Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation

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The ura4+ gene displays phenotypes consistent with variegated expression when inserted at 11 sites throughout fission yeast centromere 1. An abrupt transition occurs between the zone of centromeric repression and two adjacent expressed sites. Mutations in six genes alleviate repression of the silent-mating type loci and of ura4+ expressed from a site adjacent to the silent locus, mat3-M. Defects at all six loci affect repression of the ura4+ gene adjacent to telomeres and at the three centromeric sites tested. The clr4-SS and rik1-304 mutations cause the most dramatic derepression at two out of three sites within cen1. All six mutations had only slight or intermediate effects on a third site in the center of cen1 or on telomeric repression. Strains with lesions at the clr4, rik1, and swi6 loci have highly elevated rates of chromosome loss. We propose that the products of these genes are integral in the assembly of a heterochromatin-like structure, with distinct domains, enclosing the entire centromeric region that reduces or excludes access to transcription factors. The formation of this heterochromatic structure may be an absolute requirement for the formation of a fully functional centromere.

[Key Words: S. pombe; PEV; heterochromatin; telomere; mating type]

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is dependent on the products of the \textit{SIR3} and \textit{SIR4} genes, suggesting that they are involved in tethering RAP1p and maybe telomeres to the nuclear envelope [Palladino et al. 1993]. These factors are therefore implicated in the assembly of the silent mating-type loci and telomeres into related heterochromatin-like structures that have variable accessibility to transcription factors and other proteins [Gottschling 1992; Singh and Klar 1992].

In fission yeast, genes placed within any of the three centromeres are transcriptionally repressed and exhibit phenotypic variegation [Allshire et al. 1994]. As in many organisms, recombination across fission yeast centromeres is also suppressed [Nakaseko et al. 1986] and they contain large domains packaged into unusual chromatin [Polizzi and Clarke 1991; Takahashi et al. 1992]. Fission yeast centromeres and telomeres, except during mitosis, are associated with the nuclear periphery throughout the cell cycle [Funabiki et al. 1993]. Therefore, the centromeric regions of \textit{S. pombe} chromosomes share many characteristics in common with heterochromatin and, although not visible by conventional staining methods, can be considered as representing small domains of centromeric heterochromatin. Centromeres are not the only regions of fission yeast chromosomes that have properties akin to heterochromatin. Reversible repression is also known to occur adjacent to the silent mating-type loci [\textit{mat2} and \textit{mat3}; Thon et al. 1994] and telomeres [Nimmo et al. 1994]. Repression of the silent mating-type loci is alleviated by mutations at the \textit{clr2}+, \textit{clr3}+, \textit{clr4}+, \textit{rik1}+, or \textit{swi6}+ loci [Egel et al. 1989; Thon and Klar 1992; Ekwall and Ruusala 1994, Lorentz et al 1994; Thon et al. 1994].

The \textit{swi6}+ gene is nonessential and encodes a protein with homology to proteins containing a chromodom domain [Lorentz et al. 1994] including the \textit{Drosophila} heterochromatin protein HP1 [Powers and Eissenberg 1993], Polycomb [Paro and Hogness 1991], murine m31 and m32, and human HSM [Singh et al. 1991] and HP1HS [Saunders et al. 1993]. Mutant forms of \textit{Drosophila} HP1 act as suppressors of variegating genes, whereas overexpression results in enhanced repression [Eissenberg et al. 1992]. Several chromodom domain proteins have been found associated with centromeric heterochromatin [Powers and Eissenberg 1993, Saunders et al. 1993, Nicol and Jeppesen 1994, Wreggett et al. 1994]. Similarly, Polycomb is required to maintain repression of homeotic genes during development [Paro 1993].

The studies of telomeric silencing in \textit{S. cerevisiae} suggested that some or all of the products of the \textit{clr1}+, \textit{clr2}+, \textit{clr3}+, \textit{clr4}+, \textit{rik1}+, and \textit{swi6}+ genes might be involved in silencing not only at the \textit{mat} loci, but also at telomeres and centromeres in \textit{S. pombe}. Here, we first establish the limits of the silenced domain within \textit{S. pombe cen1} and test the effect of mutations at these six loci on \textit{ura4}+ expression at various locations: adjacent to \textit{mat3-M,} integrated at three sites within \textit{cen1}, or next to a telomere. Three of the mutations identify gene products crucial for the maintenance of centromeric gene repression and/or complete centromere function. We propose that the formation of a heterochromatin-like structure is important for centromere function in fission yeast and utilizes factors involved in silencing at other chromosomal locations.

## Results

### The assays

In the data presented below, repression of the \textit{ura4}− gene at various sites in the fission yeast genome (centromeric, telomeric, adjacent to \textit{mat3}) is assayed in two ways. First, all strains contain a convenient control \textit{ura4-DS/E} minigene allele residing at the \textit{ura4} locus that produces a truncated, nonfunctional, mRNA easily distinguishable from full-length \textit{ura4}+ mRNA by Northern analyses. After quantitation, the ratio of the full-length \textit{ura4}+ mRNA to the truncated minigene control mRNA allows the level of expression/repression from each site to be assessed. In all cases, these strains are grown under nonselective conditions so the level of \textit{ura4}+ expression seen in each strain reflects an average for the whole population. The second assay is presented in the form of serial dilution of cells from each strain plated on nonselective (N/S), selective (URA−), or counterselective (FOA) plates. In fission yeast it is not known how much \textit{ura4}+ product is required to render a cell Ura+. In addition, it is not known how much \textit{ura4}+ product can be tolerated in an FOA-resistant cell. Previously, we have shown that strains with the \textit{ura4}+ gene inserted within \textit{cen1, cen2,} or \textit{cen3} and grown in N/S, FOA, or URA− media have the same repressed level of \textit{ura4}+ message [Allshire et al. 1994]. Therefore, there must be a relatively low threshold of \textit{ura4}+ gene expression below which a cell is FOA resistant and above which it is Ura+. The plating assays appear more sensitive to small changes in the level of \textit{ura4}+ expression around this threshold. Northern analyses cannot always detect these small differences in expression but permit a distinction between slight alleviation and more dramatic derepressed states. In addition, some strains with the \textit{ura4}+ gene inserted at certain centromeric sites have, on average, a high level of \textit{ura4}+ expression but can form FOA− colonies at a relatively high frequency. Closer examination often reveals that the colonies formed are smaller than colonies formed by control strains. A plausible explanation is that the \textit{ura4}+ gene in such strains is generally expressed under nonselective growth conditions but that the repressed state is selected upon plating on FOA. These strains can be thought of as bearing a repressible \textit{ura4}+ gene. Similarly, other strains that have low levels of \textit{ura4}+ mRNA under nonselective growth conditions but that can grow on URA− plates can be thought of as bearing an expressible \textit{ura4}+ gene. The range of repression/expression of \textit{ura4}+ at different sites of insertion is variable, presumably reflecting differential access to transcription factors at these sites.

