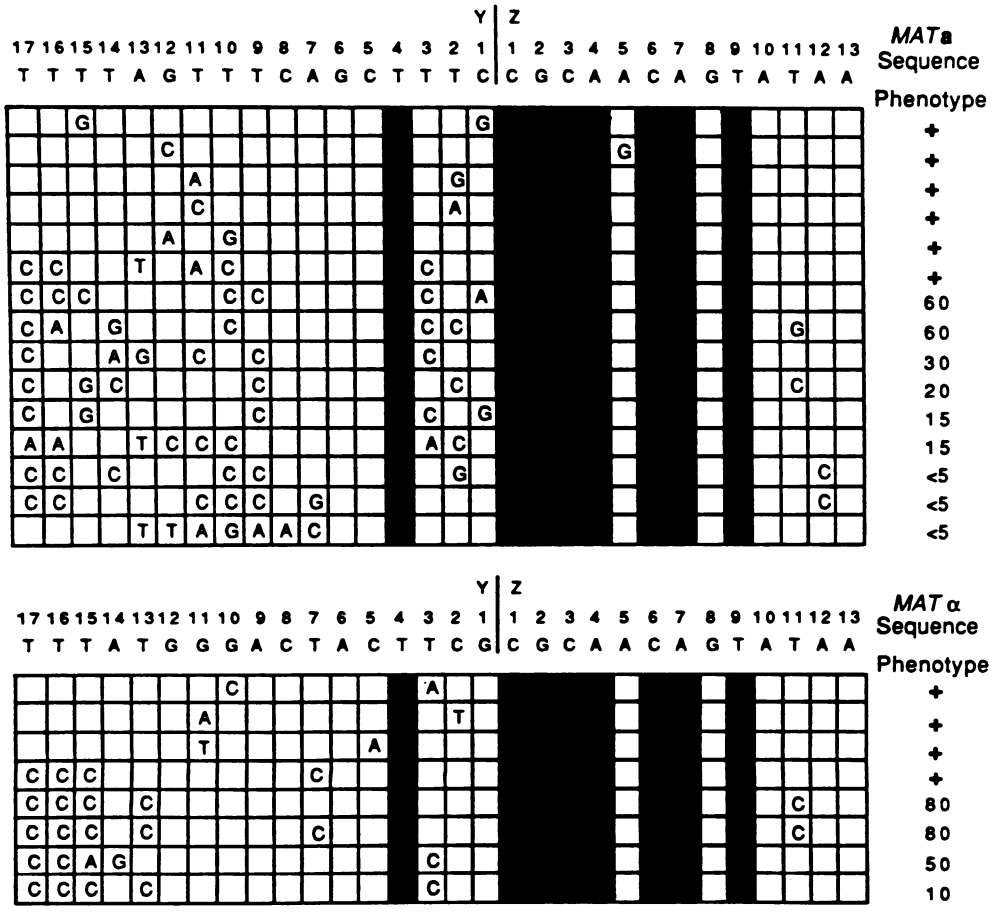

FIG. 4. Recombination levels for HO sites with multiple mutations outside the consensus sequence. Results for *MAT*a (top) and *MAT*α (bottom) HO sites are given. Consensus bases occur in the solid areas. Mutations within each 30-bp site are shown below the wild-type sequence. Phenotypes of each site, as described in the legend to Fig. 3, are shown to the right.

tion is reduced as more mutations are introduced outside the consensus sequence. Also, mutations in the region Y1 to Y3 apparently have stronger effects than those in the Y5 to Y17 region. These data show that the core sequence is necessary but not sufficient for recognition or cleavage of the HO site.

DISCUSSION

We have defined the recognition sequence of the *S. cerevisiae* HO nuclease using an in vivo assay that mimics mating-type interconversion. Previously, it was shown that the sequence recognized in vitro is at least 18 bp in length and may be 24 bp in vivo (11). In this report, we have shown there is a core of at least 8 bp necessary but not sufficient for in vivo recognition. We find that gross changes, but not point mutations, outside the core have strong effects on recognition.

Earlier results from other groups showed that mutations in *MAT* can prevent homothallic strains from undergoing mating-type interconversion at high frequency (4, 10, 18, 19). Several of these *MAT*α-*inc* mutations were shown to occur near the junction of Y and Z in *MAT*. Wieffenbach et al. (21) showed that *MAT*α-*inc* mutations occur at Z2 (G-to-A transition) and Z6 (C-to-T transition). We synthesized HO sites containing mutations at these positions and found that these mutations eliminate HO-induced recombination. Weiffenbach et al. (21) also reported that a mutation at *MAT***a** position Z11 had no effect on mating-type interconversion, in accord with our results.

Most point mutations in 30-bp HO sites that affect in vivo recognition also affect in vitro cleavage by HO (data not shown). Furthermore, contacts between HO and consensus bases Z1 to Z4, Z6, and Z8 were identified in methylation interference experiments (15; data not shown). These experiments identify purines, which when methylated, interfere with cleavage in vitro. However, there are significant differences between HO recognition in vitro and in vivo. HO recognizes a sequence longer than 18 bp in vivo, but an 18-bp site and a 4,000-bp *MAT* fragment are cleaved to the same extent in vitro (11). Increasing the length of the HO site from 24 to more than 100 bp does not significantly change the recombination enhancing or in vitro cleavage properties of the site (J.A.N. and F.H., unpublished observations). Three mutations in *MAT***a** sites gave conflicting results in in vivo and in vitro assays. These differences may be due to several factors, such as the presence of accessory factors within the cell absent in purified HO nuclease preparations, different posttranslational modifications of HO nuclease in *S. cerevisiae* and *E. coli* (the source of HO), or differences in the structure of the HO recognition site in vivo and in vitro. Clearly, HO nuclease recognition of *MAT* in yeast cells is not strictly sequence dependent, since identical sequences present in *HML* and *HMR* are not cleaved (5). Furthermore, there is some evidence for accessory proteins produced in concert with HO, which might modify HO activity. Such factors would be absent or low during galactose-induced HO expression and in agreement, we observe that galactose-induced HO stimulates mating-type switching less in *MAT***a** cells than in *MAT*α cells (J.A.N, Merl Hoekstra, and F.H., unpublished observations), but wild-type HO shows no allele preference (5). Generally, HO recognition or cleavage has reduced specificity in vitro from that seen in vivo.

