Engineered interphase chromosome loops guide intrachromosomal recombination

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How large-scale topologies regulate interphase chromosome function remains an important question in eukaryotic cell biology. Looped structures are thought to modulate transcription by pairing promoters with distant control elements and to orchestrate intrachromosomal recombination events by pairing appropriate recombination partners. To explore the effects of chromosomal topology on intrachromosomal recombination, distinct loop geometries were engineered into chromosome III of the budding yeast *Saccharomyces cerevisiae*. These topologies were created by employing pairs of *lac* operator clusters to serve as pairing sites and a modified *lac* repressor to perform the role of a protein cross-bridge. The influence of these engineered loops on the selection of donor loci during mating-type switching was evaluated using novel genetic and molecular methods. These experiments demonstrate that engineered interphase chromosome loops are biologically active—capable of influencing the course of intrachromosomal recombination. They also provide insight into the mechanism of mating-type switching by revealing a causal relationship between defined chromosomal topologies and the choice of donor locus.

Keywords: chromosome/interphase/loop/recombination/topology

Introduction

Chromosome topologies have been proposed to play regulatory roles in eukaryotes. Loops or folds are believed to exert an influence by assembling chromosomal regions that would otherwise remain apart. For instance, by sustaining the juxtaposition of a promoter and regulatory element (Muller *et al.*, 1989), a looped topology could influence gene expression, or alternatively, by causing the synapses of particular sequences, it could direct the course of a recombination event (Cremer *et al.*, 1996; Sachs *et al.*, 1997; Ostashevsky, 2000). Demonstrating these regulatory roles for interphase chromosome topologies has been problematic, due in part to the difficulty of identifying and characterizing topology when chromosomes are in a highly dispersed state.

Imaging techniques have been of limited utility in describing interphase chromosome topology. Optical microscopic techniques, such as fluorescence *in situ* hybridization (FISH) (Bickmore, 1999) and *in vivo lac*

repressor–green fluorescent protein (GFP)/operator complex formation (Robinett *et al.*, 1996), are unable to resolve structural details beyond the classical diffraction limit of light, which is in the order of 250–300 nm. And while electron microscopic techniques have better resolution and have been key to describing the looped topology of extracted condensed mitotic (Marsden and Laemmli, 1979) and meiotic chromosomes (Reznik *et al.*, 1991), they have not been as informative when it comes to elucidating the native topology of dispersed interphase chromosomes.

Our understanding of the relationship between chromosome structure and function has come mainly from genetic and molecular studies of recombination. Since recombination events require the physical synapsis of participating sequences, a gene conversion event involving a pair of sequences on the same chromosome requires, at the very least, the formation of a transient loop. In particular, studies of mating-type switching in the budding yeast *Saccharomyces cerevisiae* have provided important insight into the genetic regulation of interphase recombination and into how chromosomal structure affects function (Haber, 1998).

Wild-type haploids switch between the *a*- and α -mating types as often as once every cell division by way of gene conversion events involving three widely separated loci on chromosome III. The centrally located *MAT* locus, which specifies a cell's mating type, acts as a gene conversion recipient. It recombines with one of two distant gene conversion donors: the *HML* locus, 190 kb away near the left telomere, or the *HMR* locus, 90 kb away near the right telomere (Figure 1). Selection of the gene conversion donor is regulated by mating type in a process referred to as 'donor preference': *a*-cells preferentially use *HML*, while α -cells preferentially use *HMR*. The preferred donor is favored at an ~10:1 ratio over the other donor (Wu *et al.*, 1997). This process is not dependent either on the allelic content of the donor loci or on sequences within 1–2 kb of the donor loci (Klar *et al.*, 1982; Weiler *et al.*, 1995; Wu and Haber, 1995; Wu *et al.*, 1996). In α -cells, the left arm of chromosome III, including *HML*, is rendered inaccessible to *MAT*, so that cells use the *HMR* donor by default (Wu *et al.*, 1996). However, in *a*-cells, the left arm of the chromosome is activated, allowing *HML* to prevail over *HMR* (Wu and Haber, 1996; Wu *et al.*, 1997). Regulation is apparently achieved largely through the action of a cell-type-modulated recombination enhancer (RE) located 17 kb centromere proximal to *HML* (Wu and Haber, 1996; Szeto and Broach, 1997; Szeto *et al.*, 1997). Although the chromatin structure of the RE is modulated in a cell-type-specific manner (Weiss and Simpson, 1997), there is to date no indication that this local effect on chromatin structure extends to *HML* or influences large-scale chromosome topology.

