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MATING-TYPE GENE SWITCHING IN *SACCHAROMYCES CEREVISIAE*

James E. Haber
Department of Biology, Brandeis University, Waltham, Massachusetts 02454-9110;
e-mail: haber@hydra.rose.brandeis.edu

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ABSTRACT

Saccharomyces cerevisiae can change its mating type as often as every generation by a highly choreographed, site-specific recombination event that replaces one *MAT* allele with different DNA sequences encoding the opposite allele. The study of this process has yielded important insights into the control of cell lineage, the silencing of gene expression, and the formation of heterochromatin, as well as the molecular events of double-strand break-induced recombination. In addition, *MAT* switching provides a remarkable example of a small locus control region—the Recombination Enhancer—that controls recombination along an entire chromosome arm.

CONTENTS

INTRODUCTION 562
Cell Lineage 564
First Models of MAT Switching 564
SILENCING OF *HML* AND *HMR* 567
Establishment and Maintenance of Silencing 570
MAT SWITCHING: A MODEL FOR HOMOLOGOUS RECOMBINATION 572
A New View of Double-Strand Break Repair: Synthesis-Dependent Strand Annealing 577
DONOR PREFERENCE 578
Identification of a Recombination Enhancer 581
The RE Affects Recombination Along an Entire Chromosome Arm 581
Is RE Portable? 583
Changes in Chromatin Structure Associated with Regulating RE 584
Functional Domains of the RE 584
Role of the MATα2p-Mcm1p Operator 585
*Activation of RE in MAT***a** *Cells Depends on Mcm1 Protein* 586

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Donor Preference Genes 587
Possible Relation of Donor Preference to Mechanism in Fungi, Metazoans, and Mammals 588
EPILOGUE 590

INTRODUCTION

In *Saccharomyces cerevisiae*, mating type is determined by two different alleles of the mating-type (*MAT*) locus. Like many other fungi, budding yeast has acquired the capacity to change some cells in a colony from one haploid mating type to another. The subsequent mating of cells of opposite mating type enables these homothallic organisms to self-diploidize. The diploid state appears to provide fungi with a number of evolutionarily advantageous strategies unavailable to haploids, most notably the ability to undergo meiosis and spore formation under nutritionally limiting conditions. Mating-type gene switching in *S. cerevisiae* is a meticulously choreographed process that has taught us much about many aspects of gene regulation, chromosome structure, and homologous recombination.

The two mating-type alleles, *MATα* and *MAT***a**, differ by approximately 700 bp of sequences, designated Yα and Y**a**, respectively (Figure 1*A*). Yα and Y**a** contain the promoters and most of the open reading frames for proteins that regulate many aspects of the cell's sexual activity (reviewed in 51, 63, 84, 181). *MATα* encodes two proteins, Matα1p and Matα2p. Matα1p, in conjunction with

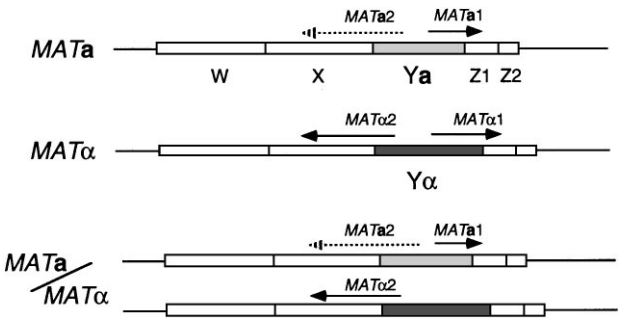

Figure 1 Structure of *MAT***a** and *MATα* alleles, distinguished by their Y**a** (650-bp) or Yα (750-bp) regions. The *MAT* locus shares X and Z1 regions of homology with a donor locus, *HMR*, while the W and Z2 regions are only shared with *HML*. *MAT***a** contains two transcripts. *MAT***a**1 encodes a co-repressor that acts, along with the homeodomain protein MATα2p, to turn off haploid-specific genes in *MAT***a**/*MATα* diploids, but *MAT***a**2 has no known function. In *MATα*, the *MATα*1 gene encodes a co-activator, with the Mcmlp, of transcription of α-specific genes. *MATα*2 encodes a co-repressor, with Mcm1p, that turns off **a**-specific genes. In a *MAT***a**/*MATα* diploid, *MATα*1 transcription is repressed.

a constitutive protein, Mcm1p, activates a set of α-specific genes (16, 56), including those encoding the mating pheromone α-factor and Ste2p, a *trans*-membrane receptor of **a**-factor (See Figure 1*B* on http://www.AnnualReviews.org, supplementary materials). *MAT*α2 encodes a repressor protein that acts with Mcm1 and also with Tup1 and Ssn6 proteins to repress the expression of **a**-specific genes (62, 80, 139), including those that produce **a**-factor and the Ste3 transmembrane receptor protein that detects α-factor. When both *MAT*α1 and *MAT*α2 are deleted, cells have **a**-like mating behavior, because **a**-specific genes are constitutively expressed and α-specific genes are not transcribed (179). *MAT***a** has two open reading frames, only one of which, *MAT***a**1, has been assigned a biological function (200). Mat**a**1p seems to play no role in haploid cells, but it combines with MATα2p to form a repressor complex that turns off so-called haploid-specific genes (47, 77, 99, 180). These genes include *RME1*, a repressor of early meiosis genes, and *HO*, which encodes the site-specific endonuclease necessary to induce mating-type gene switching. MATα2p/Mat**a**1p also represses expression of MATα1p; thus both α-specific and **a**-specific genes are turned off and diploid cells are nonmating.

There are a number of important mating-type–dependent differences. These distinctions are not simply a question of haploidy versus diploidy: *MAT***a**/*MAT*α diploids are notably different from diploids homozygous for either *MAT***a** or *MAT*α. As noted above, the most important difference is that *MAT***a**/*MAT*α cells can initiate meiosis and spore formation, whereas **a**-mating or α-mating diploids cannot. In addition, there is a distinctive difference in the pattern of bud formation (17): Haploids and diploids expressing one mating-type allele exhibit an axial pattern of budding that appears to be designed to facilitate efficient mating in homothallic cells (see below), whereas nonmating *MAT***a**/*MAT*α diploids have a polar budding pattern. Finally, some aspects of DNA repair are under mating-type control (42, 64). *MAT***a**/*MAT*α diploids are substantially more resistant to ionizing radiation than are *MAT***a**/*MAT***a** or *MAT*α/*MAT*α cells. In addition, spontaneous rates of recombination, for example, between alleles of various biosynthetic genes, are higher in *MAT***a**/*MAT*α cells. The genetic basis for these repair and recombination differences has not been established.

S. cerevisiae has evolved an elaborate set of mechanisms to enable cells to switch their mating types. Learning how these processes work has provided some fascinating observations in eukaryotic cell biology. *MAT* switching depends on four phenomena: (*a*) a cell lineage pattern such that only half of the cells in a population switch at any one time; (*b*) the presence of two unexpressed (silenced) copies of mating-type sequences that act as donors during *MAT* switching; (*c*) site-specific double-strand break-induced recombination at *MAT* that results in the replacement of Y**a** or Yα sequences at *MAT*; and (*d*) an extraordinary mechanism that regulates the selective use of the two

donors (donor preference). Each of these important mechanisms is reviewed below.

Cell Lineage

MAT switching has provided an excellent model to study the determination of cell lineage. Only half of the cells in a colony are able to switch mating type in any one cell division (Figure 2). A germinating haploid spore grows, produces a bud, and divides without changing mating type. Then, in the next cell division cycle, the older mother cell and its next (second) daughter change mating type while the first daughter buds and divides without any change (182). The axial budding pattern of haploids places two *MAT***a** cells immediately adjacent to two *MATα* cells and they readily conjugate, forming *MAT***a**/*MATα* diploids in which the HO endonuclease gene is turned off and further mating-type switching is repressed. If cells are prevented from mating, one can establish that the lineage rules persist: Any cell that has previously divided once is capable of switching *MAT*, while new daughter cells cannot (182).

Nasmyth (122) first demonstrated that the control of this lineage pattern depended on the asymmetric expression of the HO endonuclease gene, which is restricted to mother cells that have divided at least once (Figure 2). Subsequent work has led to a series of remarkable findings that can only be very briefly summarized here. Control of HO expression depends on the Swi5 transcription factor, which appears to be localized to mother cell nuclei and not to those of their daughters (123). More recent studies suggest that the absence of Swi5p in daughters is caused by the Ash1 repressor protein that exhibits a striking localization only in the daughter cell and not in the mother (12, 168). Ash1p acts upstream of Swi5p and may directly repress *SWI5* transcription, thus restricting HO expression to the mother cell in the next G1 stage of the cell cycle. The localization of the Ash1p protein occurs by localizing its mRNA prior to cell division (101). mRNA localization apparently depends on the myosin-like protein Myo4p (12, 75), as well as actin (197). This is, to date, the only example of such mRNA localization in *Saccharomyces*, and it provides the opportunity to explore this important aspect of developmental biology in great detail.