### Variegated expression of \textit{ura4}+ occurs at multiple sites within \textit{cen1}

The \textit{ura4}+ gene is variably repressed when placed at a
central location within fission yeast cen1, cen2, or cen3 as judged by reduced levels of expression and heterogeneity in the ability to form colonies on selective (URA-) or counter selective (FOA) plates [Allshire et al. 1994]. To establish the extent of the position-effected domain, further analyses have concentrated on the entire region of ~35 kb containing cenl (Fig. 1A).

The urad4 + gene was inserted at 13 sites within and immediately flanking the cenl region by transplacement with cloned fragments of cenl DNA disrupted by the urad4 + gene. Southern analyses indicated that in each case the structure of cenl was preserved, apart from the desired urad4 + insertion event (data not shown). The urad4 + gene was first inserted at seven sites (shown in Fig. 1B,C) extending from the Ncol site on the left side of imrlL (B'), across cntl (CC1) to the Ncol site at the right side of imrlR (B'). The urad4 + gene is shown inserted in both orientations at the left and right imrl Ncol sites. Only one orientation of the urad4 + gene is shown at the other insertion sites (Fig. 1B). Transcription of the urad4 + gene at each insertion site was examined by comparison with the urad4-DS/E minigene internal control (Allshire et al. 1994). Figure 1B shows a symmetrical profile with respect to urad4 + expression levels across imrlL, cntl, A cen l-

Figure 1. The insertion and state of repression of the urad4 + gene within the central region of cenl. [A] A schematic representation of S. pombe cenl. [B] Northern analyses of total RNA prepared from strains with the urad4 + insertion sites as shown grown in rich nonselective medium (YEA) at 32°C. The filter was hybridized with labeled urad4-DS/E probe washed and exposed to a PhosphorImaging screen. The resulting image is shown from which the relative level of urad4 + RNA produced from the intact gene vs. that from the urad4-DS/E minigene residing at the urad4 locus was calculated (below tracks). The position of each urad4 + insertion relative to a restriction map and schematic diagram of the central region of cenl is shown. The nomenclature of various regions is as presented by Takahashi et al. [1992], and the K'/B'/CC1 nomenclature of Hahnenberger [1991] is also outlined. The vertical lines within the imrlL and imrlR regions show the position of tRNA genes. The symbols < and > indicate the direction of transcription of the inserted urad4 + gene at each site. The sites where the urad4 + gene was inserted in both orientations are marked [*]. Restriction enzyme sites: [B] BamHI; [Bg] BglII; [C] Clal; [E] EcoRI; [RV] EcoRV; [H] HindIII; [Hp] HpaI; [K] KpnI; [N] Ncol; [Nd] NdeI; [S] SphiI; [Sp] Spel; [X] XhoI. (Not all sites for each enzyme are shown.) [C] Cultures of the same strains as shown in B above were grown to 0.5×10^7 to 2.0×10^7 cells/ml in N/S medium. Serial dilutions (1:5) of each culture were spotted onto URA-, FOA, and N/S plates and incubated at 32°C for 3–4 days. The highest density spots contained 4×10^3 cells. Strains and location of the urad4 + gene within cenl are indicated with respect to the same schematic diagram as described in B.

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and imr1R, reflecting the inverted arrangement of sequences in this region of the centromere. The ura4* gene is repressed at all sites but is apparently expressed well when placed between the tRNA^ala and tRNA^eth genes.

Growth of these strains at 32°C in the absence of uracil or presence of FOA is shown in Figure 1C. Again, there is a symmetrical relationship with respect to their ability to grow on URA^- and FOA plates. All strains grow equivalently on nonselective plates. Strains FY496, FY498, FY501, and FY502, with low levels of ura4* transcript under nonselective growth conditions, form more large colonies on FOA than on URA^- plates. However, strains such as FY524 and FY525, which show relatively high levels of ura4* transcript under nonselective conditions (0.5 and 0.7 in Fig. 1B), grow well on URA^- plates and poorly on FOA plates. In these strains the ura4* gene is inserted in the 350-bp region between two tRNA genes. It is known that this region of chromatin is DNase I hypersensitive (Takahashi et al. 1992); therefore, this site may also be more accessible to transcription factors. The fact that these strains can form small colonies on FOA plates indicates that the ura4* gene at these sites is still repressible.

The ura4* gene was also inserted at four sites within otr1 (dg1/dh1 or K'/L/K") and two sites just outside cen1 (Fig. 2A). Insertion of ura4* into otr1 was complicated by the fact that not only are there 2 copies of the repetitive elements comprising the cen1 otr, but cen2 and cen3 contain up to 4 and 13 copies of these repeats, respectively (Steiner et al. 1993). Consequently, insertion events at these sites within cen1 were infrequent, and for this reason only left-side or right-side otr1 insertion events were analyzed. Because of the symmetry of cen1, we assumed that the behavior at equivalent sites on each side would be identical. Only FY648 has the ura4* gene inserted on the right side, whereas all others are on the left as drawn. All insertion sites within otr1 showed reduced ura4* mRNA levels [Fig. 2A]. Even in strains FY939 and FY988, with insertion sites in the more distal region of otr1, expression of the inserted ura4* gene is still repressed and many colonies develop on FOA plates [Fig. 2B]. However, just 0.9 and 1.2 kb outside otr1, at the Xhol (FY937) and Hpal (FY941) sites,
the \textit{ura4} \textsuperscript{+} gene is expressed at relatively high levels (Fig. 2A) and colonies are rarely formed on FOA plates (<1x10\textsuperscript{-4}) (Fig. 2B).