Fig. 1. Diagrams of wild type and variants of chromosome III. Chromosome III is depicted in blue on which the relative locations of *HML*, *MAT* and *HMR* are shown; its centromere is indicated by a black dot. The jagged red line indicates lactose operator clusters and the green polygons represent lactose repressors, dimeric in strains LM2, RM2 and RL2, tetrameric in strains LM4, RM4 and RL4. The chromosomal loops that are conjectured to occur in strains LM4, RM4 and RL4 from synapsis of operator clusters by the tetrameric repressor are illustrated.

It has long been suspected that specific chromosomal topologies influence selection of donor loci by either facilitating or inhibiting particular recombination pathways. This notion is based on the assumption that the *MAT* locus would tend to recombine with the most accessible donor locus, and that one potent determinant of accessibility is the physical proximity of the gene conversion donor. This reasoning leads to scenarios of donor preference wherein chromosome III exists in two distinct cell-type-specific topologies that favor either selection of *HML* or *HMR* (Wu *et al.*, 1997). Chromosomal topology of an α -haploid would encourage the use of the *HML* donor by either sequestering *HMR* from *MAT* and/or by preferentially pairing *MAT* with *HML*. Conversely, the α -haploid topology would be one in which *HML* is sequestered from *MAT* and/or *MAT* is paired with *HMR*. Indirect support for the existence of alternative chromosome topologies has come from experiments exploring the effects of deletions on donor preference. When deprived of their *HML* locus, α -cells will recombine efficiently with *HMR*, while α -cells deprived of their *HMR* loci will often fail to recombine with *HML* and die as a consequence (Wu *et al.*, 1996). These observations suggest that *HML* is accessible to *MAT* in α -cells but not in α -cells. While this evidence is consistent with models that posit chromosome topologies that physically distance *HML* from *MAT*, it is also compatible with models that postulate local changes in the chromatin structure of *HML* that affect its propensity to act as a substrate for recombination (Szeto *et al.*, 1997; Wu *et al.*, 1997).

This paper describes experiments that explicitly test the impact of specific chromosome topologies on gene conversion during mating-type switching. These experiments made use of a novel method for creating defined large-scale structures in interphase chromosomes. This method is analogous to one employed in the study of

protein topology, wherein pairs of cysteine residues are introduced into a protein, permitting the formation of an intramolecular disulfide bond linking the two cysteines (Matsumura and Matthews, 1989; Matsumura *et al.*, 1989). The cysteine residues, in effect, define the endpoints of a loop in the polypeptide backbone that constrains the topology of the protein, permitting study of the structure, thermodynamics and biological activity of a particular topological isomer. The new method described in this paper provides a general strategy for creating pairing sites, analogous to cysteine residues in a peptide, at predetermined sites along the length of the chromosome backbone. Moreover, similarly to the way disulfide loops in a protein can be regulated at will—formed and dissipated chemically, by respectively oxidizing or reducing the cysteines—loop formation in chromosomes can be turned on and off genetically, by modulating the expression of a cross-bridging DNA-binding protein specific for the pairing sites.

Pairs of artificially constructed synapsis sites, composed of clusters of *Escherichia coli lac* operators, were integrated into strategic positions in chromosome III. Synapsis of these sites was achieved by the binding of a modified *lac* repressor, which is capable of forming a protein cross-bridge between clusters of operators (Kramer *et al.*, 1987). The ability of the resultant loops to influence the choice of donor locus during mating-type switching was examined using a novel genetic assay as well as a quantitative molecular method. These experiments revealed a relationship between defined topologies and donor selection, showing that large-scale interphase chromosomal topology can be a critical factor in the process of donor selection in wild-type yeast. The potency of these engineered chromosomal loops lends credence to models of donor preference which postulate that cell-type-specific chromosome topologies govern donor selection.