First Models of MAT Switching

Early studies of *MAT* switching recognized the existence of two additional key loci that were required for the replacement of *MAT* alleles: *HML* and *HMR* (161, 195, 196). A remarkably insightful hypothesis by Oshima &Takano (133) suggested that these loci were the seat of controlling elements that could transpose to *MAT* and activate opposite mating-type alleles. Coupled with the key experiments of Hawthorne (57, 58), these ideas led Hicks et al (68) to suggest

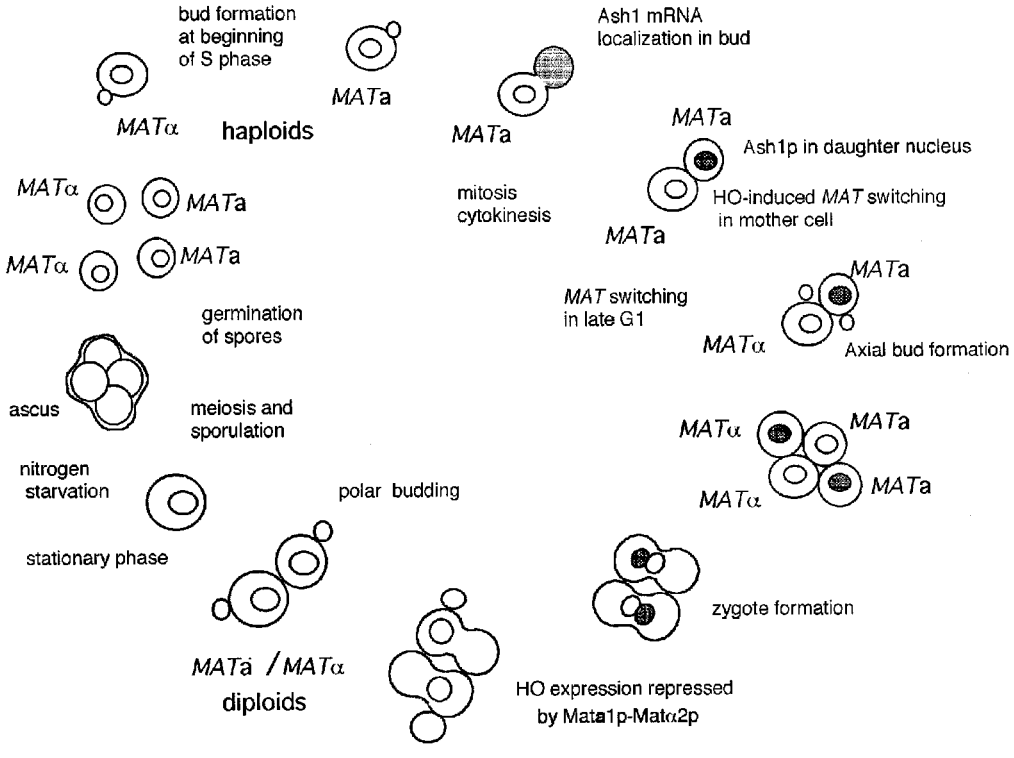

Figure 2 Homothallic life cycle of *Saccharomyces*. A *MAT***a** haploid cell that has divided can switch to the opposite mating-type and the original cell and its switched partner can conjugate to form a *MAT***a**/*MATα* diploid cell. Meiosis and sporulation will regenerate haploid cells. Homothallic switching is confined to cells that have previously divided (mother cells) by the action of the Ash1 repressor protein that is localized to the newly formed bud that gives rise to a daughter cell. Ash1p represses a positive regulator of HO endonuclease expression, Swi5p. In germinating spores, it is most likely the absence of Swi5p itself that prevents MAT switching until the cells have budded and divided.

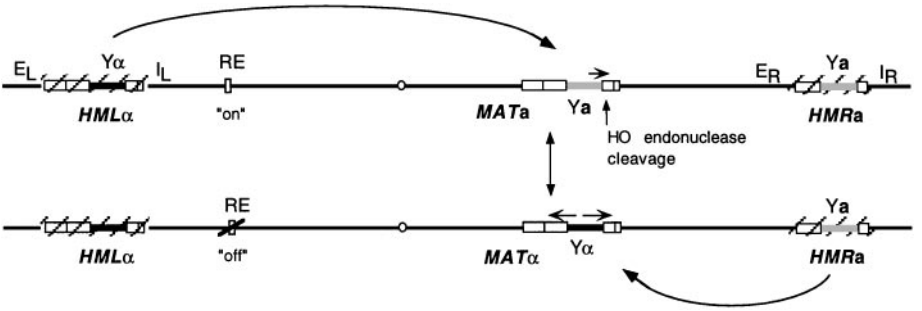

Figure 3 Mating-type loci on chromosome III. In addition to the expressed *MAT* locus, chromosome III harbors two unexpressed donor loci, *HML* and *HMR*. These donors are maintained in a heterochromatic structure (*diagonal lines*) enforced by two adjacent silencer sequences *HML*-E (E_L) and *HML*-I (I_L), and *HMR*-E (E_R) and *HMR*-I (I_R). When the HO endonuclease is expressed, MAT alleles can be switched by a gene conversion.

a specific version of the transposition model, known as the cassette model, in which an unexpressed copy of Yα sequences was located at *HML* (*HML*α) and unexpressed Y**a** sequences were found at *HMR***a**. These sequences could be transposed to the *MAT* locus, where they would be expressed (Figure 3). Subsequent studies (6, 126, 185, 200) confirmed the presence of two additional copies of mating-type information at *HML* and *HMR*. Most laboratory strains carry *HML*α and *HMR***a**, but natural variants were found that carry the opposite configuration: *HML***a** and *HMR*α (128, 195). One early surprise in the molecular analysis of *MAT*, *HML*, and *HMR* was that the two donor cassettes did not carry simply the Y**a** and Yα donor sequences but rather were intact, complete copies of mating-type genes. Somehow these genes were not transcribed. The two unexpressed cassettes differ in the extent of homology they share with *MAT*. *HMR*, *HML*, and *MAT* all share two regions flanking the Y sequences, termed X and Z1. *HML* and *MAT* share additional sequences, termed W and Z2 (see Figure 1).

During switching there is no change in either donor sequence; that is, *MAT* switching does not involve a reciprocal exchange of Y**a** and Yα sequences, but rather the sequences from either *HML*α or *HMR***a** are copied and inserted at *MAT* in place of the original *MAT* allele (66). The idea that *HML* and *HMR* repeatedly served as donors during *MAT* switching provided an explanation for an early observation of Hawthorne (58) that a mutant *MAT*α cell could be replaced by *MAT***a**, which then switched to a wild-type *MAT*α allele. Subsequent healing and wounding experiments were carried out in which mutations at *MAT* were corrected by recombination with the donor or in which a mutation at the donor was introduced into the *MAT* locus (67, 86, 174). In some cases, the replacement

of *MAT* information included not only the Y region but also at least part of the flanking X and Z1 regions shared by *MAT* and its two donors (111, 174).

The cassette model of Hicks, Strathern & Herskowitz has now withstood a 21-year test of intense experimentation. During this time we have come to understand aspects of this process in great detail.

SILENCING OF *HML* AND *HMR*

The presence of intact, but unexpressed copies of mating-type genes at *HML* and *HMR* implied that these two loci had to be maintained in an unusual, silent configuration. The mechanism of silencing of these donors has been extensively studied and has provided some important insights into the way in which chromatin structure influences gene expression and recombination (reviewed in 95, 103, 165, 176). Our current understanding can be summarized as follows. Both *HML* and *HMR* are surrounded by a pair of related but distinct silencer sequences, designated *HML*-E, *HML*-I, *HMR*-E, and *HMR*-I. These *cis*-acting elements interact, directly or indirectly, with a large number of *trans*-acting factors to repress the transcription of these genes. Among the *trans*-acting proteins that play critical roles in this process are histone proteins, the multipurpose Rap1 protein, four Silent Information Regulator (Sir) proteins, the DNA replication Origin Recognition Complex (ORC) proteins, and several protein transacetylases, deacetylases, and chromatin assembly factors. Together these gene products and *cis*-acting sequences create short regions (about 3 kb) of heterochromatin, in which the DNA sequences of *HML* and *HMR* are found as a highly ordered, but not continuous, nucleosome structure (125, 217) (Figure 4). These heterochromatic regions are transcriptionally silent for both Pol2- and

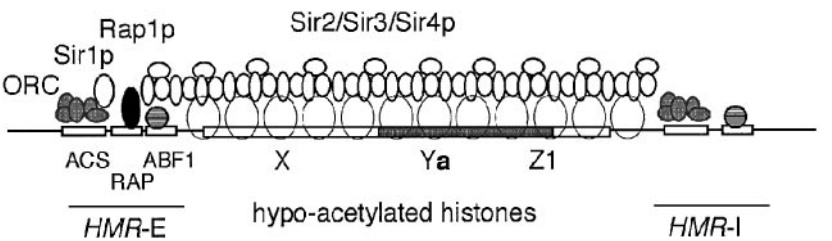

Figure 4 Heterochromatic structure of the silent locus *HMR*. A highly positioned array of nucleosomes (*large circles*) are established between E_R and I_R, which contain binding sites for the DNA replication complex, ORC, and the DNA binding proteins Rap1p and Abf1p. Silencing depends also on the deacetylation of histones and the interactions of four Sir proteins. A similar heterochromatic structure is found at *HML*, except that the positions of nucleosomes are discontinuous, with the region around the promoter sequences still accessible to nucleases (217).

Pol3-transcribed genes (14, 162) and are resistant to cleavage by several endogenously expressed endonucleases (26, 103).

Silencing also occurs adjacent to yeast telomeres, and many genes involved in *HM* gene silencing also play a role in telomeric silencing (46; see also 49, 95, 103, 104, 165, 176). There seems to be a hierarchy of silencing, with *HML* and *HMR* being more strongly silenced than telomeres. In general, telomeric silencing is more fragile, and several mutations that strongly affect telomeric silencing have either no effect on *HM* loci or only affect a partially debilitated *HMR*-E.

