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Genetics

A 24-base-pair DNA sequence from the *MAT* locus stimulates intergenic recombination in yeast

(HO endonuclease/double-strand break repair/gene conversion/mating type/*Saccharomyces cerevisiae*)

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ABSTRACT **HO nuclease is a site-specific double-strand endonuclease present in haploid *Saccharomyces cerevisiae* undergoing mating type interconversion. HO nuclease initiates mating type interconversion by making a double-strand break within the *MAT* locus. To define the recognition site for the enzyme *in vitro*, we have constructed a number of point mutations and deletions within or adjacent to the HO recognition site. Digestion of these substrates with HO *in vitro* reveals that the minimal recognition site is 18 base pairs long, although several shorter substrates and substrates containing point mutations are cleaved at low levels *in vitro*. A 24-base-pair HO recognition site stimulates homologous recombination when present in a region unrelated to *MAT*. Recombinants arise from both gene conversion and crossover events. The identification of the HO recognition site provides a way of introducing a defined initiation site for recombination.**

The mating type of a haploid cell of the yeast *Saccharomyces cerevisiae* is determined by a single genetic locus on chromosome III, the *MAT* locus. *MAT* shares DNA sequence homology with two unexpressed copies of mating type information, *HML* and *HMR* (Fig. 1; refs. 1–4). A cell of one mating type can change to the opposite mating type. During mating type interconversion one of the two silent "cassettes" donates sequence information to *MAT*. All three mating type cassettes share DNA homology flanking the allele-specific sequences. We have numbered the bases in the allele-specific DNA (Y) and the homologous sequences to the right of this (Z) starting with the Y/Z junction (Fig. 2).

Aside from *HML* and *HMR*, several additional genes are required for mating type interconversion. These genes include *HO* (homothallic; ref. 5) and three members of the *RAD52* double-strand break repair epistasis group (refs. 6 and 7; J. Game, personal communication; J. Haber, personal communication). The *SWI* genes (1–5) control *HO* expression and are required for mating type switching (8, 9).

The first evidence to suggest the mechanism of mating type interconversion was the observation of a double-strand break at *MAT* in cells undergoing mating type switching (6, 10–12). HO nuclease cleaves *MAT*, leaving a 4-base-pair (bp) 3′ extension near the Y/Z junction (Fig. 2). The location of the break found *in vitro* corresponded to the break made *in vivo* (13, 14). Mutations in *MAT* that prevent mating type interconversion were found close to the cleavage site and are more refractory to digestion *in vitro* (14, 15).

A double-strand break at *MAT*, the recipient for interconversion, is in agreement with the model for gene conversion first proposed by Stahl (ref. 16; for review, see ref. 17; for adaptation to mating type interconversion, see ref. 12). According to this model, the broken ends can invade homologous duplex DNA sequences, in this case the silent cassette,

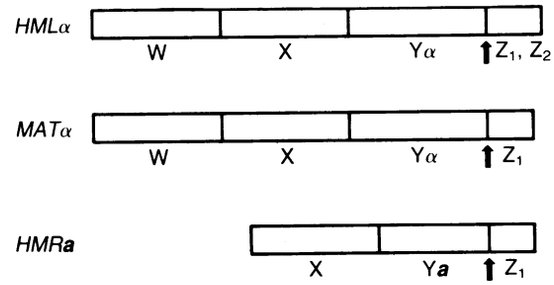

FIG. 1. Comparison of the three "cassettes" of mating type information. Unexpressed mating type information is present at *HMLα* and *HMRa*. The cassettes are homologous in the regions labeled X and Z_1, which flank the allele-specific DNA (Y). Location of the double-strand break made by HO nuclease is indicated by the arrow.

and prime DNA replication from the 3′ ends. During mating type interconversion, new copies of mating type information are transferred to *MAT* but, unlike normal conversion events, mating type switching occurs without concomitant crossing-over. We have carried out a mutational analysis of the sequences required for HO recognition. We introduced copies of the HO recognition sequence into new locations in the yeast genome. This has allowed us to examine HO-mediated mitotic recombination.