Fig. 2. Colony assay for perturbations in donor preference. Photographs of colonies produced by indicator strains expressing dimeric or tetrameric lactose repressors and having lactose operator clusters adjacent to *HML*, *MAT* or *HMR*. (A) Strain LM2, operators at *HML* and *MAT* expressing dimeric repressor. (B) Strain RM2, operators at *MAT* and *HMR* expressing dimeric repressor. (C) Strain RL2, operators at *HML* and *HMR* expressing dimeric repressor. (D) Strain LM4, operators at *HML* and *MAT* expressing tetrameric repressor. (E) Strain RM4, operators at *MAT* and *HMR* expressing tetrameric repressor. (F) Strain RL4, operators at *HML* and *HMR* expressing tetrameric repressor. Colonies were grown in parallel on YPD plates at 30°C for 8 days; they are shown at the same magnification and are ~4 mm in diameter.

In addition, these results demonstrate that engineered loops can be an effective tool for use in the study of the relationship between defined chromosome structures and chromosome function.

Results

Genetic screen

A special haploid yeast genotype was developed to facilitate monitoring donor preference in the presence of engineered chromosome loops. It lacks both the *STE2* and *STE3* genes, which encode receptors for α - and *a*-mating pheromones, respectively, rendering the genotype non-mating. Unlike most laboratory yeast strains, it has the wild-type *HO* gene, making it capable of high frequency mating-type switching. It has *HMLa* and *HMRa* alleles rather than the wild-type *HML α* and *HMR α* alleles. Because the inherent selection bias of donor loci causes *a*-cells to choose the *HMLa* donor and α -cells to use the *HMRa* donor, most of the recombination events involving *MAT* leave its mating-type allele unchanged (Klar *et al.*, 1982). Switches of mating type occur as a consequence of occasional failures of donor bias, which occur approximately once in 10 gene conversion events. The genotype also incorporates an *ADE2* gene placed under the control of an *a*-specific promoter (*PSTE2::ADE2*) causing α -cells to accumulate red pigment, characteristic of *ade2* cells, while leaving *a*-cells white, typical of *ADE2* cells. The combination of these genetic features produces a genotype that grows as variegated red and white colonies (Figure 2) (occasional stable red or white sectors arise due to the spontaneous formation of non-switching mutants). Color

variegation is the result of clusters of cells having the same mating type that persist due to the action of donor preference in the presence of the *HMLa* and *HMRa* alleles. The character of the variegation allows perturbations of donor preference to be identified and diagnosed visually.

The use of these switching strains to monitor donor preference presents some limitations. For instance, these strains are not suitable for monitoring the effect of $\alpha 2$ overproduction on donor preference. Overproduction of $\alpha 2$ using a galactose-inducible promoter causes the strains described in this paper to become non-switching, thus failing to provide meaningful insight into the effect of excess $\alpha 2$ on donor preference (data not shown). Expression of $\alpha 2$ in the population of cells having the *MATa* allele renders these cells non-switching because the *a1*– $\alpha 2$ complexes repress expression of *HO*, a gene whose expression is required for mating-type switching. As more and more of the *MATa* cells switch to *MATa*, the strain rapidly becomes composed entirely of non-switching *MATa* cells.

Chromosome modifications

Clusters of 256 *E. coli lac* operators (Robinett *et al.*, 1996) were inserted adjacent to *HML* and *MAT*, *MAT* and *HMR*, or *HML* and *HMR*, to create strains LM, RM and RL, respectively (see Materials and methods). Derivatives of these three strains were created that express *E. coli lac* repressor variants. Native repressor is bidentate, a tetramer capable of simultaneously binding two distant operators to form a protein-mediated 'looped complex' (Kramer *et al.*, 1987; Brenowitz and Jamison, 1993; Mehta and Kahn, 1999). Dimeric repressor variants, in contrast, have been

Fig. 3. Allele occupancy of the *MAT* locus. The proportion of the *MAT* locus having the *a*-allele was determined for the various strains described in this study (see Materials and methods). 'Wild type' refers to strain RKY150, which neither expresses lactose repressor nor has lactose operator clusters inserted into chromosome III.

developed that retain their ability to bind operators but are incapable of bridging pairs of *lac* operators (Chen and Matthews, 1992). Derivative strains LM4, RM4 and RL4 express the tetrameric form of the repressor (Straight *et al.*, 1996) able to form cross-bridges between pairs of distant operator clusters (Figure 1). Derivative control strains LM2, RM2 and RL2, constructed to serve as control strains, express a dimeric variant of the repressor (Straight *et al.*, 1998) that does not form cross-bridges (Figure 1).