CIS-ACTING SILENCER SEQUENCES There appear to be some differences in the silencing of *HML* and *HMR*. Analysis of *HMR*-E (essential) and *HMR*-I (important) sequences showed that *HMR*-E was sufficient for silencing of *HMR***a** or of other PolII- or PolIII-transcribed genes, whereas *HMR*-I could not silence completely without *HMR*-E (1, 14, 65). Mutations of *HMR*-E that weaken silencing can be tightened by the presence of *HMR*-I. In contrast, either *HML*-E or *HML*-I is sufficient to silence *HML* or other genes placed nearby (106). Silencing also appears to be enforced by the fact that both *HMR* and *HML* lie relatively near chromosome ends (telomeres) that also exhibit gene silencing. When *HML*-E or *HMR*-E silencer sequences are inserted at other chromosome locations, further from telomeres, their ability to silence various adjacent genes is less strong (107, 109, 164, 201). This may explain why a circular chromosomal fusion of *MAT***a** and *HML*α (i.e. containing *HML*-I but lacking *HML*-E and lacking telomeres) is expressed (184).

The distance over which E and I silencers can act to completely silence genes is not very great. Normally, the distance between E and I is less than 3 kb; however, silencing is weakened if that distance is increased. For example, if most of the mating-type gene sequences lying between *HMR*-E and *HMR*-I are deleted and replaced by a 2.2-kb *LEU2* gene fragment, *LEU2* is completely silent; however, if the same *LEU2* gene is simply inserted into the middle of the mating-type sequences, thus moving *HMR*-E and *HMR*-I further apart, *LEU2* gene expression is sufficient to allow *leu2* cells to grow (B Connolly & JEH, unpublished data). Similarly, Weinstock et al (215) discovered that arrays of multiple insertions of the Ty1 retrotransposon within *HML* unsilenced the locus, with the degree of expression correlated to the size of the array and thus the distance between E and I sites. However, heterochromatin organized from telomeric regions and partially silencing genes can extend more than 10 kb (177).

A striking feature of all four silencer sequences is that each is capable of acting as an autonomously replicating sequence (ARS) on a plasmid, thus allowing it to replicate (1, 34, 83). Yet when these same sequences on the chromosome

are examined by two-dimensional gel electrophoresis to detect structures characteristic of an origin of DNA replication, neither *HML*-E nor *HML*-I is active, while *HMR*-E appears to act as a chromosomal origin, though only in a fraction of cell cycles (25, 29, 155). Whether silencing depends on origin activity has been the subject of much debate; current evidence suggests that the binding of the ORC proteins to silencer regions is a key step in establishing silencing, but it is not necessary that replication be initiated at that site (30, 39, 40; see below).

Silencer sequences also confer centromere-like behavior on plasmids, allowing them to segregate properly in most cell divisions (83). One explanation for this behavior is that silencer sequences may be anchored to some nuclear structure involved in chromosome partitioning. Evidence that silencers are anchored has been presented in studies of scaffold-attachment regions by Roberge & Gasser (156) and in elegant topological experiments by Ansari & Gartenberg (4), showing that DNA bound by a lexA-Sir4 fusion protein is anchored within the nucleus.

The most detailed dissection of a silencer has been carried out with *HMR*-E. A combination of deletion analysis and protein binding experiments demonstrated that *HMR*-E contains three distinct subdomains (Figure 4): ARS consensus sequence to which ORC proteins bind; a binding site for the ARS binding factor (ABF1) protein; and a binding site for Rap1p, which plays a complex role in both gene activation and in gene silencing (114). Deletion of any one region still allows incomplete silencing, but deletion of any two removes all repression of transcription. The ORC binding domain can also be replaced by a lexA binding domain to serve as a target to localize a lexA-Sir1 fusion protein, suggesting that one important role for ORC binding is in tethering another protein such as Sir1p to facilitate the establishment of silencing (20, 40). The arrangement of DNA binding sites varies at each silencer; for example, *HMR*-I does not contain a Rap1 binding domain.

TRANS-ACTING SILENCING PROTEINS The establishment and maintenance of silencing requires a large number of proteins. The first *trans*-acting silencing gene was identified by Klar (91), based on the fact that co-expression of both mating types in a haploid cell produced a nonmating phenotype. Eventually, four *SIR* genes were identified (52, 153, 154). A deletion of *SIR2*, *SIR3*, or *SIR4* completely abolished silencing, whereas loss of *SIR1* had a less extreme phenotype (see below). We still do not know the precise role of any of these proteins. None of them apparently binds directly to DNA. Sir3p and Sir4p appear to associate with each other and with the ordered histone octamers of silent regions to create a heterochromatic structure (see Figure 4) at *HML* and *HMR*, and also at telomeres (48, 60, 61, 79). Sir4p also directly interacts with

Sir2p (118). The stoichiometry of these proteins and organization of this silent structure is still not fully established. Sir2p may help stabilize this structure, again both at *HML*, *HMR*, and at telomeres (177).

However, the Sir proteins clearly play a much more complex role than was imagined from their effects on silencing. A *sir2* deletion also affects mitotic chromatin structure and recombination in rDNA, implying a broader role in chromosome organization (43, 45). There is also transcriptional repression in the rDNA locus, but here *sir2* depresses transcription of a polII-transcribed *URA3* gene, whereas *sir3* has little effect and *sir4* actually represses transcription (172). Recent work suggests that Sir4p modulates the pool of available Sir2p, whose distribution in the cell affects rDNA silencing (173). Overexpression of Sir3p or Sir4p also causes a significant increase in chromosome instability (70), possibly because of its effects on telomeres and their position in the nucleus (134). A deletion of *SIR3* also increases mitotic recombination on chromosome V about fourfold, while a *sir4* deletion apparently increases chromosome loss about the same extent (134). Even more striking is that the *SIR2*, *SIR3*, and *SIR4* genes also affect longevity (81, 82, 170). At least one aspect of yeast cell aging concerns the stability of rDNA and the accumulation of rDNA circles (169).

The Sir proteins are also found in the end-joining repair of double-strand breaks (DSB) (209). Even re-ligation of 4-bp complementary overhanging ends of a linearized plasmid, transformed into yeast, requires *SIR2*, *SIR3*, and *SIR4*, along with the yeast Ku proteins (Hdf1p and yKu80) and the recombination complex Mre11/Rad50/Xrs2p.

However, four other genes exhibit homology to *SIR2* (*HST* genes) (13, 28). Individual deletions of these *HST* genes do not affect silencing with an intact *HMR*, but *hst3* and *hst4* mutants partially derepress telomere silencing and overexpression of *HST1* suppresses a *sir2* mutation in *HM* gene silencing. Moreover, *hst3* and *hst4* mutations are also radiation-sensitive and show significant increases in chromosome instability (13).

Establishment and Maintenance of Silencing

Silencing at *HML* and *HMR* requires the protein transacetylase encoded by *NAT1* and *ARD1*. The Nat1/Ard1 complex plays many roles in addition to silencing, including its participation in G1 arrest after starvation (120, 220). Silencing is influenced by two other protein transacetylases encoded by *SAS2* and *SAS3* (31, 151). The pleiotropic deletion of the *RPD3* deacetylase also influences silencing (27, 159). There is evidence that at least some protein modification occurs at the silent locus itself. Acetylation of the N-terminal tails of histones H3 and H4 is directly implicated in silencing by mutations that replace the four evolutionarily conserved lysine residues (36, 60, 136, 202). More direct

evidence has come from the fractionation of chromatin based on the state of acetylation of lysine-16 of histone H4 (15), showing that *HML* and *HMR* are preferentially recovered in the hypoacetylated fraction. A similar analysis, taking advantage of affinity chromatography that detects an alteration in histone H3, also showed a difference in chromatin structure between *MAT* and the two silent loci (18).

One key question in silencing is whether the establishment of silencing differs from its subsequent maintenance. Although tethering Sir1p in place of ORC will indeed establish silencing (20, 40), in a wild-type cell its role is more subtle. Cells carrying a *sir1* deletion exhibit a striking epigenetic variegation in *HML* silencing (142). Some cells express *HML* and others do not, but each state is persistent through many cell divisions. A *sir1* cell with *HML* in a silent state gives rise predominantly to silent *HML* for many generations, but occasionally a cell will arise where *HML* is not silenced, and this unsilenced state will also persist for many generations, until a cell re-establishes silencing, and so on. This epigenetic inheritance suggests that the establishment of silencing and its subsequent maintenance are separable, an idea strongly supported by other observations (reviewed in 176).

An early study by Miller & Nasmyth (115) showed that raising a temperature-sensitive *sir3* mutant to its restrictive temperature caused immediate loss of silencing, but returning cells to their permissive temperature did not restore silencing until cells had passed through the next S phase. However, this experiment does not mean that DNA replication per se is required; silencing may only require an enzyme whose synthesis is confined to the S phase of the cell cycle. More recent experiments from Broach's lab might support this latter idea (10, 69). Site-specific recombination was used to pop out a DNA circle from *HML* either with or without a silencer sequence. Silencing (as reflected in a more negative supercoiled state) can be maintained in the absence of *HML*-E or *HML*-I, but only so long as the cells do not pass though S phase. Surprisingly, the popped-out circle does not contain an origin of replication, so that the loss of silencing is apparently not directly caused by the partitioning of nucleosomes to newly synthesized DNA. This result suggests that some events in maintaining silencing depend on progressing though the S phase part of the cell cycle but are not intimately involved in replication itself. Nevertheless, in normal cells, when replication occurs, the maintenance of silencing may well be closely connected to chromatin assembly. Indeed, Enomoto & Berman (33) showed that a deletion of the Caf1 subunit of chromatin assembly factor affects the maintenance of *HML* silencing but apparently not its establishment.