MATERIALS AND METHODS

Plasmids and Bacterial Strains. pRK128 is a plasmid that contains the *HO* gene fused to the bacteriophage λ *cro* gene. pDC287 and pDC307 are *Eco*RI–*Hin*dIII subclones of *MAT* containing the *a* and *α* alleles, respectively, cloned into pBR322 (12, 18). All *MATa* alleles used in this study are "survivor" alleles (19). pRK113 is a derivative of pUC9 that contains the *Bgl* II–*Hin*cII fragment of *MATa*. This fragment includes the Y/Z junction of *MAT*. pSE271 is a shuttle vector containing *TRP1/ARS1/CEN4* in pUC19 (courtesy of P. Hieter and R. Davis, Stanford University). pGAL-HO is a shuttle plasmid containing a transcriptional fusion between the *GAL10* promoter and *HO* kindly supplied by R. Jensen and I. Herskowitz (6).

Construction of BAL-31 Deletion Mutants. Deletion mutants of the Z region of *MAT* were constructed from plasmid pRK113 (see above) by use of BAL-31 nuclease (13). BAL-31 deletion mutants of Y were constructed starting with a subclone of *MAT* that contains 12 bp of Z (pFH700) but 62 bp of Y.

Purification of HO Nuclease and Reaction Conditions. *Escherichia coli* strain DH1 containing pRK128, a plasmid specifying a thermoinducible *HO* gene (13), was grown at 31°C to log phase (OD_{600} 0.36) in 400 liters of Luria–Bertani (LB)

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Abbreviation: bp, base pair(s).

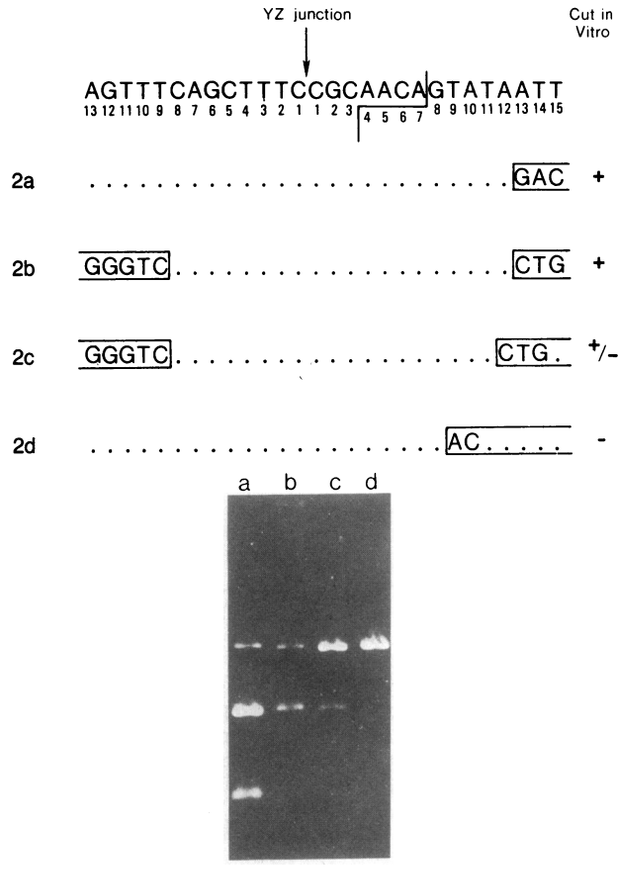

FIG. 2. Sequences in Z required for HO recognition. Substrate 2a contains 67 base pairs (bp) of Y*a* and 12 bp of Z (pFH700). (In Figs. 2–4, dots indicate identity with the sequence shown at the top, and sequences within boxes indicate vector sequences. At the bottom of each figure is shown an agarose gel; the lanes of each gel correspond to the substrates listed above.) pFH700 is digested (±) by HO. Substrate 2b, containing bases Y8–Z12, is also digested by HO. Substrate 2c, containing bases Y8–Z11, is digested less completely (±) than pFH700. Substrate 2d is refractory to digestion (−) by HO. At least 12 bp of Z are required for recognition by HO.