These data suggest that there is a rapid change between the repressed region inside the distal BglII site in \textit{otrl} to the expressing XhoI site 0.9 kb outside the centromeric domain. Repression of the \textit{ura4} \textsuperscript{+} gene outside the distal region of the large inverted repeat is minimal. Consistent with this abrupt transition in centromeric repression, plating experiments suggest that at the distal BglII insertion site the \textit{ura4} \textsuperscript{+} gene is more repressed when its promoter is adjacent to \textit{otrl} (FY939) than when it is adjacent to the flanking outer sequences (FY988). Fewer and smaller FOA \textsuperscript{+} colonies result from the strain FY988 with the \textit{ura4} \textsuperscript{+} gene transcribed toward the centromere (Fig. 2B). No effect of gene orientation is seen at other sites where tested (cf. FY965 and FY967).

\textbf{Mutations at six loci alleviate silencing of \textit{ura4} \textsuperscript{+} adjacent to mat3 and telomes}

In \textit{S. cerevisiae}, mutations in genes that suppress silencing at the silent mating-type loci also alleviate repression of telomere adjacent genes (Aparicio et al. 1991). Six previously isolated mutations, \textit{clrl-5}, \textit{clr2-E22}, \textit{clr3-E36}, \textit{clr4-S5}, \textit{rik1-304}, or \textit{swi6-115}, appear to alleviate repression of the \textit{S. pombe} silent mating-type loci, \textit{mat2-P} and \textit{mat3-M} (Egel et al. 1989; Thon and Klar 1992; Ekwall and Ruusala 1994; Lorentz et al. 1994). When the \textit{ura4} \textsuperscript{+} gene is inserted adjacent to the \textit{mat3-M} locus (\textit{mat3-M::ura4} \textsuperscript{+}) its expression is severely repressed, but this is alleviated by mutations at the \textit{clrl}, \textit{clr2}, \textit{clr3}, \textit{clr4}, \textit{rik1}, and \textit{swi6} loci (Ekwall and Ruusala 1994; Thon et al. 1994). Figure 3A demonstrates that the \textit{mat3::ura4} \textsuperscript{+} gene results in only a few small colonies on URA\textsuperscript{-} plates but many large colonies on FOA plates in a wild-type background. In contrast, the six mutant backgrounds reduce or lose this ability to grow in the presence of FOA and gain the ability to grow normally on URA\textsuperscript{-} plates. Control strains bearing the \textit{ura4-DS/E} locus alone or in addition to a functional \textit{ura4} \textsuperscript{+} gene integrated at a random, nonrepressed site (Allshire et al. 1994) are included for comparison.

Transcription of the \textit{mat3-M::ura4} \textsuperscript{+} gene was compared with that from the \textit{ura4-DS/E} minigene by Northern analyses (Fig. 3B). Very little \textit{ura4} \textsuperscript{+} transcript is de-
tected in a wild-type background, but the presence of any of the six mutations increases the level of ura4+ message to levels approximately half that of the minigene control. Thus, although these mutations suppress the ability of a mat3::ura4+ strain to grow on FOA, they do not completely abolish all repression imposed on this ura4+ gene. Complete deletion of the nonessential swi6+ gene had identical effects as the swi6-115 allele on expression from mat3::ura4+ (data not shown).

The six mutations were also tested for their effect on telomere-mediated repression of ura4+ using a strain with the gene adjacent to and transcribed toward a telomere constructed by telomere-associated chromosome breakage of a nonessential minichromosome Chl6 (Nimmo et al. 1994). Serial dilution assays and Northern blot analyses were performed (Fig. 3C,D). In the wild-type background, the telomere-adjacent ura4+ gene (Chl6 m23-ura4+–TEL) is repressed, because this strain (FY520) displays reduced growth on URA- plates, whereas many large colonies are visible on FOA plates. This situation is reversed by the presence of any of the six mutations, indicating that defects in any of these genes can alleviate the repressive effects imposed by the nearby telomere (Fig. 3C). However, the levels of ura4+ transcript produced from the telomeric site indicate that these mutations have only slight effects on repression (Fig. 3D). Deletion of the entire swi6+ gene had the same minimal effect on expression of this telomeric ura4+ gene (data not shown). Therefore, none of the six mutations tested abolishes repression imposed by telomeres on the adjacent ura4+ gene.

As a control, Northern analyses of a set of strains containing TM1-ura4+ integrated at a random site (Allshire et al. 1994) in wild-type and mutant backgrounds is shown (Fig. 3E). The ura4+ gene is expressed at normal levels, and none of the six mutations tested alters the expression of this ura4+ gene.

The clr4 and rik1 mutations abolish ura4+ repression at certain sites within cen1.

Strains with mutations in any of the six genes were tested to see if they alter repression of ura4+ genes inserted within fission yeast centromeres. Three sites of ura4+ insertion within cen1 (shown in Figs. 1 and 2) were analyzed: the central NcoI site within ctn1/TM1 (FY336), the NcoI site at the right end of imr1R (FY498), and the SplI site in otR1 (FY648). Plating assays of strains bearing one of these insertion sites, the clr1-5, clr2-E22, clr3-E36, clr4-S5, rik1-304, swi6-115, or swi6Δ::his1+ lesion, and the ura4-DS/E minigene control are shown in Figure 4. Wild-type controls and strains with a randomly integrated ura4+ gene or no functional ura4+ gene are included.

Most mutations alleviate ura4+ repression regardless of the site of insertion in the centromere, as shown by the impaired ability of these cen1 ura4+ insertion strains to form colonies on FOA plates. This suggests that the level of expression from ura4+ genes inserted within the centromere, at any of the sites tested, may increase when there is a defective clr1, clr2, clr3, clr4, rik1, or swi6 gene. Mutations in clr1, clr2, or clr3 clearly have a weaker effect at the imr1R[NcoI]:ura4+ and otR1[SplI]:ura4+ insertion sites than the clr4, rik1, and swi6 mutations. The clr1-5, clr2-E22, swi6-115, and swi6Δ::his1+ mutations appear to have little or no effect on the ctn1/TM1[NcoI]:ura4+ insertion site (Fig. 4).