The list of genes affecting silencing continues to grow. Two mutations affecting the ubiquitination of proteins affect silencing (71, 117). The case of Ubp3 protein is especially interesting, as it has been reported to be affinity purified in a

complex with Sir4p (117). (The number of proteins that apparently interact with Sir4p is surprisingly large—including Sir2p and Sir3p (118), the yeast Ku70 homologue, Hdf1p (208), and the antisilencing protein Ris1p (226)). Recently, Ris1p[1]—a member of the Swi2/Snf2p family of proteins implicated in chromatin remodeling—has been shown to tighten silencing when it is deleted and to weaken silencing when a truncated gene is overexpressed (226). Similar phenotypes have been found for still another antisilencing factor (*ASF1*) (K Mills & L Guarente, personal communication). In addition, temperature-sensitive mutations in two essential genes, *NLP3* and *YCL54*, also perturb silencing by unknown means (102). The *CDC7* gene, encoding a protein kinase necessary to initiate chromosomal DNA replication, also plays a role in silencing (7), though how this relates to the role of ORC proteins is unknown. Finally, recent studies have shown that the MAP kinase pathway(s) responsible for mating pheromone, starvation, and heat-shock response all cause the hyperphosphorylation of Sir3p (175), though how phosphorylation affects normal silencing is not yet established. Even more fascinating is the *SUM1* gene, where a dominant (gain-of-function?) mutation will re-establish silencing even in the absence of the *SIR* genes (19, 88, 94). Despite intensive work in this area, many mysteries remain.

MAT SWITCHING: A MODEL FOR HOMOLOGOUS RECOMBINATION

The conversion of one mating type to the other involves the replacement at the *MAT* locus of Y**a** or Yα by a gene conversion induced by a DSB (93, 183). The process is highly directional, in that the sequences at *MAT* are replaced by copying new sequences from either *HML*α or *HMR*a, while the two donor loci remain unchanged by the transaction. Directional gene conversion reflects the fact that HO endonuclease cannot cleave its recognition sequence at either *HML* or *HMR*, as these sites are apparently occluded by nucleosomes in silent DNA (125, 217). Thus the *MAT* locus is cleaved and becomes the recipient in this gene conversion process. A very weak cleavage of *HML* has been observed when HO endonuclease is overexpressed (26) and rare illegal switches, where *MAT* is the donor and the silent locus is switched, have been observed when *MAT* itself cannot be cut because of a change in the HO recognition site (53). In Sir$^-$ cells where *HML* or *HMR* is expressed, HO can readily cut these loci and they become recipients (90).

Normally, the *HO* gene is tightly regulated to be expressed only in haploid mother cells and only at the G1 stage of the cell cycle (124); however, the

[1]Initially published as *DIS1* (disruption of silencing), this gene has been renamed *RIS1* (role in silencing, or "*SIR*" reversed) because the original name was already taken.

creation of a galactose-inducible HO gene enabled *HO* to be expressed at all stages of the cell cycle and in all cells (76). This made it possible to deliver a DSB to all cells simultaneously and to follow the appearance of intermediates and final products by physical analysis of DNA extracted at times after HO induction (26, 148, 219). Physical monitoring of recombination at *MAT* has yielded much of what we know about DSB-induced mitotic recombination (reviewed in 50). However, some related studies have been done with small HO endonuclease recognition sites inserted at other locations and from the induction of other site-specific endonucleases (112, 131, 137, 143, 149, 157, 218). Additional information has been gleaned from the analysis of DSB-induced recombination in meiotic cells (reviewed in 92). By and large, the results are sequence independent, though some interesting aspects particular to *MAT* switching are noted below.

HO endonuclease cleaves a degenerate recognition site of 24 bp in vitro (130), although sites of 33 bp or 117 bp are generally used when the HO recognition site is inserted at other locations. With HO enzyme purified from bacteria, cleavage appears to be stoichiometric; that is, the enzyme cleaves once and is then inactive (78). HO cleavage in vitro appears to be influenced by a protein that binds in the *MAT*-Z region, but its biological role is not yet known (211). Single-bp *MAT-inc* (inconvertible) or *MAT-stk* (stuck) substitutions in the recognition site abolish or greatly reduce switching (150, 212). HO cutting generates 4-bp, 3′ overhanging ends, both of which are accessible to exonucleases in vitro (93). In vivo, however, the DSB is processed almost exclusively by one or more 5′ to 3′ exonucleases to create long 3′-ended tails (219). As discussed more fully below, the 3′ end is remarkably resistant to exonucleolytic removal. It is possible that there are no 3′ to 5′ exonucleases that act on a 3′-overhanging end, or that the end is protected in vivo. Physical analysis of the rate of 5′ to 3′ degradation has implicated the trio of interacting proteins, Rad50p, Mre11p, and Xrs2p, in this process (74, 186, 207). There is good circumstantial evidence that Rad50p and Mre11p comprise a nuclease (163). However, the loss of these components only reduces the rate of exonuclease digestion by two- to fourfold, suggesting that there may be other exonucleases important in this process. The Exo1p exonuclease involved in nucleotide excision repair has been suggested as one possible alternative (207).

Once long 3′ tails have been generated, they can associate with recombination proteins that facilitate a search for homologous regions, to initiate recombination (Figure 5*C*). Because the Y region is not homologous between *MAT* and its normal donor, this end does not seem to engage in any of the early events in recombination. The 3′ end distal to the cleavage site is fully homologous to the Z1 region of the donor and thus can invade one of the donors in the Z1 region. The 3′ end of the invading strand is presumed to act as a primer to initiate new DNA synthesis, copying the Y region of the donor (Figure 5*C*). This newly

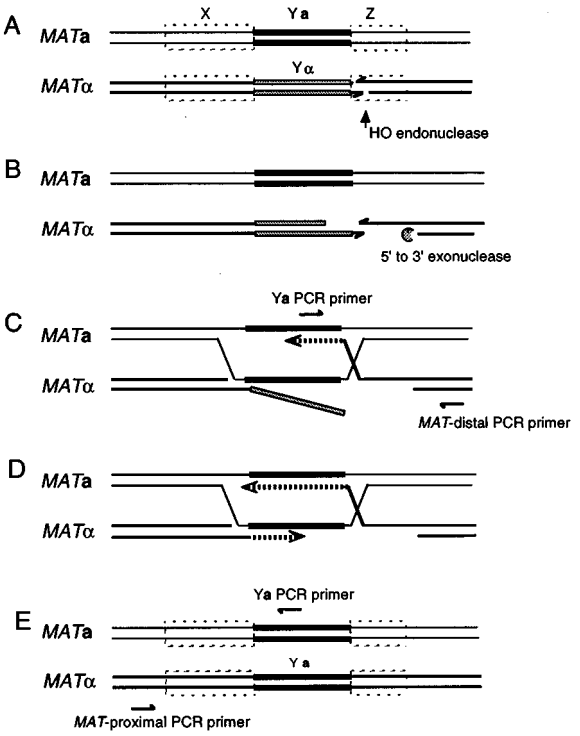

Figure 5 A basic model for *MAT* switching, based on the DSB repair model of Szostak et al (195). After HO endonuclease cleavage of the recipient locus (*A*), a 3′ end in Z1 is exposed by 5′ to 3′ exonuclease (*B*). Strand invasion initiates new DNA synthesis, copying the donor locus (*C*). This intermediate step can be monitored by PCR amplification that is only possible when there is a covalent intermediate. Removal of the original Y region allows a second strand of new DNA synthesis (*D*). The completion of switching can be detected by Southern blot hybridization or by a second PCR primer set (*E*).

copied sequence will eventually replace the Y region at *MAT*. *MAT* switching, induced by a galactose-regulated HO endonuclease, is a surprisingly slow process, requiring 1 h to complete, independent of the time of induction during the cell cycle (26, 148, 219). However, normal *MAT* switching may be more rapid, when HO is expressed in G1, only in mother cells, and in a coordinated fashion with other genes; this may be inferred from the low level of steady-state HO-cleaved *MAT* DNA in cells that can continually switch (i.e. *MAT***a** cells with *HML***a** and *HMR***a** cassettes) (183).

A polymerase chain reaction (PCR) can be used to detect the early steps in recombination by using two primers—one complementary to sequences distal

to *MAT* and one in the Y region of the donor (Figure 5*C*). PCR amplification can only take place after the invasion of the 3′-ended single strand into the Z region of the donor locus and the beginning of new DNA synthesis, primed from the 3′ end, thus creating a strand of DNA that covers both primers. This step occurs 30 min before gene conversion is complete, as monitored both by Southern blots and by a second PCR assay, detecting the time when the donor Y sequences are joined to the proximal side of *MAT* (Figure 5*C*) (219).

The region replaced during *MAT* switching is substantially longer than the Y region itself. McGill et al (111) used artificial restriction sites inserted at different places in the X and Z regions to show that replacement of the Y segment often extends well into the flanking homologous regions. They further showed that there was no reciprocal transfer of markers from *MAT* to the donor. This observation was supported by studies of the mismatch repair of a single bp mutation only 8 bp from the 3′ of the HO cut, in the Z region (150). In the absence of mismatch repair, this mutation was most often retained during switching (thus confirming physical studies showing that there was almost no 3′ to 5′ removal of the 3′-ended tail). Usually, only one of the two daughter cells carried the mutation. This type of postswitching segregation is analogous to postmeiotic segregation observed among meiotic segregants when the DNA inherited into one spore is heteroduplex (mutant/wild-type) in the absence of mismatch correction. A kinetic analysis (54) further demonstrated that, in repair-proficient cells, mismatch correction occurred very rapidly (as quickly as the PCR-amplified intermediate could be detected), suggesting that correction occurred soon after the strand invaded the donor locus. Moreover, the heteroduplex DNA was corrected in a highly biased way, such that mutant sequence in the invading Z DNA was corrected to the genotype of the donor. This observation is probably the most direct in vivo demonstration of the idea that mismatch repair will preferentially correct a mismatch adjacent to a nick (in this case, the 3′ end of an invading strand) (98, 144).