On the basis of our results, we have developed a model for HO nuclease site recognition. Circular dichroism studies with synthetic HO sites show that the HO site occurs in solution as B-form DNA (J.A.N., J.D.S., and F.H., unpublished observations). Figure 5 shows two views of a space-filling model of bases Y8 to Z10 for *MAT***a**. Essential bases are shown in light gray, and the contact points, determined in methylation interference experiments, are shown in white. These contacts occur in the major groove at Z1, where only purines are allowed, and at the guanine N7 position at Z2 (top strand), Z3 (bottom strand), and Z8 (top strand). A weak contact is made in the minor groove at the guanine N5 position at Z4. Note that contact points occur on both sides of the helix and are primarily in the major groove. Methylation interference indicated that Z8 makes a strong contact with HO. However, a Z8 G-to-T transversion does not affect HO-induced recombination. This transversion did, however, reduce cleavage by HO in vitro (data not shown). Footprint analysis showed that a large region including bases Y21 to Z14 is protected by HO nuclease (J.D.S. and F.H., unpublished observations.

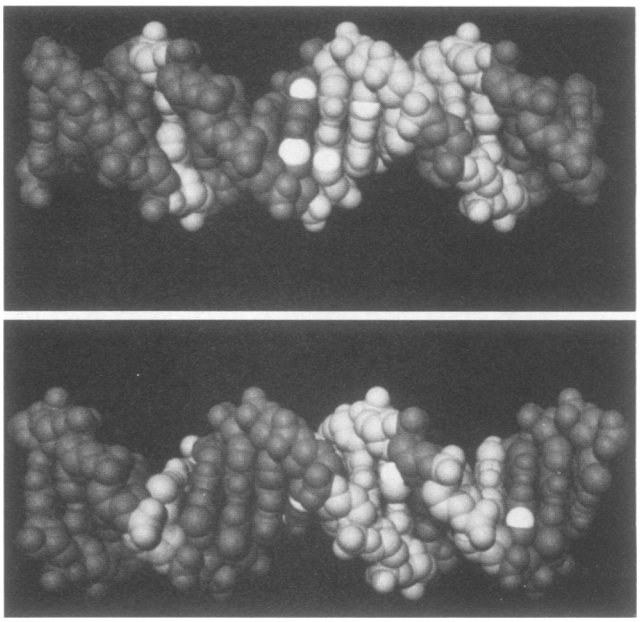

FIG. 5. Computer graphic model of the HO recognition site. Two views of duplex B-form space-filling DNA molecule including bases Y8 to Z10 of *MAT***a** are shown. The model was produced by using Biosym software on a Silicon Graphics model computer. Invariant bases are shown by the lighter shade of gray, and contact points between HO nuclease and the site, determined by mutational data and methylation interference experiments, are white. See text for details.

Recognition of the HO site by HO nuclease is not analogous to procaryotic type II restriction endonuclease site recognition. The HO site is about three times longer than a procaryotic restriction site, and many point mutations within the HO recognition sequence are silent. If recognition involved only the bases found in the consensus sequence then this site would be found at the same frequency as a six-base restriction site which is 1 in 4,096 or approximately 4×10^3 times in the yeast genome. Since this site is not sufficient for recognition, the actual recognition site occurs at random at a much lower frequency. Our results show that HO interacts with bases on both sides of the Y-Z junction. Although almost all point mutations in Y are silent, gross changes in Y can affect recognition. Furthermore, we found several cases in which identical mutations in Z have different phenotypes in *MAT***a** and *MAT*α alleles. Taken together, these results reveal the complex nature of HO site recognition and suggest that the allele-specific DNA in Y influences how the site is

recognized. We can speculate that the binding in the two regions is of two types. In this view, individual contacts in Y do not contribute to recognition, but the net effect of the combined Y contacts is significant. Bases in Y may be involved in site recognition and binding (and have somewhat relaxed specificities), while those in Z are involved in cutting (with more stringent requirements). Another possibility is that HO has separate binding domains for *MAT***a** and *MAT*α sites. Within the predicted amino acid sequence for HO are several sequences related to zinc fingers found in a wide variety of DNA-binding proteins (3, 15). Mutational studies on these potential DNA-binding domains are in progress.

HO site recognition may be similar to the way promoter and operator sequences are recognized by transcription factors, RNA polymerase, and repressors (14, 16). In these systems, minor changes in the DNA sequence often do not affect binding or activity. Instead, a few nucleotides buried within a long sequence are responsible for most of the protein-DNA interactions and changes at these positions have strong effects. While only a few bases in the HO site strongly affect HO recognition, it is probable that many bases within the region from Y12 to Z12 interact in a cooperative manner to increase the specificity of the recognition and cleavage.

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