Northern analyses (Fig. 5) show that in many cases the presence of a mutant locus leads to slight increases in the level of expression from the cen1 inserted ura4+ genes. However, certain mutations have their greatest effect on certain ura4+ insertion sites. In strains with the ctn1/TM1::ura4+ insertion, none of the mutations tested caused substantial derepression [expression of ura4+ is up to a maximum of 0.2 of the minigene control]. At the imr1R[NcoI]:ura4+ and otR1[SplI]:ura4+ insertions sites, the clr1, clr2, clr3, and swi6 mutations only result in partial derepression of the ura4+ gene. In contrast, at both of these sites, the presence of the clr4-S5 or rik1-304 mutations almost abolishes repression so that the level of ura4+ expression is elevated to between 0.85 and 0.95 of the minigene control. This suggests that there are distinct domains within cen1 that are highly sensitive to the clr4-S5 and rik1-304 lesions and other domains, such as the central region of cen1, which are not (see Fig. 7, below).

These data indicate that the products of the clr1+, clr2+, clr3+, clr4+, rik1+, and swi6+ genes all participate in the formation of structures that mediate gene repression at the silent mating-type loci, adjacent to telomeres and within centromeres. Mutations at two of these loci, clr4, and rik1, have the greatest effect on the repressive mechanisms but only at certain sites within the centromere. The products of the clr4+ and rik1+ genes must therefore play a critical role in the formation of a heterochromatin-like structure at fission yeast centromeres. As only three sites of ura4+ insertion within cen1 were tested in all mutant backgrounds, it is possible that the clr1, clr2, clr3, and swi6 mutations cause greater derepression at other untested centromeric sites.

The clr4, rik1, and swi6 mutations reduce the fidelity of linear and circular chromosome segregation.

It is possible that the repressed state within fission yeast centromeres is a manifestation of higher order structures required for the formation of a functional centromere. Because the clr1-5, clr2-E22, clr3-E36, clr4-S5, rik1-304, swi6-115, and swi6Δ::his1+ mutations can all affect transcriptional repression within cen1, these mutations may interfere with the structures formed at the centromere and thereby impair centromere function.

The fission yeast minichromosome Chl6 is a 530-kb derivative of chromosome III and is lost in ~0.2% of all cells [Niwa et al. 1989]. When Chl6 is lost from cells, they form red rather than white colonies on limited adenine plates. If loss occurs in the first division of a single cell on such a plate, then half of the resulting colony will retain the minichromosome and form a white sector,
Figure 4. The effect of mutations at the clrl, clrl2, clrl3, clrl4, rik1, and swi6 loci on FOA resistance and uracil prototrophy at three sites of ura4 + insertion within cen1. Cultures with the ura4 + gene inserted at cnt1/TM1 (NcoI) [top], imrl1 (NcoI) [middle], and otrl1 (SphI) [bottom] in wild-type and mutant backgrounds as shown were grown to 0.5x10^7 to 2.0x10^7 cells/ml in N/S medium. Serial dilutions (1:5 of each culture were spotted onto URA-, FOA, and N/S plates and incubated at 32°C for 3-4 days. The highest density spots contained 4x10^3 cells. FY340, which carries the ura4 + gene at a random site and grows as a fully ura + strain, is included for comparison in each panel. FY489, which has no functional ura4 + gene, only the ura4-DS/E allele, is also included.

whereas the other half, with no minichromosome, will form a red sector. By scoring the frequency of half-sectored colonies, an accurate rate of chromosome loss per division can be calculated.

Strains bearing the minichromosome Chl6 and one of each of the mutations were constructed, and the rate of Chl6 loss compared with that in a wild-type background [Table 1A]. The loss rate/division in wild type strains is not influenced by mating type and is similar to estimates published previously [Niwa et al. 1989]. Strains bearing lesions at clrl1, clrl2, or clrl3, which had weak effects on centromeric transcriptional repression, display rates of loss similar to wild type. In contrast, strains with the clrl4-S5 or rik1-304 mutations missegregate the minichromosome in ~5% of cell divisions, almost a 100-fold increase over wild type. These same two loci have the greatest effect on transcriptional repression within centromeres [Fig. 5]. Mutations at the swi6 locus also affect chromosome segregation. In strains bearing swi6-115 or swi6Δ::his1 +, abnormal segregation is elevated 35- and 95-fold so that the minichromosome is lost in 1.8% and 4.9% of cell divisions, respectively.

The effect of these mutations on the segregation of normal chromosomes was also tested. Diploids homozygous for each mutation were forced by intragenic complementation between the ade6-210 [red] and ade6-216 [pink] alleles. In the absence of selection for Ade + cells, loss of a chromosome causes the diploid to revert rapidly to the haploid state forming red or pink sectors, allowing the rate of whole chromosome loss to be estimated. Table 1B demonstrates that the clrl4-S5, rik1-304, and swi6-115 mutations also cause elevated rates of loss of endogenous chromosomes.

Because the clrl1-5, clrl2-E22, clrl3-E36, clrl4-S5, rik1-304, and swi6-115 mutations may affect gene repression at telomeres [i.e., little or no growth on FOA, Fig. 4C], it is possible that the elevated loss rate of the linear minichromosome Chl6 and whole chromosomes above was mediated through a defect in telomere function rather than changes in centromere structure and function. To test this possibility, the rate of loss of the 36-kb centromeric circular minichromosome CM3112 [Matsumoto et al. 1990] was measured in the presence and absence of all these mutations [Table 1C]. Again the rate of chromosome loss is independent of mating type and clrl1-5, clrl2-E22, or clrl3-E36 have little or no effect on the CM3112 loss rate, which remains at between 1% and 2% of all divisions. The mis-segregation of this circular minichromosome occurs at such a high rate in strains bearing clrl4-S5, rik1-304, and swi6-115 mutations that an-
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**Discussion**

A zone of repression covering the centromeric domain

Transcription of the *ura4*+ gene is repressed at all 11 sites tested within the domain occupied by fission yeast *cen1*, as demarcated by the large inverted repeat surrounding the central region. At two sites outside this domain, the *ura4*+ gene is not repressed (Figs. 1 and 2). These two sites are 0.9 kb [Xhol] and 1.2 kb [HpaI] outside the large inverted repeat as defined by Takahashi et al.

Other method, more suited to estimating high loss rates, was employed [Kipling and Kearsey 1990]. Table 1C shows that in strains with *clr4-S5, rik1-304, or swi6-115* mutations the minichromosome CM3112 is lost in 18%, 21%, and 21% of divisions, respectively. This dramatic difference in the stability of CM3112 in wild-type versus *clr4-S5, rik1-304, and swi6-115* backgrounds is illustrated in Figure 6. In *S. pombe*, acentric minichromosomes or *ars* containing plasmids are, in general, lost in 20–50% of cell divisions [Heyer et al. 1986; Matsumoto et al. 1990]. Therefore, these results demonstrate that mitotic centromere function of the already compromised circular minichromosome CM3112 is almost completely obliterated in *clr4-S5, rik1-304, and swi6-115* backgrounds. This implies that the factors encoded by these genes are essential for the function of this minimal centromere.