As a control for the effects of tetrameric repressor on single clusters of *lac* operators, clusters were inserted adjacent to *HML*, *MAT* and *HMR* into a strain expressing the tetrameric *lac* repressor to create strains L4, M4 and R4, respectively (see Materials and methods).

In vivo* visualization of *HML*, *MAT* and *HMR

Microscopic examination of GFP fluorescence in strains LM2, RM2 and RL2 was performed to explore the spatial relationship of the *HML*, *HMR* and *MAT* loci. In all instances, pairs of *lac* operator clusters—whether they are located at L and M, M and R, or L and R—were not resolvable as separate fluorescent spots during the G₁ phase of the cell cycle (data not shown).

Effects on donor selection

Colonies produced by LM2, RM2 and RL2 (Figure 2) have the color and sectoring characteristic of normal donor preference, indicating that the introduction of operator clusters and their occupancy by repressor do not interfere appreciably with donor selection. The consequence of cross-bridging operator clusters was revealed by the colony phenotypes of strains LM4, RM4 and RL4. Strains having pairs of operator clusters adjacent to *HMR* and *MAT* (RM4) or adjacent to *HMR* and *HML* (RL4) produced sectorized colonies characteristic of normal donor preference (Figure 2). In contrast, the conspicuous colony phenotype of a strain having operator clusters adjacent to *HML* and *MAT* (LM4) indicates significantly perturbed donor selection. LM4 colonies had smaller and fewer red sectors than LM2 colonies, indicating a preponderance of *MATa* cells. Since the donor *a*-allele is resident at the *HML* locus in these strains, the pale colony phenotype of LM4 shows that *lac* repressor-mediated

pairing of *HML* to *MAT* leads to preferential selection of the *HML* donor.

The proportion of *a*- and α -alleles at the *MAT* locus in the engineered strains was quantitated (Figure 3) using a technique (see Materials and methods) that proved to be more sensitive than the colony assay. It revealed subtle effects on donor selection, such as a slight inhibition of *HML* selection that occurred when operator clusters were inserted adjacent to it (compare LM2 and RL2 with RM2; Figure 3). Comparison of LM4 with LM2 revealed that expression of the cross-bridging tetrameric form of lactose repressor increased the occupancy of the *MAT* locus with the allele resident at the *HML* locus by 160% when operator clusters were positioned at *HML* and *MAT*. In contrast, tetrameric repressor had a negligible effect on donor selection when operator clusters were adjacent to *HMR* and *MAT*. Curiously, when operators were positioned adjacent to *HMR* and *HML*, the tetrameric repressor caused a small but significant (40%) bias toward the allele resident at the *HML* locus, relative to the dimeric repressor.

Discussion

Locus proximity

The *in vivo* spatial relationship of *lac* operator clusters located adjacent to the *HML*, *HMR* and *MAT* loci was determined by fluorescent microscopic examination of bound *lac* repressor–GFP. Strains LM2, RM2 and RL2 displayed single fluorescent spots during the G₁ phase of the cell cycle, indicating that these loci are too close to be resolved as visibly separate fluorescent spots. The resolution of these observations was determined by the Rayleigh diffraction limit—the minimum distance at which two point sources can be resolved as separate—a function of the wavelength of light being imaged and the numerical aperture (NA) of the objective lens. These experiments utilized a 1.4 NA objective to visualize 500 nm green light, thereby giving a Rayleigh limit of 240 nm. Although this optical technique is incapable of demonstrating synapsis, it does show that these loci typically reside within 240 nm of one another, a distance that is approximately one-quarter of the diameter of the haploid G₁ phase nucleus.

Interphase chromosome loops

While the word 'loop' is a synonym of ring and hoop, the term 'chromosome loop' has sometimes been used to refer to a chromosomal domain delimited by sites of attachment to the nuclear matrix (Manuelidis, 1990). But since the nuclear matrix is dispersed throughout the nucleus during interphase, rather than highly condensed as it is in metaphase, consecutive matrix attachment sites need not be physically near one another. Thus, the term 'chromosome loop' has been used on occasion to refer to structures in interphase cells that are not necessarily loop shaped (Mirkovitch *et al.*, 1984; Nelson *et al.*, 1986; Jackson *et al.*, 1992). The engineered loops described in this paper are formed from the synapsis of two *lac* operator clusters by the *lac* repressor tetramer and thus have an authentic looped topology. It is clear from detailed structural studies of the tetrameric *lac* repressor–operator complexes that the

repressor cross-bridge brings operators within a few nanometers of one another (Lewis *et al.*, 1996).