medium, induced for 90 min at 42°C, harvested by centrifugation, and stored at −70°C. Cells (125 g) were lysed and HO endonuclease was purified through the phosphocellulose step as described (13, 20). HO activity was assayed in buffer containing 10% (vol/vol) glycerol, 100 mM NaCl, 5 mM dithiothreitol, 10 mM $MgCl_2$, and substrate DNA.

To assay for HO digestion *in vitro*, we first linearized plasmids and then treated the DNA with HO nuclease. This digestion served two purposes: each substrate was tested for the presence of any potential inhibitors to the HO digestion, and the size of the fragments was diagnostic for cutting at the correct site.

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 380A. The strands were annealed and cloned as described (13).

Construction of Yeast Strains. Strain YP52 (*MATa, ho, trp1Δ901, his3Δ200, ura3-52, lys2-801, ade2-101*) was obtained courtesy of P. Hieter and R. Davis and used to construct strains JY13, JY14, and JY15. A deletion of *MAT* was constructed by cloning *MATa* into pUC19 and deleting two internal *Hin*cII fragments. *URA3* was inserted into this plasmid and used as the selective marker in the two-step gene-disruption procedure of Scherer and Davis (21).

The tandem *ura3* heteroalleles were constructed in three steps. The *ura3-52* allele was replaced with a wild-type copy of *URA3* by transformation with linear *URA3* DNA. A pUC19 derivative was constructed containing *URA3* (cloned as a *Hin*dIII fragment) and *HIS3* (cloned as a *Bam*HI fragment). The *Xho* I linker mutation was introduced at the *Stu* I site of *URA3* (to produce *ura3-XhoI*) in this plasmid, and the copy of *URA3* in the yeast strain was replaced with *ura3-XhoI* using the two-step gene-disruption procedure (21). The second *ura3* alleles containing either *Eco*RI or HO nuclease sites were constructed in the same pUC19/*URA3*/*HIS3* derivative by converting the *Nco* I site within *URA3* to an *Eco*RI site. Two complementary oligonucleotides containing the 24-bp HO recognition site were inserted into this *Eco*RI site (11 bp in Y and 13 bp in Z). Derivatives containing the HO site in either orientation were identified by DNA sequencing and transformed into yeast, and the structure of the tandem repeat was confirmed by Southern (22) hybridization. Strain JY13 contains only the *Eco*RI site; JY14 and JY15 contain HO sites in opposite orientations (see Fig. 5). A *gal10-HO* gene fusion was excised from pGAL:HO and inserted into pSE271, yielding pFH800, the source of HO nuclease in JY13, JY14, and JY15.

RESULTS

Deletion analysis had suggested that HO nuclease recognized at most a 40-bp sequence adjacent to the Y/Z junction in *MAT* (12–15). We used HO nuclease to analyze the sequence requirements of the enzyme *in vitro*. These enzyme preparations are free of other endonuclease activities, based on a comparison with extracts made from the same strain but missing the HO-producing plasmid pRK128 (data not shown). The enzyme produced by pRK128 in *E. coli* has the same amino acid sequence as that made in *S. cerevisiae* because the ATG of the λ *cro* gene precisely replaces the ATG of *HO* (13). The relative ability of various mutant and wild-type substrates to be digested by the nuclease is not affected by salt or glycerol concentration (data not shown).

Sequences in Z Required for Digestion by HO Nuclease *in Vitro*. We have tested each of the substrates shown in Fig. 2 for digestion by HO nuclease *in vitro*. The deletions in Z were constructed in one of two ways. Substrates 2a and 2d were constructed by BAL-31 digestion of the *MATa* allele and include Y sequences to the *Bgl* II site 66 bp from the Y/Z junction. Substrates 2b and 2c contain synthetic oligonucleotides with *Sma* I–*Pst* I ends inserted in pUC19. Sequences were verified by Maxam–Gilbert DNA sequencing (23). The boxed sequences in Figs. 2–4 correspond to the vector which, in a few cases, extends the homology to *MAT* as discussed below.