### Table 1. Effect of *clr1, clr2, clr3, clr4, rik1, and swi6* mutations on the fidelity of chromosome transmission

#### A. The 530-kb linear minichromosome Ch16

| Strain | Background | per division | percent divisions |
|--------|------------|--------------|------------------|
| FY521  | WT h+      | 4.4 x 10^-4  | 0.044            |
| FY721  | WT h-      | 5.8 x 10^-4  | 0.058            |
| FY723  | clr1-5     | 1.7 x 10^-3  | 0.17             |
| FY725  | clr2-E22   | 1.9 x 10^-3  | 0.19             |
| FY727  | clr3-E36   | 1.1 x 10^-3  | 0.11             |
| FY729  | clr4-S5    | 4.9 x 10^-2  | 4.90             |
| FY731  | rik1-304   | 4.8 x 10^-2  | 4.8              |
| FY733  | swi6-115   | 1.8 x 10^-2  | 1.8              |
| FY1036 | swi6A::his1+ | 4.9 x 10^-2 | 4.9              |

#### B. Whole chromosomes monitored by breakdown of diploids

| Strain | Background | per division | percent divisions |
|--------|------------|--------------|------------------|
| FY1100 | WT         | <1 x 10^-3   | <0.1*            |
| FY1102 | clr1-5     | <1 x 10^-3   | <0.1*            |
| FY1104 | clr2-E22   | <1 x 10^-3   | <0.1*            |
| FY1106 | clr3-E36   | <1 x 10^-3   | <0.1*            |
| FY1108 | clr4-S5    | 4.7 x 10^-2  | 4.7*             |
| FY1110 | rik1-304   | 3.2 x 10^-2  | 3.2*             |
| FY1112 | swi6-115   | 2.4 x 10^-2  | 2.4*             |

#### C. The 30-kb circular minichromosome CM3112

| Strain | Background | per division | percent divisions |
|--------|------------|--------------|------------------|
| FY759  | WT h+      | 1.1 x 10^-2  | 1.1              |
| FY865  | WT h-      | 9.0 x 10^-3  | 0.9              |
| FY853  | clr1-5     | 1.5 x 10^-2  | 1.2              |
| FY787  | clr2-E22   | 7.9 x 10^-3  | 0.8              |
| FY790  | clr3-E36   | 1.7 x 10^-1  | 1.7              |
| FY856  | clr4-S5    | 1.8 x 10^-1  | 18.0*            |
| FY859  | rik1-304   | 1.7 x 10^-1  | 17.0*            |
| FY862  | swi6-115   | 2.1 x 10^-1  | 21.0*            |

*The loss rate was determined by the method of Kipling and Kearsey [1990].
Figure 6. Mutations at clr4, rik1, and swi6 disrupt segregation of a circular minichromosome. Wild-type [FY759], clr4-S5 [FY856], rik1-304 [FY859], and swi6-115 [FY862] colonies containing the circular minichromosome CM3112 were picked from plates selecting for the minichromosome (Ade−), resuspended in N/S medium, and plated onto YE + 0.15A plates. Colonies retaining the minichromosome are white; those without it are red. Minichromosome loss during colony outgrowth results in white colonies with red sectors. Little or no white sectors are visible in the strains bearing either the rik1-304, clr4-S5, or swi6-115 lesions, indicating that centromere function is severely disrupted in the presence any of these mutations.

al. (1992), although other data (Steiner et al. 1993; R. Allshire and G. Cranston, unpbl.) suggest that this inverted repeat extends at least as far as the HpaI site [Fig. 2A]. At the BgIII insertion site at the end of the otr region, urad4+ transcription is repressed. The fact that at a site only 0.9 kb [XhoI, FY937] further away little repression is observed indicates that the centromeric zone of repression must terminate abruptly within this 0.9-kb region. It is conceivable that the 0.9-kb region contains a specific sequence that acts as a barrier to the formation of repressive structures outside this region. It has been postulated that specific terminators are required to halt the assembly of heterochromatin along a chromosome [see Eissenberg 1989]. Specific DNA fragments have been identified that, in Drosophila, insulate genes from position effects [Kellum and Schedl 1991; Chung et al. 1993]. The 0.9-kb cen1 region may have a similar role in insulating neighboring genes from centromeric repression. Alternatively, the large inverted repeat within the centromere may form a higher order structure, such as a hairpin [see Fig. 7c], which mediates repression. In this case, the HpaI and XhoI sites would lie at the base of this structure and might be less prone to repression.

Repression, unusual centromeric chromatin, and repeats

The central domain of fission yeast centromeres forms unusual chromatin (Polizzi and Clarke 1991; Takahashi et al. 1992), which we have shown influences the chromatin structure of the urad4 gene when placed in the center of cen1 or cen2 (Allshire et al. 1994). We proposed that this unusual chromatin was causally related to the repression of urad4 observed at these sites. It was therefore surprising to find that the urad4 gene is also repressed in the outer domains of cen1 where the DNA is packaged into regular nucleosomes. This suggests that either different mechanisms of repression operate in these different domains or that the alteration in urad4+ chromatin structure within cen1 and cen2 does not mediate repression.