Recombination requires a number of proteins initially identified because of their role in repairing X-ray–induced damage. Recent experiments have shown multiple interactions among the Rad51, Rad52, Rad54, Rad55, and Rad57 proteins (24, 59, 116). Whether these proteins act in a single recombinosome is not known, but deletions of each of these genes clearly yield different phenotypes (50, 141, 146, 147). Rad51p shows strong homology, both in sequence and in in vitro assays, to the RecA strand exchange protein of *Escherichia coli* (132, 166, 191). Rad55p and Rad57p may act as accessory proteins to improve the loading of Rad51p onto single-stranded DNA to initiate recombination (190). Recent experiments suggest that one role of the Rad52 protein is also to facilitate Rad51p activity in the presence of the single-stranded binding

protein, RPA (9, 129, 167, 189). Rad52p also appears to facilitate single-strand annealing (119).

However, mutations of these *RAD* genes have very different effects on recombination in general and, in some circumstances, on HO-induced recombination. For example, spontaneous recombination is much less strongly reduced by *rad51* or *rad54* mutations than by *rad52* (141, 146, 147) and a similar difference is seen in recombination events where one end of a HO-induced DSB can lead to copy-choice or break-induced replication (108). This difference among *rad* mutations is also evident in HO-induced recombination using model substrates lacking the complexities of the *MAT* system. For example, when HO cleavage initiates gene conversion on a plasmid carrying two copies of the *E. coli* LacZ sequence, one of which is cleaved by HO, recombination can occur in the absence of *RAD51* and *RAD54*, but is abolished in a *rad52* deletion (73). Similar results are obtained when a plasmid contains two copies of *MAT*, one of which can be cleaved, but when the donor is not silent (187); however, when a similar plasmid is tested but where the donor is silenced by adjacent E and I sites, *RAD51* is required. These results have been interpreted to mean that Rad51p and Rad54p may play roles in opening up silent chromatin, to allow strand invasion, as well as in recombination per se. Note that strand invasion into the donor takes place at the same DNA sequence that is occluded from HO cleavage by the closed chromatin structure, so that the inaccessibility of the locus is a barrier to highly efficient recombination.

By physical monitoring of *MAT* switching, it has been possible to identify the early steps in this process, but exactly what happens in some later steps remains obscure. For example, how are the original Y sequences, which are not homologous to the incoming Y sequences, removed? An initial physical analysis suggested that neither strand of Y**a** was degraded until just before switching is completed, whereupon both strands disappeared (219). However, more recent analysis suggests that, as on the distal side of the DSB, one strand is degraded 5′ to 3′, producing long single-stranded tails (N Sugawara & JEH, unpublished data; 225). Removal of nonhomologous DNA strand tails that arise as intermediates of HO-induced recombination appears to occur by an endonucleolytic cleavage of the tail at its junction with paired, homologous sequences (37). This depends on two proteins from the nucleotide excision repair pathway (Rad1p/Rad10p) and two proteins from the mismatch repair pathway (Msh2p/Msh3p); however, none of the many other proteins in these two pathways is required (72, 188). It appears that the mismatch repair proteins Msh2p/Msh3p recognize branched structure such as would be formed between the old Y region and the newly synthesized DNA (Figure 5*C*), allowing the Rad1p/Rad10p endonuclease to clip it off. In cells lacking Rad1p, *MAT* switching is delayed for several hours until an apparently redundant, but inefficient, process removes the Y region (A Holmes & JEH, unpublished data).

Physical analysis has also made it possible to analyze conditional lethal mutants to ask what DNA replication enzymes are required for *MAT* switching. *MAT* conversion can occur in cells arrested in G1, when there is no active replication (148). Surprisingly, virtually all of the proteins necessary for origin-dependent DNA replication are also required for *MAT* switching, which include the single-strand binding protein RPA (210), the clamp protein PCNA, and most likely all three major DNA polymerases (A Holmes & JEH, unpublished data). The most striking result is that *MAT* switching appears to involve both leading and lagging strand synthesis. Thus mutations in primase and polymerase α drastically impair the completion of switching. In contrast, a temperature-sensitive *orc5* mutation that prevents initiation of DNA replication does not prevent switching to occur normally (A Holmes & JEH, unpublished data). This result and those discussed below have led to the idea that the mechanism of DSB repair is different from that proposed by Szostak et al (194), which predicts only two leading strand syntheses, each primed by a 3′ end (Figure 5). With recently developed techniques such as chromatin precipitation that have been applied to the study of DNA replication initiation (5), it should be possible to demonstrate more precisely which proteins are directly engaged in interacting with donor and recipient DNA strands.

Another striking aspect of *MAT* switching is that it is very rarely accompanied by crossing-over. In fact when found, crossing-over seems to have resulted from a lesion that persisted after switching (in a G1 cell) was complete and only led to crossing-over after DNA replication (in G2) (89). Studies of other recombination events indicated that intrachromosomal recombination in general is suppressed for crossing-over, but *MAT* appears to be unusually restricted (141). In plasmids, however, crossing-over accompanying intrachromosomal recombination is less constrained. Thus in a plasmid carrying two inverted copies of the *E. coli* lacZ sequence, one of which has an HO cleavage site, repair of the DSB is accompanied by crossing-over 30% to 50% of the time (158, 188). Similar results are found with a plasmid carrying two yeast *LEU2* sequences (M Coláiacóvo & JEH, unpublished data). In contrast, a similar plasmid carrying *MATα* and an un-cuttable *MATα-inc* locus crossed over in only 5% of all gene conversions (187; M Coláiacóvo & JEH, unpublished data). This suggests that there may be sequence-specific constraints on cross-overs at *MAT*, and/or there may be a real difference in the mechanism of repair.

A New View of Double-Strand Break Repair: Synthesis-Dependent Strand Annealing

The absence of crossing-over, the involvement of lagging strand synthesis, and the lack of transfer of genetic information from *MAT* to the donors during recombination are all consistent with a new family of DSB repair models, known

collectively as synthesis-dependent strand annealing (SDSA) (35, 111, 112, 121, 127, 135, 218). Furthermore, other studies of HO-induced recombination also suggest that gene conversion involves the conservative inheritance of both newly synthesized DNA strands at the recipient, leaving a completely unchanged donor (111, 112, 135). One version of this mechanism that accounts for the involvement of lagging strand synthesis is illustrated in Figure 6. In this mechanism, the strand invasion sets up a replication fork in which there is both leading and lagging strand synthesis. Unlike normal origin-dependent DNA replication, however, branch migration is postulated to displace both newly synthesized strands so that they are both inherited by the recipient (*MAT*) locus, leaving the donor completely unchanged. Note that this mechanism does not lead automatically to the formation of a pair of Holliday junctions that can be resolved by crossing-over, so that crossing-over would rarely accompany *MAT* switching.

DONOR PREFERENCE

In addition to the inherent directionality of switching—namely, that *HML* or *HMR* donate sequences to *MAT*, there is a very elaborate mechanism that enables yeast to choose between its two donors. It makes sense that *MAT***a** should seek out and recombine with *HML*α rather than with *HMR***a**, so that the recombinational repair of the DSB will lead to a switch to the opposite mating type. Donor selection is, however, not dictated by the Y**a** or Yα content of the donors; a strain with reversed silent information (*HML***a** *MAT***a** *HMR*α) still chooses *HML* about 85% to 90% of the time (87, 213). Weiler & Broach (213) showed that replacing the entire *HML* region, including its silencers, with a cloned *HMR* locus did not change donor preference, so it is the location of the donor, not the sequence differences between *HML* and *HMR*, that directs donor selection. Therefore, one or more *cis*-acting sequences, outside of the donors themselves, must activate or repress one or both donors, depending on mating type.

MATα cells choose *HMR***a** about 85% to 90% of the time. This occurs independently of the *MAT*α1 gene but is strongly dependent on *MAT*α2, the gene that acts as a repressor of **a**-specific genes (67, 193, 199, 214, 224). *MAT***a** donor preference does not depend on a functional *MAT***a**1 gene (222). These observations might suggest that *MAT***a** cells activate *HML* for switching through one or more **a**-specific gene products, and this is turned off in *MAT*α cells, where α-specific proteins might activate *HMR*. In fact, there appear to be two donor preference mechanisms at work, but they are not mirror images of one another (223). The work described below suggests that *MAT***a** cells activate *HML* to recombine preferentially with *MAT*, although the other donor at *HMR* is available

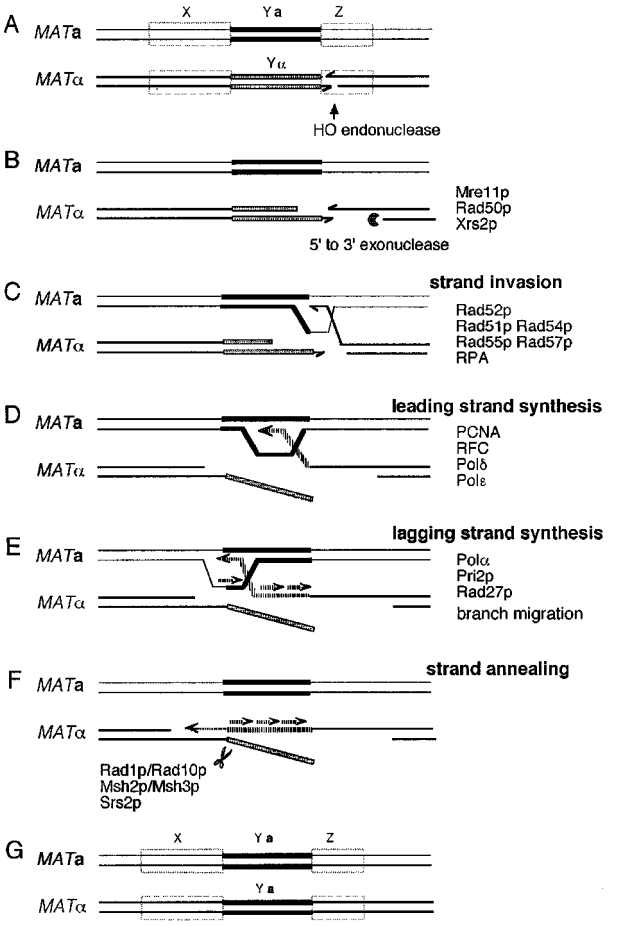

Figure 6 A synthesis-dependent strand annealing model for *MAT* switching. Proteins involved at different stages of the process are shown. In this mechanism, *RAD52*-dependent strand invasion initiates new DNA synthesis, requiring RFC, PCNA, and either Polδ or Polε. The newly synthesized strand is displaced and eventually anneals with the second broken end, without the formation of a Holliday junction that would permit crossing-over to accompany *MAT* switching. A second strand may be synthesized by lagging strand replication. Removal of the original Y region is excised by a flap endonuclease including Rad1p/Rad10p, Msh2p/Msh3p, and Srs2p.