Substrate 2a, which leaves bases Z1–Z12 intact, is digested by HO as well as an intact clone of *MATa* (determined by codigestion of the two substrates). Substrate 2b is digested one-fifth to one-half as well as a complete copy of *MATa*. Substrate 2c is digested one-tenth as well as a complete copy of *MATa*. Substitution of cytosine at position Z11 or Z12 also results in one-tenth as much digestion (data not shown). Substrate 2d, containing only 7 bp of Z, is completely refractory to digestion (Fig. 2, lane d). A sequence polymorphism at base Z11 was found among several isolates of mating type loci (1). The polymorphism has an adenine at Z11 instead of a thymine. This change makes the sequence more refractory to cutting in short substrates but has no effect when sequence homology to *MATa* is extended by 4 bp. Thus, for the *MATa* allele shown, at least 12 bp of Z are required for digestion by HO nuclease *in vitro*.

Sequences in Y Required for Digestion by HO Nuclease. The same strategy was followed in analyzing the sequences in Y required for HO digestion. Substrates 3b, 3f, and 3g (Fig. 3) were constructed by BAL-31 digestion of Y in plasmid pFH700. The remaining substrates in Fig. 3 were constructed by inserting annealed oligonucleotides into pUC19. When

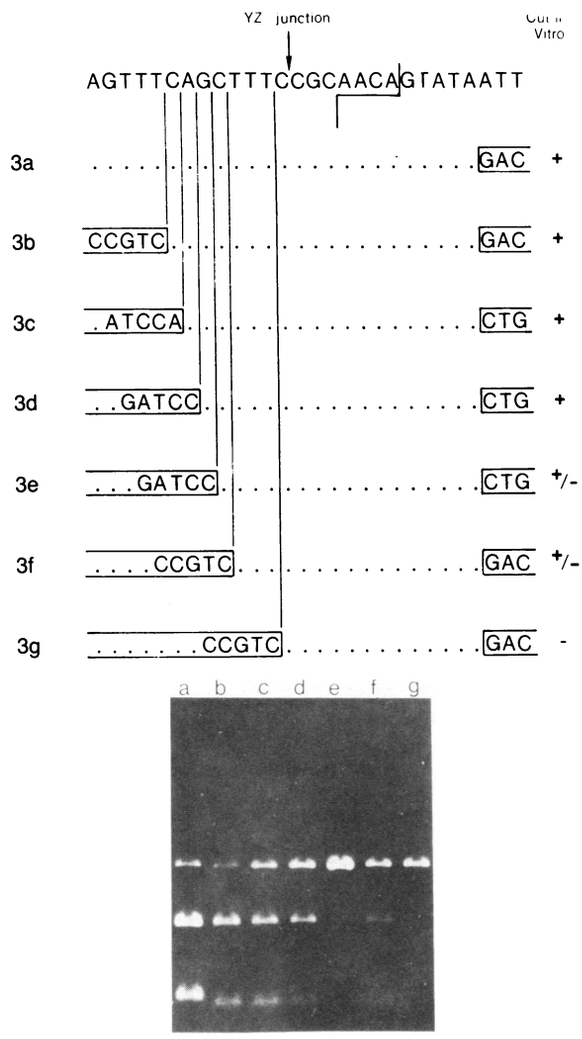

FIG. 3. Sequences in Y required for digestion by HO. Substrate 3a is pFH700 (substrate 2a in Fig. 2). The remaining substrates (3b–3g) contain bases Z1–Z12 and various lengths of Y*a*. Substrates 3b, 3c, and 3d contain bases Y1–Y8, Y1–Y7, and Y1–Y6, respectively; these substrates are cleaved by HO. Altering bases Y1–Y6 reduces digestion by HO. This is shown for substrates 3e, 3f, and 3g (lanes e–g), which contain 5, 4, and 1 bp of homology to Y*a*, respectively. Thus, 6 bp of Y*a* are required for HO recognition.