Clarke et al. (1993) have observed that all functional centromere constructs assemble unusual chromatin in the central domain, whereas nonfunctional constructs do not. This suggests a link between specialized chromatin structure and centromere function. It is also possible that a higher order structure, for example a hairpin configuration [such as that proposed by Takahashi et al. 1992, Clarke et al. 1993; see Fig. 7c], is required to mediate both centromeric repression and centromere function. Recent observations in Drosophila indicate that tandem or inverted repeats of a minigene have a propensity to interact and mediate their own repression. In some configurations this repression is sensitive to common suppressors of variegating position effects such as mutations in the Suvar(2)5 gene encoding HP1 (Dorer and Henikoff 1994). It has been proposed that repression is brought about by the pairing of copies of the minigene so as to exclude transcription factors. Other phenomena related to PEV, such as trans-sensing, are also thought to rely on somatic pairing (Henikoff 1994). It is therefore intriguing that, apart from containing large inverted repeats of 18 kb or more, all fission yeast centromeres cluster together at the nuclear periphery in interphase (Funabiki et al. 1993) and that genes are also repressed at fission yeast telomeres (Nimmo et al. 1994) which also form aggregates at the nuclear periphery (Funabiki et al. 1993). Credence to the idea that a higher order structure is required for fission yeast centromere function comes from the observation that certain minichromosome con-
Factors affecting position effects at fission yeast centromeres

Transcriptional repression linked to centromere function in fission yeast

We have shown that mutations at clr4 and rik1 derepress the ura4+ gene at certain sites within fission yeast cen1 much more than those at clr1, clr2, clr3, or swi6. All of these mutations have minimal effects on the expression of the ura4+ gene at telomeres and no effect on expression of the ura4+ gene from a random integration site. Strains bearing either clr4 or rik1 mutations also display highly elevated rates of chromosome loss. In contrast, the clr1, clr2, and clr3 mutations have a negligible effect on chromosome segregation. This strongly suggests that the zone of repression formed within fission yeast centromeres has an integral role in the formation of fully functional centromeres. In Figure 7a this zone of repression is depicted as the centromeric DNA coated with a heterochromatin-like structure that is refractory to transcription factors. This centromeric zone of repression appears to be divided into separate domains. The central region remains resistant to transcription in strains bearing any of the mutations tested. However, in the presence of the clr4-S5 or rik1-304 mutation, the flanking domains are released and become accessible for transcription (Fig. 7b). It is envisaged that this heterochromatin-like structure is required, directly or indirectly, to either maintain the attachment of sister chromatids during mitosis up until the onset of anaphase or form the foundations for the assembly of a functional kinetochore and its attachment to the microtubules emanating from the spindle pole body.

Strains bearing mutations at the swi6 locus also exhibit high rates of chromosome loss even though they have weaker effects on repression at the three centromeric sites tested. It is possible that these swi6 mutations cause complete derepression at other untested regions of the centromere, although complete derepression per se is not necessarily a prerequisite for defective centromere function. Alternatively, the swi6 protein might be involved in facilitating the formation of a higher order structure on the underlying heterochromatin but is not directly involved in mediating transcriptional repression at centromeres (Fig. 7c). To explain the effects of the swi6-115 mutation on the directionality of mating-type switching, it has been proposed previously that the swi6 product promotes intrachromosomal folding of the silent mating-type loci onto mat1 (Thon and Klar 1993). In addition to clr4+, rik1+, and swi6+, it is conceivable that the products of the clr1+, clr2+, and clr3+ genes have an auxiliary role in the formation of centromeric heterochromatin, but they are not critical for centromere function.

Heterochromatin and centromere function in other organisms

In many eukaryotes, the centromeric region is associated with arrays of repetitive DNA such as aliphoid satellite in humans, minor satellite in mouse, and simple and complex satellite DNA in Drosophila (Miklos and Cotsell...
These regions are normally packaged as heterochromatin and therefore tend to be late replicating and suppressed for recombination and to show frequent associations with the nuclear periphery (see introductory section). Although the exact role of these heterochromatic regions in centromere function and kinetochore assembly remains to be elucidated, several studies indicate that they contribute to centromere function. At human centromeres, although CENP-B is associated with both active and inactive centromeres, aliphid repeats associated with CENP-B underlie the kinetochore (Cooke et al. 1990) and injection of dividing cells with predominantly anti-CENP-B antibodies leads to some disruption of kinetochore structure, which may be the result of alteration in the compaction of the underlying chromatin (Bernat et al. 1991). Aliphid repeats inserted at noncentromeric sites may display some aspects of centromere function (Haaf et al. 1992; Larin et al. 1994). In Drosophila it is known that certain alleles of Suvar(3)6 (encoding protein phosphatase 1), which suppresses variegation of white adjacent to heterochromatin, can result in abnormal chromosome segregation (Baska et al. 1993). Again in Drosophila, a marker chromosome responds to suppressors of variegating position effects, so that in genetic backgrounds where there is less repression of reporter genes, and presumably less heterochromatin, the chromosome is less stable. Analyses of aberrant mitotic configurations in these flies suggested that the nondisjunction events resulted from premature sister chromatid separation (Wines and Henikoff 1992). This implies that the amount of DNA packaged as heterochromatin within a centromeric domain directly influences the functionality of that centromere.

Of the three genes that we have shown to disrupt centromere function (clr4+, rik1+, and swi6+), only the swi6+ gene has been cloned and sequenced. It is intriguing that swi6+ encodes a protein containing a chromodomain and plays a role in transcriptional silencing and perhaps in the formation of a folded structure at the mat loci (Thon and Klar 1993; Lorentz et al. 1994). Other chromodomain-containing proteins, such as HP1, are known to be associated with centromeric heterochromatin (see introductory section). Mutations in the Drosophila chromodomain protein HP1 [Suvar(2)5] alleviate repression of variegating genes, whereas increased dosage of the wild-type gene enhances this repression (Eisenberg et al. 1992). Recently, it has been observed that aberrant chromosome segregation occurs in Drosophila embryos homozygous for HP1 mutations. These embryos contain a high frequency of nuclei with lagging chromosomes and anaphase/telophase bridges [R. Kellen and B. Alberts, pers. comm.]. Clearly, this also implies that normal chromosome segregation is dependent on the integrity of centromeric heterochromatin.

Concluding remarks

Further analyses of the clr4+, rik1+, and swi6+ genes and their products should lead to a more complete understanding of their role in centromere function. At present, the product of swi6+ is a good candidate for a component of a heterochromatin-like structure at centromeres and at other sites in fission yeast. The products of clr4+ and rik1+ could also be components of heterochromatin-like structures or may play a role in the regulation of such structures. Our analyses indicate that the clr4+, rik1+, and swi6+ genes have important roles at both centromeres and the silent mating-type loci, whereas the clr1+, clr2+, and clr3+ genes have a less critical role at the former. Further genetic and molecular analyses may reveal other factors that only play a critical role in centromeric transcriptional repression and centromere function. Such factors might include DNA-binding proteins that interact specifically with certain DNA sequences at centromeres. Alternatively, other centromere-specific non-DNA-binding factors, required for the assembly of a functional kinetochore, may be identified. Such factors might simultaneously associate with the products of the clr4+, rik1+, or swi6+ genes to form heterochromatin at fission yeast centromeres that promotes the assembly of a functional kinetochore.
ura4⁻ HindIII fragment was inserted at both sites generating pPhe-otrI[XhoI]-ura4 and pPhe-otrI[HpaI]-ura4.
pPhe-otrI/dh1Xhol/SphI/BglII]-ura4 Oligonucleotides were designed to amplify the region between the Phe tRNA (same oligo as above) and part of dh1a/K' CCAGCTTTATGCGCAAAACATGCATG. The 2.2-kb PCR product was digested with Xhol and Sphl, and the 1.4-kb fragment was cloned in pGEM7. The ura4⁺ gene on a 1.75-kb BamHI fragment from puc13/18-ura4 was cloned in both orientations [oriI and oriII] into the BglII site at the end of dh1a/K'.