Since switching occurs in haploids at the end of the G₁ phase of the cell cycle (Nasmyth and Shore, 1987), a time when a single copy of each chromosome is present, *lac* repressor-mediated pairing must occur between operator clusters on the same chromosome rather than between clusters on sister chromatids present during the S, G₂ or M phases of the cell cycle. Therefore, as far as effects on donor selection described in these experiments go, synapsis and chromosome loop formation are contemporaneous and inseparable. Also, since cleavage by the HO endonuclease occurs after G₁ phase-engineered loops have had a chance to form, this DNA strand scission should not affect loop function.

Loops can influence donor selection

Pairing *MAT* with *HML* via an engineered loop (strain LM4) had a pronounced and unambiguous influence on donor choice, substantially enhancing selection of *HML*. This result is at odds with proposed mechanisms of donor selection in which the chromatin of *HML* is rendered immune to recombination in α -cells. Engineered pairing of *HML* with *MAT* would not be expected to override the effect of chromatin and cause an enhanced selection of *HML*. The potency of the *MAT*–*HML* loop lends support to the notion that a similar mechanism for donor preference occurs during mating-type switching in wild-type yeast. Were such looping a part of the normal mechanism of donor selection, one would expect it to be cell-type specific, present in *a*-haploids to enhance selection of *HML* but absent in α -haploids to lessen selection of *HML*.

The tetrameric repressor in conjunction with operator clusters at *HMR* and *MAT* did not measurably influence donor selection. This result could be interpreted in two ways. One possibility is that the pairing system employed in these experiments is unable to pair *MAT* with *HMR*, perhaps because the engineered pairing is incompatible with the particular structural properties of the right arm of chromosome III. A second possibility is that the *HMR* locus is normally positioned adjacent to *MAT* in both *a*- and α -cells, in which case the engineered synapsis would be structurally redundant and ineffectual. Support for this second possibility is provided by the enhanced selection of *HML* that occurs in the presence of an *HMR*–*HML* loop (RL4 versus RL2; Figure 3). Such an enhancement would be expected if, by virtue of its intimate association with *HMR*, the *HML* locus were brought in close proximity to *MAT*.

Mechanistic implications

The disparate effects that loops connecting *HML* to *MAT* and *HMR* to *MAT* have on donor selection is perhaps not surprising in light of the inherent asymmetry in the regulation of donor preference. The *HMR* locus behaves as a default donor, one that is selected in the absence of RE activation of the *HML* locus. Deprived of the preferred *HML* locus, *a*-cells will readily use *HMR*, whereas α -cells deprived of an *HMR* locus often fail to complete the switching process and die as a consequence of the improperly resolved gene conversion event (Wu *et al.*, 1996). These observations, along with those presented in this paper, suggest that activation of the RE results in

synapse of *HML* and *MAT*, thereby contributing to the preferential selection of the *HML* donor by *a*-cells. In α -cells, inactivation of the RE allows *HML* and *MAT* to remain sufficiently distant from one another so that *HMR* becomes the preferred donor. Juxtaposition of *HML* with *MAT* by engineered pairing sites enhances selection of the *HML* donor by achieving the required chromosome topology while bypassing the usual requirement for RE activation. The ability of engineered loops to influence donor selection demonstrates the importance of chromosome topology in regulating this recombination process.

Steric consideration

A loop can promote physical interactions between distant parts of a chromosome and by doing so can constrain genetic exchange between particular recombination partners. It is reasonable to expect that a loop would be most effective in this role if its endpoints were located immediately adjacent to the recombination partners. Given the inherent flexibility of chromatin fibers, a loop's effectiveness in constraining recombination partners should be sensitive to the partners' distance from the loop endpoints.

In the experiments described here, clusters of *lac* operators were positioned as close to *HML* and *HMR* as possible without compromising the regulatory functions of the flanking *cis*-acting sites required for silencing transcription and switching (Abraham *et al.*, 1984; Feldman *et al.*, 1984). This constraint required that the operator clusters be placed 3 kb from the center of *HML* and 4.5 kb from the center of *HMR*. Placement of the operator cluster adjacent to *MAT* was made 6 kb from its center in order to avoid disrupting the essential *TSM1* gene (Walker *et al.*, 1996). The degree to which loop endpoint positioning influences donor selection remains a point of speculation in the absence of a systematic study.