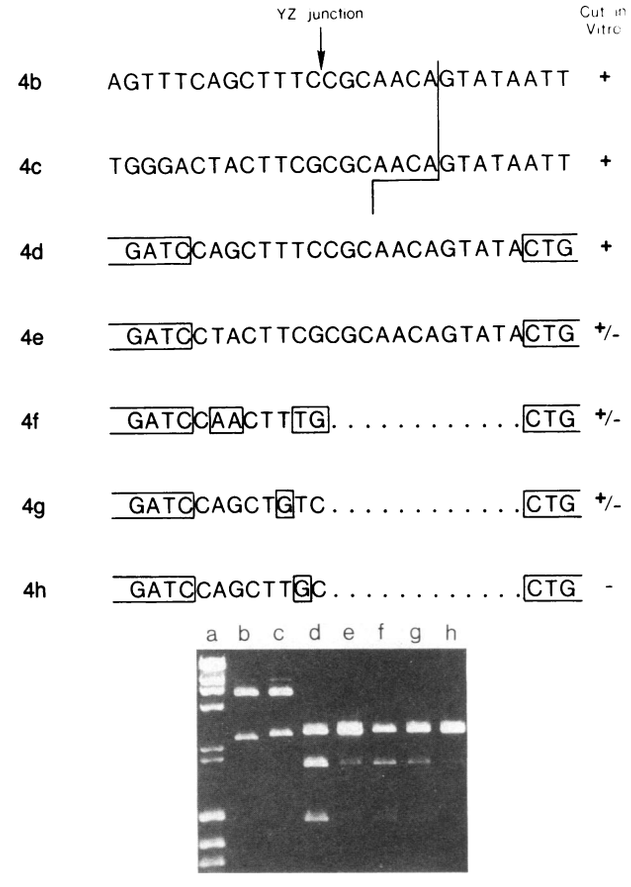

FIG. 4. Comparison of digestion of substrates containing single or multiple base changes within Y. Lane a shows bacteriophage λ DNA digested with *Hin*dIII. Lines 4b and 4c show the sequences of *MAT**a*** and *MATα*, respectively, near the Y/Z junction. Both alleles are recognized by HO when complete copies of *MAT* are used (lanes b and c). Lane d shows the minimal substrate for a Y*a* allele (bases Y6–Z12; see Figs. 2 and 3). The remaining substrates (4e–4h) contain base changes within Y and are refractory to cleavage by HO (lanes e–h). Included is substrate 4e, which contains the same length of *MAT* homology as 4d but contains Y*α* allelic bases instead of Y*a*. Substrate 4h contains a transversion at base Y2. This base is a pyrimidine in both Y*a* and Y*α* sequences.

only bases Y1–Y8 are intact, *in vitro* digestion by HO endonuclease is reduced, at most, by a factor of 2–5. A transversion mutation at either base Y7 or base Y8 gives a substrate that is digested at least one-half to one-fifth as well as a complete copy of *MAT* (Fig. 3, lanes c and d).

Mutations at base Y6 have a more pronounced effect upon *in vitro* digestion. Substrates 3e and 3f are identical for base pairs Y1–Y5 but contain mutations at base Y6. Substrate 3e contains a G→C transversion and is digested not more than one-tenth as well as an intact substrate. Substrate 3f contains a G→T transversion and also shows greatly reduced digestion. A plasmid containing only Y1 and Z1–Z12 was not digested appreciably (Fig. 3, lane g). Thus, Y6 appears to be the first base in Y that has a pronounced effect on digestion by HO.