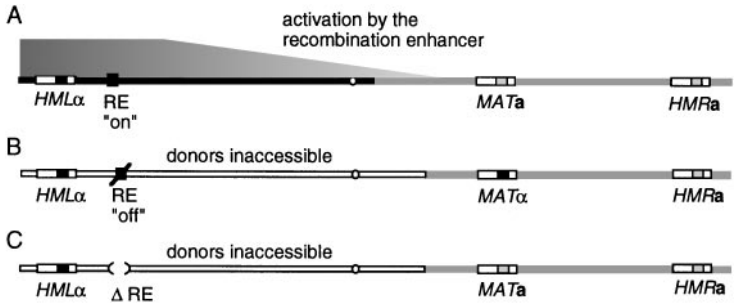

Figure 7 Donor preference in *MAT* switching. *A.* In *MAT***a** cells, *HML* or a donor placed at other sites along the left arm of chromosome III is activated to be the preferred donor. Activation depends on the Recombination Enhancer (RE). *B.* In *MATα* cells, the RE is turned off and the entire left arm and part of the right arm become cold, allowing *HMR* to be the preferred donor. *C.* When the RE is deleted in a *MAT***a** cell, the left arm becomes inaccessible and *HMR* becomes the preferred donor.

as a back-up to repair the DSB (Figure 7). In contrast, *MATα* cells do not simply activate *HMR* in a similar fashion. Instead they inactivate donors on the left arm, making *HMR* essentially the only available donor. Thus a *MAT***a** cell deleted for *HML* can easily use *HMR*, but approximately one third of *MATα* cells died when their only choice of a donor was *HML* (223–225). The failure of many *MATα* cells to use the wrong donor occurs despite the fact that cells experiencing an unrepaired DSB become arrested at a G2/M checkpoint (160). This should theoretically have allowed cells time to locate a donor and repair the DSB by gene conversion, and the death of so many *MATα* cells when *HML* is the only donor suggests that *HML* is very inaccessible in *MATα* cells.

The second important observation is that the activation of *HML* in *MAT***a** cells and its inactivation in *MATα* cells are not confined to the *HML* locus itself (or to *HMR* in place of *HML*). In a series of strains in which *HML* was deleted and a donor (either *HML* or *HMR*) was inserted at other chromosome locations along chromosome III, many sites along the entire left arm of chromosome III became activated in *MAT***a** cells and inactivated in *MATα* cells (222, 224). The inactivation in *MATα* cells extended along the right arm of the chromosome (Figure 7). A donor 20 kb distal to the centromere on the right arm was poorly used, whereas one 49 kb from the centromere (and 37 kb proximal to *MAT* on the right arm) was used efficiently. There does not seem to be a sequence-specific boundary to the cold region to the left of *MAT*, as an insertion of 25-kb of phage λ DNA that moved the donor from 37 kb to the left of *MAT*, where it was efficiently used, to a point 62 kb to the left of *MAT*, resulted in its inefficient

use (224). Donor preference does not depend on the presence of telomeres, as the same rules were found in a circular chromosomal derivative (222).

Identification of a Recombination Enhancer

These findings led to a search for *cis*-acting sequences on the left arm of the chromosome that were responsible for the activation of donors in *MAT***a** cells. Thus, Wu & Haber (223) inserted a donor 41 kb from the left end of the chromosome (*HML* is normally located 12 kb from the end) and then created a series of in vitro–generated terminal truncations of the left arm of chromosome III by integrative transformation of a telomere sequence; one of these lost the ability to activate the donor at 41 kb. In strains carrying the normal *HML*, a set of internal deletions was created to pinpoint the key *cis*-acting element. A 2.5-kb deletion located 17 kb proximal to *HML* completely reversed donor preference, so that a *MAT***a** cell now used *HML* only 10% of the time instead of 90% (Figure 8*Aii*). Deletion of this sequence also abolished *MAT***a** donor preference for donors located 41 kb, 62 kb, and 92 kb from the left end. Again, in the absence of this sequence, donors at these positions were not used 50:50 with *HMR*; rather, they were selected only 10% of the time. Deletion of this region had no effect on *MATα* cells, which continued to use *HMR* most of the time.

Into the deletion were then inserted subfragments of the missing sequences to restore donor preference (Figure 8*Aiii*, *iv*). The result was the identification of a 700-bp Recombination Enhancer (RE) sequence that restored *MAT***a** donor preference almost to wild-type levels. Subsequent work, described below, has narrowed the most important sequences to ∼250 bp.

The RE Affects Recombination Along an Entire Chromosome Arm

The regulation of *HML* is not specific to these particular donor sequences nor to HO-mediated recombination. This was shown by replacing *HML* with an allele of the *LEU2* gene (223). When a second *leu2* allele was placed elsewhere on chromosome III, or even on another chromosome, the rate of Leu^+ spontaneous recombination was 25 to 30 times higher in *MAT***a** cells than in *MATα*. This difference is lost when the RE is deleted. That RE should stimulate recombination even between chromosomes ruled out the idea of specific pairing sequences that would bring *MAT* and a left-arm donor together. No significant mating type–dependent difference was detected when a similar experiment was done with one *leu2* allele in place of *HMR*, thus supporting the conclusion that donor preference was effected through changes in the left arm of the chromosome, with *HMR* being a more passive participant. The accessibility of the site for recombination is obviously different from its accessibility for transcription factors.

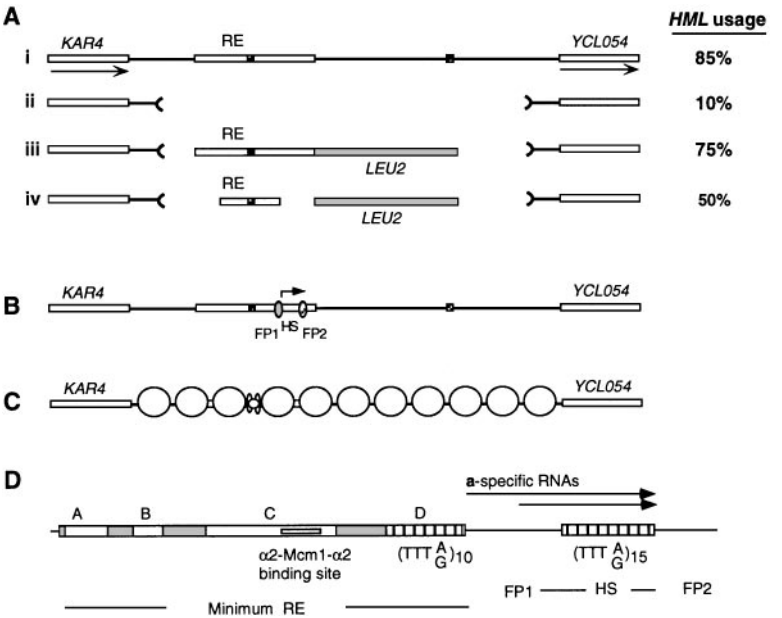

Figure 8 Identification and dissection of the RE. *A.i*. The 2.5-kb interval between *KAR4* and *YCL54* contains the RE and a second MATα2p-Mcm1p binding site (dark squares). *ii*. Deletion of the entire region abolishes RE activity, which can be restored by insertion of PCR-derived subfragments, in either orientation (*iii* and *iv*). *B*. In *MAT***a**, the RE is active and is distinguished by two distinctive footprints and a notable DNaseI hypersensitive (HS) region. There are also a pair of sterile transcripts in *MAT***a** cells. *C*. In *MAT*α, the entire region is occupied by highly positioned nucleosomes, whose sites are apparently established by binding of the MATα2p-Mcm1p co-repressor to its operator. *D*. A blow-up of the RE region showing a 270-bp minimum enhancer and an adjacent set of TTT(A/G) repeats. The conserved regions, A, B, C, and D, were identified by their conservation between *S. cerevisiae* and *S. carlsbergensis* (222).

The RE does not appear to affect the overall chromatin structure of the left arm of chromosome III. For example, there is no significant mating type– dependent difference in mRNA levels for the *LEU2* gene inserted in place of *HML* (which shows a 30-fold recombination difference) (223). Another assay, based on the position-effect variegation of expression of a *URA3* gene placed close to the *HML*-E silencer, also showed no mating-type–dependent difference (X Wu & JEH, unpublished data). Second, a direct examination of the chromatin structure of the *HML* locus by micrococcal nuclease and DNaseI digests revealed no significant mating-type–dependent difference (217). Finally, even when the *HML*α locus is unsilenced by deleting the adjacent silencers, thus having a much more accessible chromatin structure, it fails to be used efficiently as a

donor in *MATα* cells (224). A similar experiment in an *HMLα MAT***a** cell with an unsilenced *HMR***a** locus gave a very different result: *HMR* was now used more than half the time (X Wu & JEH, unpublished data). Thus opening up the closed chromatin structure of *HMR* had the expected result of improving its usage. The failure of this to happen when *HML* was unsilenced in *MATα* cells strongly reinforces the idea that the left arm donor becomes somehow sequestered or constrained such that it is unable to participate effectively in recombination with *MAT*. The action of the RE is not confined to intrachromosomal interactions. The efficiency of interchromosomal switching is generally much lower than intrachromosomal events (55, 152), but the use of *HML* in such interactions is almost completely dependent on the presence of the RE (225).