puc21otr1-diNdcl/BglII/HindIII]-ura4 Oligonucleotides were designed to generate a 1.51-kb PCR product spanning the Ndcl-BglII region of dh1a/K' of cen1 [GAACATTTCTCAGATTTAAGGCTC] and GCCATGTAACTAAAAACTTTACCG). The product was digested with Ndcl and BglII, and the 1.08-kb fragment was inserted into puc21. The ura4⁺ gene on a 1.75-kb HindIII fragment was cloned in both orientations [oriI and oriII] into the HindIII site located in the insert.

potr1-dgl1a/K' [HindIII]-ura4 A 2.22-kb PCR product generated with oligonucleotides CAGGGCCTGGATCAAGATC and GGCATGCGATGATGTCTCA spanning the Asp718 and Sphl sites within dgl1a/K' of cen1 was cut with these enzymes and cloned in puc21. The ura4⁺ gene on a 1.75-kb HindIII fragment was cloned in the HindIII site located in the insert.

S. pombe media

Media were essentially as described by Moreno at al. (1991) and in Allshire et al. (1994). For chromosome loss tests, YE plates were supplemented with 0.15 the normal amount of adenine [YE + 0.15A, 12 mg/liter]. Comparative plating and serial dilution experiments were performed as described previously [Allshire et al. 1994; Nimmo et al. 1994].

Strain construction

For transformation, a simplified version of the lithium acetate pH 4.9 procedure was used [Moreno et al. 1991]. The genotype of all strains used is presented in Table 2. FY489 contains a 280-bp deletion in the ura4⁺ gene at the ura4 locus and was derived by crossing FY290 with FY200 [Allshire et al. 1994]. To insert the ura4⁺ gene at sites within cen1, the same general procedure was followed in each case. A linear DNA fragment with the region of interest disrupted by the ura4⁺ gene was released from various plasmids, gel-purified, and used to transform the strain SP813.

Ura⁺ transformants (200–2000) were then subjected to a simple stability test by streaking for single colonies on FOA plates. Single colonies were picked from each original colony that grew in the presence of FOA and again restreaked on FOA plates. These two rounds of streaking for FOA⁺ colonies ensured the loss of episomal forms. Colonies that grew were then streaked onto URA⁻ plates to identify cells retaining the ura4⁺ gene. DNA analyses confirmed the presence of the ura4⁺ gene at the desired site within the centromere. Within the imr1 and ctn1 regions, digestion with ClaI allowed insertions into the left and right sides of the inverted structure to be distinguished. Digestion with Hpal allowed insertion within the left and right Phe-dh1a/K' regions to be distinguished. The starting strain, SP813, carries a complete deletion of the ura4⁺ gene [ura4-D18]. After confirming that transformants had an insertion at the correct site on the left or right side of cen1, all were crossed with FY489 to introduce the truncated allele of ura4 [ura4-DS/E], allowing quantification of ura4⁺ RNA levels produced from each site. Pulse-field gel electrophoresis after Sall digestion of large DNA prepared in agarose plugs from strains with ura4⁺ insertions within cen1 demonstrated that no gross structural alterations had occurred. After hybridization with a ura4⁺ probe, the expected Sall fragment of ~56 kb containing the whole cen1 region could be detected in each strain [data not shown]. Other digests also indicated that the normal structure of cen1 was maintained.

FY336 and FY340 were described by Allshire et al. (1994). FY520 and FY521 were described by Nimmo et al. (1994). FY597 was derived from a cross between PG9 [Thon and Klar 1992] and FY489. FY1028, the swi6⁺ gene, was replaced with his1⁺ [lain Hagan, University of Manchester, UK], resulting in swi6Δ:his1⁺, similar to that described previously [Lorentz et al. 1994]. The clt-1, clt2-222, clt3-136, clt4-SS, rkl1-304, and swi6-115 mutations were followed in crosses by iodine staining and visual examination of cells by phase-contrast microscopy for those undergoing haploid meiosis [Thon and Klar 1992; Ekwall and Ruusala 1994; Lorentz et al. 1994]. After crossing with FY489, the strains FY530, FY602, FY637, FY514, FY512, FY528, and FY1028 were generated, and these strains that bear the clt-1, clt2-222, clt3-136, clt4-SS, rkl1-304, swi6-115, or swi6Δ:his1⁺ mutations and the ura4-DS/E allele were crossed with FY336, FY498, FY648, FY527, FY520, FY597, FY340, FY521, and FY757, generating a series of isogenic strains [see Table 2].

Mitotic chromosome stability assays

Cells (500–1000) from Ade⁺ colonies were plated on YE + 0.15 adenine plates and incubated at 32°C for 3–5 days followed by 2–3 days at 4°C to allow the red color to deepen. The number of colonies with a red sector covering at least half of the colony was counted. The number of chromosome loss events per division is the number of these half-sectored colonies divided by the total number of white colonies plus half-sectored colonies. The rate of loss of the circular minichromosome, CM3112, was too high to use the above method. Another method was employed [Kipling and Kearsey 1990]. Briefly, an Ade⁺ colony was picked, resuspended in a small volume of medium, and the cell density was measured. Cells (500–1000) were plated on Ade⁻ plates to estimate the proportion of cells in the colony containing the minichromosome. Cells (1 × 10⁸) from the same colony were grown in the absence of selection [Ade⁺ plates] for 15–20 generations, after which time the proportion of cells retaining the minichromosome was again estimated by plating on Ade⁻ plates at 32°C. The rate of chromosome loss per division was estimated using the formula: loss rate = 1 - (F/I)², where F is the final percentage of cells bearing the minichromosome, I is the initial percentage of cells carrying the minichromosome, and N is the number of generations between I and F [Kipling and Kearsey 1990].