HO Digestion of Substrates Containing Single and Multiple Base-Pair Changes Within Y. In Fig. 4, lanes b and c, we compare the digestion of intact *MAT**a*** and *MATα* substrates. Both appear to be equally good substrates. Digestion of two substrates corresponding to either *MAT**a*** (lane d) or *MATα* (lane e) for bases Y8–Z12 was compared. The *MATα* substrate is digested not more than one-fifth as completely as the *MAT**a*** substrate. This result suggests that HO nuclease requires a longer *MATα* recognition sequence than a *MAT**a*** sequence.

MATα and *MAT**a*** have four base-pair differences within the first eight bases of Y. We constructed a hybrid *MAT* substrate containing a mixture of ***a*** and *α* allelic bases (Fig. 3, substrate 3f). This substrate is digested at a level intermediate between that of either parent, indicating that the enzyme does not simply recognize ***a*** or *α* alleles. A *MAT**a*** substrate containing a transversion at base Y3 (3g) is a poor substrate. Similarly, a clone containing a transversion at base Y2 is also a poor substrate (3h). These results should be considered in light of the earlier results for sequence requirements in Z. Both of these results suggest that the further away a mutation is located from the Y/Z boundary, the less effect it has on *in vitro* digestion by HO.

HO Nuclease Will Stimulate Recombination at a 24-bp HO Recognition Site. We inserted a synthetic HO recognition site within one of two *ura3* heteroalleles to determine whether a site recognized by HO *in vitro* will stimulate mitotic recombination *in vivo*. The structures of the *ura3* heteroalleles are shown in Fig. 5. These strains contain a galactose-inducible *HO* gene.

JY13 is a control strain. One copy of *ura3* in JY13 has had the *Nco* I site converted to an *Eco*RI site (Fig. 5). The second

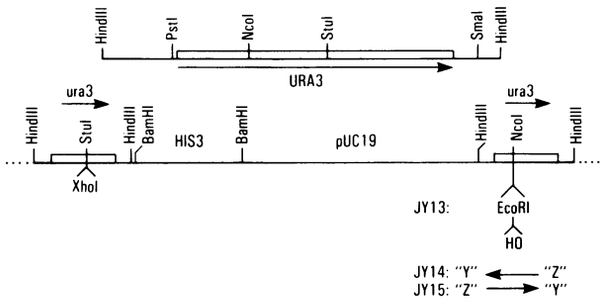

FIG. 5. Structures of *ura3* heteroalleles. (*Upper*) Structure of *URA3* gene region. (*Lower*) Structures of heteroalleles in strains JY13, JY14, and JY15. All strains contain an *Xho* I linker mutation in the *Stu* I site of one *ura3* gene. JY13 contains an *Eco*RI linker mutation in the *Nco* I site of the second gene. JY14 and JY15 contain a synthetic HO recognition site inserted in the *Eco*RI site. The orientation of the HO recognition site is indicated below.

copy of *ura3* in JY13 has had the *Stu* I site converted to an *Xho* I site. JY14 and JY15 are identical to JY13 but contain a synthetic HO recognition site inserted in opposite orientations in the *Eco*RI site (Fig. 5).

The three strains were grown to mid-log phase (about 5×10^7 cells per ml) in medium containing glycerol as a carbon source. Cells were diluted 1:2 and grown for 6 hr in fresh media containing either glucose or galactose as a carbon source to provide either repression or induction of *HO*, respectively. To determine whether induction of *HO* causes cell death, we compared the number of viable cells, determined with a hemacytometer, and the number of colony-forming units. The ratio of hemacytometer counts to plate counts was 75–80% for both cultures as normally observed, and thus there was no appreciable cell death on induction.

Following growth, cells were harvested, washed, and plated on synthetic defined medium lacking uracil as well as on complete (YPD) plates. Ura^+ colonies were counted and tested for their His phenotype. His^- colonies were identified by replica-plating colonies from complete medium to defined synthetic medium to identify His^- Ura^- recombinants. The recombination frequencies determined by selecting Ura^+ colonies or screening unselected colonies are similar, indicating that there were not a significant number of mixed colonies, which might be expected if recombination events occurred in phase G_2 of the cell cycle. Approximately 30% of the cells had lost pFH800 (the source of HO nuclease) before induction. The values given in Table 1 are normalized for this loss.