If the RE does not exert a direct effect on the local chromatin structure of the donor locus, what might it do? We propose that the RE changes the localization or the higher-order folding of the entire left arm of chromosome III to make it more flexible in locating and pairing with the recipient site in *MAT***a** cells. In this view, the chromosome arm in *MATα* cells would be sequestered or immobilized (perhaps by being bound to the nuclear envelope) in such a way that *HML* was unavailable in *MATα* cells even though the chromatin structure at *HML* itself was unchanged. One test of this model is to examine *leu2* recombination where one *leu2* allele resides in place of *HML*, while the other *leu2* allele is on a plasmid. If a small plasmid can find *hml*Δ::*leu2* more readily than would a distant site on the same or another chromosome, then any mating type–dependent difference in the mobility of *hml*Δ::*leu2* should be minimized. The results of this experiment support this idea, as donor preference drops from 30:1 (*MAT***a**:*MATα*) to 3:1 (W-Y Leung, M Barlow & JEH, unpublished data). Another experimental approach that may prove valuable would be to tether a green fluorescent protein (GFP) to *HML* and to examine its movement and subnuclear localization in living cells, as has been done to examine centromere movement (110, 178).

Is RE Portable?

Is the action of RE dependent on the specific sequence context in which it is found, or is it portable? When RE is deleted from its normal location and inserted approximately 30 kb more centromere-proximal (i.e. between *HIS4* and *LEU2*), activation of *HML* still occurs, but at a reduced level, whereas activation of a donor inserted near *HIS4* is unchanged (G-F Richard & JEH, unpublished observations). Whether other chromosomes will respond to RE is now under investigation.

Perhaps more surprising is that the 700-bp RE exerts an effect even at the opposite end of chromosome III (223). Insertion of an additional RE element adjacent to *HMR* increased its use in *MAT***a** cells to nearly 50%, from 10% to

15%. One explanation would be that all of chromosome III is unusual and that even the right arm is somewhat cold and thus responsive to the RE.

Changes in Chromatin Structure Associated with Regulating RE

The genetic studies of how RE is regulated are strongly supported by analysis of the chromatin structure of the RE. In *MATα* cells, there appears to be a very highly ordered set of nucleosomes on either side of the (occupied) MATα2p-Mcm1p binding site (216, 221). These cover the entire RE and extend between two flanking open reading frames, *KAR4* and *YCL54* (Figure 8*B*). In *MAT***a** cells, the highly positioned nucleosomes disappear and the RE exhibits several distinctive footprints indicative of protein binding and a notable region of closely spaced (almost every 4 bp) DNaseI hypersensitive sites that cover one of two regions of TTTA/G repeats (216). The identity of the proteins responsible for the footprints or the significance of the unusual hypersensitive region is not yet known. Presumably the positioned nucleosomes in *MATα* cells turn the RE off, but note that this repression does not extend into adjacent open reading frames, nor is there any evidence of any mating-type dependent change in *HML* (K Weiss & RT Simpson, personal communication).

Functional Domains of the RE

A detailed analysis of the DNA sequences controlling donor preference raises the same philosophical dilemma that confronts students of complex enhancers. One can pare down the region to identify a minimum enhancer essential for activity; but these sequences are likely to be significantly less active than the full region, and one may lose sight of some of the redundancies and complexities of the region as a whole. Nevertheless, it seems to be the most direct way to find the core sequences required for enhancer activity. A complementary approach is to compare evolutionarily diverged enhancers to identify the most highly conserved regions. A combination of these approaches has provided considerable insight into the most important elements of the RE and, in some cases, an indication of their roles.

Although a 700-bp RE has substantial RE activity, there appear to be additional sequences in the surrounding region that are also important for full RE activity. Thus while deleting the region containing the 700-bp RE region abolishes *MAT***a** donor preference (i.e. more than 90% of cells use *HMR* instead of *HML*), a deletion that removes only the remaining centromere-proximal part of the 2.5-kb region between *KAR4* and *YCL54* (See Figure 8*A*) also causes a twofold reduction in MATa selectivity (*HML* usage falls from 80% to 50%) (193). This adjacent region shares one DNA sequence in common with the 700-bp RE: a consensus binding site for the operator region that controls

a-specific genes such as *STE6*. The importance of this operator sequence will become evident below.

By comparing the RE sequences of *S. cerevisiae* and *S. carlsbergensis* (which is functional in *S. cerevisiae*) (221), the RE has been narrowed down to 270 contiguous base pairs in *S. cerevisiae* or 244 in *S. carlsbergensis*, within which are 4 well-conserved subdomains (Figure 8*D*). Of these, domain B appears to be unimportant because it can be deleted without significant effect on *MAT***a** or *MAT*α donor preference. Deletions of subdomains A, C, or D all abolish *MAT***a** donor preference, causing cells to use *HMR* 90% of the time. Subdomains C and D can be inverted relative to A and B and still function properly.

The importance of subdomain A in the minimum enhancer illustrates the advantages and disadvantages of defining a minimum enhancer. In the minimum enhancer, several 2-bp site-directed mutations of subdomains A have been shown to abolish *MAT***a** donor preference (C Wu & JEH, unpublished data). The availability of wild-type and mutant sequences should greatly facilitate attempts to identify the protein or proteins that bind to this region. On the other hand, it is startling to discover that subdomain A was completely missing from the 700-bp RE defined by Wu & Haber (223). Clearly, other sequences in the larger region must carry out redundant functions that the subdomain performs in the minimum RE.

Subdomain D is intriguing because of its unusual sequence: 10 perfect repeats of TTT(G/A). Truncation of this region, leaving only 8 repeats, reduces donor preference by about 50% and further truncations have no RE activity (C Wu & JEH, unpublished data). It is not yet known what proteins bind to this region. In the 700-bp RE (but outside the minimum enhancer region), there is a second near-perfect array of 15 TTT(G/A) repeats that has distinctive DNaseI hypersensitivity, surrounded by two strong footprints indicative of protein binding (Figure 8*D*). Although this set of repeats and its surrounding sequences are clearly not essential for minimum RE activity, they may be important for full activity. However, this feature is poorly conserved in *S. carlsbergensis*, whose 813-bp RE fully substitutes for that of a 753-bp *S. cerevisiae* RE.

Role of the MATα*2p-Mcm1p Operator*

Most of our understanding of the control of RE function comes from analyzing part of subdomain C. This 90-bp region harbors a 31-bp consensus MATα2p-Mcm1p binding site. This site was defined as the operator to which two MATα2p and two Mcm1p proteins, in conjunction with Tup1p and to a lesser extent Ssn6p, bind to repress **a**-specific gene transcription (171). The three-dimensional structure of this corepressor, bound to an operator, is now solved (198). This same repressor binding site acts to turn off the RE in *MAT*α cells, again in conjunction with Tup1p (192). Szeto et al (193) have recently

reported that a mutation of one of the two MATα2p binding domains in subdomain C (which they call DPS1) is sufficient to alter donor preference in *MAT*α cells, so that *HML* usage is increased. When a second such mutation was created in the second operator region outside the 700-bp RE (termed DPS2), *HML* usage in the double mutant increased to 55%, compared to the normal 5–10% of *MAT*α cells. Wu et al (221) carried out similar studies on the 700- and 270-bp RE sequences, where there is only one MATα2p-Mcm1p operator. They found that mutating either one of the two MATα2p binding sites raised *HML* usage in *MAT*α cells (to about 40%), but altering both binding sites in subdomain C caused *MAT*α cells to use *HML* 55% of the time. These results also suggest that any **a**-specific gene products are unlikely to play an essential role in activating RE, since these genes should still be repressed by MATα2p-Mcm1p in the *MAT*α cell. However, the difference between *HML* use in *MAT***a** (80%) and MATα (55%) in this mutant RE could be attributed to **a**-specific genes.

With the exception of the RE, all MATα2p-Mcm1p binding sites are located just upstream of **a**-specific genes. The RE contains no open reading frame, but there are indeed two sterile (i.e. noncoding) transcripts of the RE region that are transcribed in *MAT***a**, but not *MAT*α cells (193). This invokes connections to sterile RNAs found in X chromosome inactivation in mammals, and it is an intriguing observation. However, it is unlikely that the sequence of the RNA transcript is important for RE activity, as truncations of RE that remove most of the normally transcribed sequence have full or substantial activity (221, 223). However, the act of transcription itself could still be the key regulatory feature.