Novelty and significance of engineered loops

The experimental results described in this paper demonstrate that engineered synapses can override the endogenous mechanism of donor selection during mating-type switching in the yeast *S.cerevisiae*. In doing so, this study goes beyond correlative studies and directly tests the causal relationship between large-scale chromosome topology and function. It demonstrates that biologically active interphase loops can be engineered into an otherwise wild-type chromosome using a well characterized prokaryotic operator–repressor system, and that these looped topologies can provide insight into the importance of higher order chromosome structures in nuclear processes.

Materials and methods

Strains

The parent strain genotype is *MAT(a or α), HMLa, HMR α , HO, trp1, ura3, his3, ste2, ste3::LEU2*, and the coding region of the *ADE2* gene (Stotz and Linder, 1990) is placed under the control of the *a*-cell-specific *STE2* promoter (Tan *et al.*, 1988) (*PSTE2::ADE2*). LM, operator clusters integrated adjacent to *HML* and *MAT*. RM, operator clusters integrated adjacent to *MAT* and *HMR*. RL, operator clusters integrated adjacent to *HML* and *HMR*. LM2, LM with dimeric repressor::*HIS3*, pAFS144 (Straight *et al.*, 1998). LM4, LM with tetrameric repressor::*HIS3*, pAFS67 (Straight *et al.*, 1996). MR2, RM with dimeric

repressor::HIS3. MR4, RM with tetrameric repressor::HIS3. RL2, RL with dimeric repressor::HIS3. RL4, RL with tetrameric repressor::HIS3.

Chromosome modifications

Oligonucleotide pairs were used to amplify portions adjacent to: *HML*, nucleotides 16 100–16 550 using GGAGCTCTGGTGTGTTATTTG-CGAGC and CCTCGAGGTTATCGTGAGTGGTGCCT; *MAT*, nucleotides 205 010–206 180 using ACATGTTGGAGCTCTAAATCA-CCT and ATGTTGGCGTCGACAGCAAGCT; *HMR*, nucleotides 297 070–298 520 using GACACTTTTGAGCTCTAGTTTTCT and ATAGTAGCTCGAGTCAAGAACATG. The amplified products were cloned into the *SacI*–*XhoI* sites of pAFS2 as *SacI*–*XhoI*, *SacI*–*SalI* and *SacI*–*XhoI* fragments, respectively, to create *lac* operator integrating constructs for *HML*, *MAT* and *HMR*. The *MAT* and *HMR* plasmids, cut with *XhoI* and *BamHI*, respectively, were integrated into the parent strain to generate intermediate strains M and R. The integrated *TRP1* markers of M and R were converted to *URA3* by transformation with *EcoRI*–*SacI*-cleaved YIplac211 (Gietz and Sugino, 1988). The resultant M derivative was further modified by integration of operator clusters at *HML*, cut with *BamHI*, to create strain LM. The R derivative was further modified by integration of operator clusters at *HML*, cut with *BamHI*, to create strain RL and by integration at *MAT*, cut with *XhoI*, to create strain RM.

Allele occupancy

The allele occupancy of *MAT* was determined using a competitive PCR assay. The assay utilized three oligonucleotides: one specific for *MAT* (ACCCAAGAAGCGCAATAAGA), a second for the α -allele (CACAGCGGTTTCAAAAAAGCT) and a third for the *a*-allele (TGTGGC-ATTACTCCACTTCAA). All three oligos were added to a PCR where the template consisted of genomic DNA from a strain to be analyzed. The *a*- and α -allele oligos were present at a concentration of 1 μ M while the *MAT* oligo was 5' end labeled with 32 P and present at a limiting concentration of 0.1 μ M. The amplification product was separated on a 2% agarose gel. The radiolabeled DNA present in the 781 bp *a*-allele product and in the 959 bp α -allele product was quantitated. This assay was calibrated with template standards having known ratios of *MATa* and *MAT α* . Measurements were performed in triplicate on template DNA isolated from logarithmically growing strains in liquid YPAD medium that had achieved steady state with respect to *MAT* occupancy.

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