A 24-bp sequence (Y11–Z13) stimulates mitotic recombination 100- to 150-fold over spontaneous levels for both Ura^+ and Ura^- His^- recombination events. No stimulation was observed for a strain that contains an *Eco*RI linker mutation alone. JY14 and JY15 contain HO sites in opposite orientations within *ura3* but show no difference in recombination frequency. When *HO* is repressed, 20–25% of the Ura^+ recombinants are His^+ and must have arisen by gene-conversion events. When *HO* is induced, this fraction increases to 30–50%.

Following HO induction, the majority of colonies appear to have arisen from cells that have not undergone recombination. To verify that the structure of the tandem *ura3* repeat in these cells is identical to the parent, we tested whether reinduction leads to an increase in Ura^+ colonies. One hundred colonies each from repressed and induced JY13, JY14, and JY15 cells were "patched" to synthetic defined medium lacking histidine, replica-plated to media containing either glucose or galactose, then replica-plated to synthetic defined medium lacking uracil to score for Ura^+ recombinants. All JY14 and JY15 Ura^- His^+ colonies gave an increased frequency of Ura^+ papillae on galactose medium as compared to glucose medium. JY13 showed no difference. Thus, the 95% of induced cells that are still Ura^- His^+ have a functional copy of the HO recognition site and can give rise to Ura^+ recombinants.

To confirm the genetic results, we performed Southern hybridization experiments. DNA was isolated from a total of 40 independent Ura^+ colonies from JY14 and JY15 following induction or repression and analyzed to determine the physical structures of the recombinants. All Ura^+ His^- recombinants arose via a simple cross-over to generate a single *URA3* gene. Ura^+ His^+ colonies arose in one of three ways. In *HO*-repressed cells, either copy of *ura3* was converted to *URA3*. In *HO*-induced cells, only the copy of *ura3* containing the HO site was converted. One colony, from repressed cells, contained a triplication of the region and may have arisen from unequal sister-chromatid exchange. About 1% of induced cells are Ura^- His^- (Table 1). Eighteen Ura^- His^- colonies from JY14 and JY15 were analyzed by Southern hybridization; all had a single *ura3* gene containing the *Xho* I site.

DISCUSSION

We have determined that the recognition site for HO nuclease includes bases that are 18 bp apart. The recognition site includes bases within the Y or allele-specific region of *MAT*. The recognition sequence includes most of the internal bases, particularly those immediately adjacent to the Y/Z junction (this work; M. Zoller and M. Smith, personal communication; refs. 13 and 14). Most of the single base-pair changes we have tested show reduced but appreciable digestion by the enzyme. This suggests that, *in vitro*, the enzyme does not have a precise recognition site, unlike a prokaryotic type II restriction enzyme. Recognition of HO sites by HO nuclease may be more closely related to other DNA-binding proteins, such as catabolite-activator protein (CAP) and λ repressor, which recognize a consensus sequence and must make contact at a certain number of bases before a reaction can take place.

A 24-bp sequence (Y11–Z13) stimulates recombination when it is introduced into an *HO* haploid strain of yeast. A shorter sequence (Y8–Z12) does not stimulate recombination (J.A.N. and F.H., unpublished observation). Thus, there is a

Table 1. Effect of HO nuclease on mitotic recombination

	Recombination frequency					
	Glucose		Galactose		His^+ Ura^+/Ura^+	
Strain	Ura^+	Ura^- His^-	Ura^+	Ura^- His^-	Glucose	Galactose
JY13	3.4×10^{-4}	$<6.4 \times 10^{-4}$	2.9×10^{-4}	$<7.8 \times 10^{-4}$	0.36 (864)	0.39 (983)
JY14	6.6×10^{-4}	$<5.9 \times 10^{-4}$	9.5×10^{-2}	3.2×10^{-2}	0.25 (767)	0.48 (1047)
JY15	6.0×10^{-4}	$<7.3 \times 10^{-4}$	6.8×10^{-2}	2.9×10^{-2}	0.25 (1370)	0.32 (1085)