*Activation of RE in MAT***a** *Cells Depends on Mcm1 Protein*

The Mcm1 protein is not only a co-repressor; it can also act as a co-activator of transcription for both **a**-specific (32) and α-specific genes (16, 56). In the case of α-specific genes, Mcm1p acts as a heterodimer with MATα1p (Figure 1*B*); no possible co-activator with Mcm1p in *MAT***a** cells has been identified. Indeed, the MATα2p-Mcm1p operator within the RE will act as an enhancer to promote transcription of a reporter gene (193); thus Mcm1p may activate RE in *MAT***a** cells. The idea that Mcm1p may play a decisive activating role at RE is supported by two recent observations (221). First, a 2-bp mutation that eliminates Mcm1p binding in the MATα2p-Mcm1p operator sequence abolishes *MAT***a** donor preference; *HML* is used only 10–20% of the time, even in the case where the 2-bp mutation is introduced into an otherwise unmodified chromosome III. Thus although the minimum RE is approximately 250 bp long and apparently contains some regions of redundant function, the elimination of Mcm1p binding is sufficient to completely inactivate RE. In support of this important observation, Wu et al (221) found that a single amino acid substitution mutation in the *MCM1* gene (*mcm1-R89A*) had a similar effect. The crystal

structure of MATα2p/Mcm1p/DNA reveals that this arginine lies at a contact point between Mcm1p and MATα2p (198). The mutant Mcm1p binds less strongly to the operator, as measured by UV photofootprinting (221). Thus it would appear that Mcm1 protein, which is an essential protein that activates transcription of both mating-type specific genes and a variety of other genes, is also the central player in activating RE. Whether Mcm1p has a co-activating protein in activating RE is not yet known, nor is it clear that transcription per se is required, but it is clear that Mcm1-dependent activation of **a**-specific genes depends not only on the approximately 10-bp Mcm1p binding site itself, but on at least 250 bp of surrounding sequences.

The analysis of Mcm1p binding has led to another surprising finding concerning the role of MATα2 and Mcm1 protein binding in the establishment of repression. The 2-bp mutation that prevents Mcm1p binding also causes a dramatic change in the chromatin structure of the RE. Even in *MAT***a** cells, where there is no MATα2 protein, the mutant RE has an array of highly positioned nucleosomes very similar to that seen in normal *MAT*α cells (221). Apparently, other sequences within RE can organize a phased nucleosome structure in the absence of MATα2p-Mcm1p binding, although the repressor proteins seem to more precisely position and lock in the repressing chromatin structure. Whether this is also true at the MATα2p-Mcm1p operators upstream of **a**-specific genes is now under investigation.

The idea that the sequences surrounding the Mcm1p binding site play important roles in both activating and inactivating RE finds support in the otherwise paradoxical observations that a deletion of nearly the entire 31-bp MATα2p-Mcm1p operator has a less profound effect on reducing *HML* usage in *MAT***a** cells than does the simple 2-bp deletion of the Mcm1p binding site (193, 221). The adjacent sequences may be important in determining how cold the left arm of the chromosome is in the absence of RE activation.

Donor Preference Genes

A mutation in *MCM1* significantly alters donor preference. But what other *trans*-acting gene products are needed, both to activate RE in *MAT***a** cells and—equally fascinating—to inactivate the entire left arm of the chromosome in *MAT*α cells? The coldness of the left arm in *MAT*α cells does not depend on the presence of RE; in RE-deleted strains, both *MAT***a** and *MAT*α cells use *HML* only 10% of the time. Presumably, there are *cis*-acting sequences necessary to make the left arm inaccessible, but these have yet to be identified.

Until recently, only one *trans*-acting gene had been implicated in donor preference. A deletion of the *CHL1* gene reduces donor preference in *MAT***a** switching from 80% to 60% (where 10% would be the complete loss of *HML* preference); but it has no effect on *MAT*α (214). *CHL1* is not a donor

preference–specific gene; it was identified more than 20 years ago because it causes a 200-fold increase in the loss of chromosomes (100). All chromosomes tested were affected, with smaller chromosomes being more sensitive to loss. Subsequent work showed that *chl1* also promoted chromosome gain, and is therefore most likely a nondisjunction-promoting mutation (44). *CHL1* encodes a nuclear protein with presumed but undemonstrated helicase activity. It has a human homologue that is growth factor–regulated and localized predominantly in the nucleolus (3, 41). What the connection is between chromosome stability and donor preference is not yet obvious.

Screening directly for donor preference mutations has not been very productive, largely because mutations that affect HO expression and *cis*-acting mutations that reduce HO cleavage tend to interfere with the evaluation of donor choice scored at the colony level. Only *chl1* has emerged in this way. An alternative (besides just making good guesses) is to take advantage of another assay that is less subject to unwanted mutations. The much higher level of Leu^+ papillae in *MAT***a** cells than in *MATα* cells that arise by recombination between a *leu2-R* allele placed near *HML* and *leu2-K* inserted near *MAT* should provide such an assay (222, 223). Transposon mutagenesis of *MAT*a and *MATα* strains allows one to look for mutations that reduce Leu^+ recombination in *MAT***a** cells, but that have no effect in *MATα* cells; conversely, one can look for increased papillation in *MATα* cells, with no effect in *MAT***a** cells. Then the effect of these insertion mutations can be tested on *MAT* switching per se. Such screens are under way.

Possible Relation of Donor Preference to Mechanism in Fungi, Metazoans, and Mammals

The regulation of an entire chromosome arm by a small *cis*-acting sequence for the purpose of donor preference in *Saccharomyces* may prove to share common features with other long-range regulatory events.

Some echoes of the *Saccharomyces* donor preference mechanism can be found in accounts of mating-type gene switching in the distantly related fission yeast, *Schizosaccharomyces pombe* (85, 206). In *S. pombe*, the *mat1* locus, which can carry either a P or a (nonhomologous) M cassette, can switch, using one of two silent donors (*mat2* and *mat3*) located only a short distance away on the same chromosome arm. M cells switch preferentially to P, and vice versa. *mat* switching in *S. pombe* is exactly like that in *S. cerevisiae*—except in every detail. There are almost no sequence homologies between the *MAT* loci of the two organisms, or in the silencer sequences, in the major proteins that establish silencing, or in the proteins that initiate switching. An HO-like endonuclease has never been identified, and it now appears that the double-strand breaks that apparently initiate *S. pombe mat* switching arise not by double-strand cleavage

but by replication of a nicked or gapped molecule, yielding one broken chromosome and an unbroken sister chromatid (5a). Mutations that alter donor preference also alter silencing, which is not the case for *S. cerevisiae* (203–206). Although the two systems seem to share some common features, they may be the consequence of convergent evolution.

Both *Drosophila* and *Caenorhabditis* exhibit X chromosome dosage compensation, but they accomplish this by quite different mechanisms. In both organisms, many genes on the single X chromosome of males are transcribed at twice the rate as the genes on the female's two X chromosomes. In flies, dosage compensation arises by an increase in transcription of the single X in males (8, 11). The activation of transcription in flies depends on a number of *cis*-acting sites and *trans*-acting factors (145). The number of sites is not known. The activation of recombination in flies possibly works through a series of long-range enhancers similar to the RE. Note that some Drosophila genes are the 100-kb length of the entire chromosome arm that yeast's RE controls. Moreover, although there is no profound effect on transcription on the left arm controlled by RE, a difference as subtle as twofold would not have been detected.

In worms, regulation depends on a partial condensation of the two female X chromosomes, thus reducing gene expression by a factor of about 2 (138). The genes involved in this tightening of chromosome structure include SMC proteins that are conserved from yeast to humans (21, 22). The coldness of the left arm in the absence of RE or in *MAT*α cells might share common features with *Caenorhabditis* X-regulation. However, neither yeast *smc1* nor *smc2* mutants affect donor preference (X Wu & JEH, unpublished data), but *smc3* and especially *smc4*—the gene most homologous to the worm dosage compensation gene Dpy27—have not been tested. Nevertheless, it is tempting to think that these dosage compensation mechanisms have some relationship with those that regulate yeast's sex chromosome III.

There are a number of examples of long-range regulation of gene expression, DNA replication, and recombination in mammals that may share some features with the action of budding yeast's RE. The most thoroughly studied is the locus control region (LCR) that regulates β-globin gene expression from a distance of 50 kb. Unlike the RE, however, deletion of this region causes major changes in chromatin structure across the entire β-globin locus (38). The LCR clearly does more than behave as a specific long-distance enhancer of transcription, because the LCR also controls the ability of cells to activate an origin of DNA replication in this vicinity (2).

The immune system of mice and humans involves recombinational joining of V, D, and J regions that are located as far as 200 kb apart. The stimulation of recombination is strongly controlled by enhancer sequences located 3′ to the

constant region, and thus downstream of the closest sequences, J, involved in recombination (105, 113). Whether this stimulation is dependent on transcriptional activation or more generally activates the region for recombination has not yet been firmly established.

Finally, one casts an eye at X chromosome inactivation in mammals (23, 140), where a *cis*-acting sequence and an RNA that it encodes controls the activity of an entire, big chromosome. But in yeast, sterile transcripts are only seen in the activated *MAT***a** state and not in the inactivated *MATα* case. Yeast is also devoid of the methylation that is the hallmark of mammalian chromosome inactivation.

Nevertheless, it seems unlikely that evolution has developed such a complex mechanism of donor selection and not found further uses for the basic mechanism that underlies it. There are many unanswered questions about donor preference that will absorb our attention into the next millennium.

EPILOGUE

In reviewing what we have learned about the processes of recombination that govern mating-type gene switching, one tends to focus on recent experiments that have dramatically illuminated the subject. In the process some of the pioneering work that laid the foundation becomes obscured. In some cases, gene names have been changed to reflect a more comprehensive understanding of their function. What we now know about *MAT* switching owes particular debts of gratitude to Donald Hawthorne, who published little but carried out the first pedigree analysis of *MAT* switching and also created fusions of *MAT* and *HMR* (before *HMR* had been defined) that provided essential keys to the model proposed by James Hicks, Jeffery Strathern, and Ira Herskowitz; Isamu Takano and Yasuji Oshima, whose early studies of *MAT* switching in the early 1970s included the formulation of their seminal transposable controlling element model; Vivian MacKay and Thomas Manney, whose sterile mutations, including those in *MATα*1 and *MATα*2, provided not only insights about signal transduction but also most of the genetic reagents used to demonstrate the way *MAT* alleles were replaced during homothallic switching; Michael Resnick, John Game, and Robert Mortimer, who identified most of the key *RAD* genes needed for recombination and who, along with the late Seymour Fogel, established most of the basic rules about gene conversion.

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