DNA preparation, digestion, and analyses

DNA was extracted essentially as described in Moreno et al. (1991). High-molecular-weight DNA in agarose blocks for analysis by PFGE was prepared as described in Allshire (1992). DNA was digested according to the manufacturer's recommended conditions and analyzed by electrophoresis on 0.7–1.5% agarose gels in 0.5x TAE buffer. For PFGE analyses, samples were loaded on a 1% agarose gel in 0.5x TAE buffer. To separate all three centromeric Sall fragments, the gel was run on a Biorad CHEFL system for 22 hr at 200 V with a pulse time starting at 2 sec and ending at 20 sec. DNA was transferred and hybridiza-
### Table 2. S. pombe strains used in this study

| Strain         | Position and *ura4* gene | Genotype              |
|----------------|--------------------------|-----------------------|
| 972            | wild-type                | h^-                   |
| SP813          | deleted                  | h^- leu1-32 ade6-210, ade4-DS/E |
| FY489          | *ura4*-DS/E              | h^- leu1-32, ade6-210, ade4-DS/E |
| FY340          | TMI1[Ncol]-*ura4* Random int. | h^- leu1-32, ade6-210, ade4-DS/E |
| FY496          | imtR1[Ncol]-*ura4* orlI | h^- leu1-32, ade6-210, ade4-DS/E |
| FY501          | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY502          | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY524          | imtR1[aeHindIII]-*ura4* | h^- leu1-32, ade6-210, ade4-DS/E |
| FY525          | imtR1[aeHindIII]-*ura4* | h^- leu1-32, ade6-210, ade4-DS/E |
| FY527          | imtR1[aeHindIII]-*ura4* | h^- leu1-32, ade6-210, ade4-DS/E |
| FY534          | imtR1[aeHindIII]-*ura4* | h^- leu1-32, ade6-210, ade4-DS/E |
| FY941          | Phe-otr1L[Hpal]-*ura4*  | h^- leu1-32, ade6-210, ade4-DS/E |
| FY937          | Phe-otr1L[XhoI]-*ura4*  | h^- leu1-32, ade6-210, ade4-DS/E |
| FY939          | ottr1L[dh-BglII]-*ura4* orlI | h^- leu1-32, ade6-210, ade4-DS/E |
| FY988          | ottr1L[dh-BglII]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY965          | ottr1L[dh-HindIII]-*ura4* orlI | h^- leu1-32, ade6-210, ade4-DS/E |
| FY967          | ottr1L[dh-HindIII]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY986          | ottr1L[deHindIII]-*ura4* orlI | h^- leu1-32, ade6-210, ade4-DS/E |
| FY336          | cnt1/TM1[Ncol]-*ura4*   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY576          | cnt1/TM1[Ncol]-*ura4*   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY645          | cnt1/TM1[Ncol]-*ura4*   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY555          | cnt1/TM1[Ncol]-*ura4*   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY693          | imtR1[Ncol]-*ura4* orlI | h^- leu1-32, ade6-210, ade4-DS/E |
| FY695          | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY697          | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY699          | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY1032         | imtR1[Ncol]-*ura4* orlII | h^- leu1-32, ade6-210, ade4-DS/E |
| FY648          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY701          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY703          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY705          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY707          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY711          | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY1034         | ottr1R[SpI]-*ura4*      | h^- leu1-32, ade6-210, ade4-DS/E |
| FY597          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY624          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY633          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY627          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY629          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY631          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY622          | mat3-M[EcORV]-*ura4*    | h^0 leu1-32, ade6-210, ade4-DS/E |
| FY520          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY612          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY736          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY613          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY738          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY898          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY611          | Ch16 m23:ura4-TEL[72]   | h^- leu1-32, ade6-210, ade4-DS/E |
| FY521          | Ch16 m23:ura4            | h^- leu1-32, ade6-210, ade4-DS/E |
| FY721          | Ch16 m23:ura4            | h^- leu1-32, ade6-210, ade4-DS/E |
| FY723          | Ch16 m23:ura4            | h^- leu1-32, ade6-210, ade4-DS/E |
| FY725          | Ch16 m23:ura4            | h^- leu1-32, ade6-210, ade4-DS/E |
| FY727          | Ch16 m23:ura4            | h^- leu1-32, ade6-210, ade4-DS/E |

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Table 2. (Continued)

| Strain | Position and ura4 gene | Genotype |
|--------|------------------------|----------|
| FY729  | Ch16 m23::ura4         | h<sup>a</sup> leu1-32, ade6-210, ura4-DS/E (Ch16 ade6-216) clr4-S5 |
| FY731  | Ch16 m23::ura4         | h<sup>a</sup> leu1-32, ade6-210, ura4-DS/E (Ch16 ade6-216) rik1-304 |
| FY733  | Ch16 m23::ura4         | h<sup>a</sup> leu1-32, ade6-210, ura4-DS/E (Ch16 ade6-216) swi6-115 |
| FY1036 | Ch16 m23::ura4         | h<sup>a</sup> leu1-32, ade6-210, ura4-DS/E (Ch16 his1-102) swi6<sup>A</sup>:his1<sup>+</sup> |
| FY757  | h<sup>a</sup> ad6-704, (CM3112 sup-3) |
| FY759  | h<sup>a</sup> ad6-704, (CM3112 sup-3) |
| FY853  | h<sup>a</sup> ad6-704, (CM3112 sup-3) clr1-5 |
| FY787  | h<sup>a</sup> ad6-704, (CM3112 sup-3) clr2-E22 |
| FY790  | h<sup>a</sup> ad6-704, (CM3112 sup-3) clrl-E36 |
| FY856  | h<sup>a</sup> ad6-704, (CM3112 sup-3) clr4-S5 |
| FY859  | h<sup>a</sup> ad6-704, (CM3112 sup-3) rik1-304 |
| FY862  | h<sup>a</sup> ad6-704, (CM3112 sup-3) swi6-115 |
| FY1038 | h<sup>a</sup> ad6-704, (CM3112 sup-3) his1-102 swi6<sup>A</sup>:his1<sup>+</sup> |

(h<sup>a</sup>) Mating type is ambiguous because the silent mating-type loci are deregulated in this background.

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