Recombination frequencies are expressed as the number of Ura^+ colonies or His^- Ura^- colonies per cell plated for cells grown in medium containing either glucose or galactose. His^+ Ura^+/Ura^+ is the ratio of His^+ Ura^+ colonies to Ura^+ colonies. Numbers in parentheses indicate the number of colonies tested.

discrepancy between the size of the recognition site defined *in vitro* and that observed to stimulate recombination *in vivo*. Similarly, a single base-pair change at *MAT* can make the strain inconvertible and relieves the lethality of *MATα* in an *HO rad52-1* background (15, 19), but these same point mutations appear to be weakly digested by HO nuclease *in vitro* (data not shown). The facts that lethality is relieved in this strain background and HO has not been observed to stimulate recombination at any sites other than *MAT* suggest that no other HO recognition sites are present within the yeast genome. However, we have observed weak digestion within a number of cloned yeast genes (R. Kostriken and F.H., unpublished data). There are several explanations for these discrepancies. Perhaps the enzyme has a lower specificity *in vitro* than *in vivo* due to the lack of interactions with additional cis- or trans-acting factors. Alternatively, HO nuclease may be unstable when partially purified and the breakdown product has a lower specificity than the native protein.

A *MATa* cell preferentially replaces the allele-specific sequences from *MAT* with those present at *HML* (24). The greater number of bases required for recognition of *MATα* than of *MATa* suggests that HO nuclease must be capable of discriminating between the two alleles and thus might play a role in determining the directionality of switching. Previously it was observed that *MATa* and *MATα* alleles were differentially recognized in the mutant HO-1 (25).

We find that a 24-bp HO site stimulates mitotic recombination between tandem *ura3* heteroalleles by 2 orders of magnitude over the level of spontaneous recombination. This frequency is much lower than the frequency of mating type interconversion. Preliminary evidence suggests that this difference is due to the length of the *MAT* sequence present (J.A.N. and F.H., unpublished data). It is important to note that we used the *MATa* survivor allele. It was suggested by Malone and Hyman (19) that this allele may not be cleaved by HO, thus allowing *HO rad52-1* strains to survive. We find that HO cleaves the *MATa* survivor allele *in vitro* and that this allele stimulates recombination in *HO* cells.

During mating type interconversion, a double-strand break is introduced at *MAT* in the Z region, immediately adjacent to the allele-specific sequences. Thus the initiating event in mating type interconversion takes place on the recipient for gene conversion as predicted by the double-strand break repair model (16). When an HO site is present within one of two *ura3* alleles and *HO* is induced, we find that the *ura3* gene containing the HO site is the recipient gene during conversion, in agreement with this model.

We find that 25–35% of spontaneous *ura3* heteroallele recombinants result from gene-conversion events. This contrasts with results reported by Jackson and Fink (26), who studied spontaneous recombination between direct duplications of a 24-kbp region containing the *his4* locus. They found that 75–88% of the recombinants resulted from gene-conversion events. This difference could be due to differences in the length of homologous sequences, the type of mutations present, the sequences involved, or a combination of these factors.

Following induction of *HO*, Ura^+ recombinants were isolated that had arisen both by crossing-over and by gene conversion. Both types of recombination events are stimulated by HO nuclease. This recombination is similar to that observed for spontaneous mitotic recombination and recombination stimulated by transformation of yeast with linear DNA. Although the HO-stimulated recombination we describe exhibits an increase in the relative frequency of gene conversion to cross-over events when compared to spontaneous recombination events, it is different from what is observed during mating type interconversion, which always occurs without crossing-over and loss of the genes between *MAT* and the silent cassettes. We hypothesize that there must be additional sequences or trans-acting factors that allow mating type interconversion to occur without crossing-over.

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