RESEARCH COMMUNICATION

Early-replicating heterochromatin

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Euchromatin, which has an open structure and is frequently transcribed, tends to replicate in early S phase. Heterochromatin, which is more condensed and rarely transcribed, usually replicates in late S phase. Here, we report significant deviation from this correlation in the fission yeast, *Schizosaccharomyces pombe*. We found that heterochromatic centromeres and silent mating-type cassettes replicate in early S phase. Only heterochromatic telomeres replicate in late S phase. Research in other laboratories has shown that occasionally other organisms also replicate some of their heterochromatin in early S phase. Thus, late replication is not an obligatory feature of heterochromatin.

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"Heterochromatin" was originally defined as chromatin that remains condensed during interphase, whereas "euchromatin" decondenses during interphase (Heitz 1928, 1929). Later, the discovery that genes from euchromatin can become epigenetically inactivated if they are translocated into heterochromatin (for review, see Grewal and Elgin 2002) provided an additional, functional definition for heterochromatin. Recent studies have begun to provide a molecular definition as well (for review, see Grewal and Elgin 2002). Many forms of heterochromatin are characterized by hypermethylation of Lys 9 on histone H3 (H3-K9). In these cases, a protein with a chromodomain and a chromo-shadow domain (similar to *Drosophila* Hp1 and fission yeast Swi6) binds to methylated H3-K9 with its chromodomain, and then recruits additional proteins to the heterochromatic region with its chromo-shadow domain.

Until now, replication in late S phase has been considered to be another distinguishing feature of heterochromatin. The paradigm of late heterochromatin replication was first articulated by Lima-de-Faria and Jaworska (1968) on the basis of studies in a wide range of eukaryotic organisms. With minor exceptions, this paradigm has withstood the test of time (for review, see Gilbert 2002). Now, however, we report significant deviation from this paradigm in fission yeast.

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Results and Discussion

Previous results: telomeres

Chromatin near fission yeast telomeres is heterochromatic, because bringing normally active genes into the proximity of telomeres epigenetically inactivates the genes, and this inactivation is partially dependent on standard heterochromatin proteins including Swi6 (Nimmo et al. 1994; Allshire et al. 1995; Ekwall et al. 1995, 1996). Epigenetic inactivation of euchromatic genes introduced into telomere-proximal positions is called Telomere Position Effect (TPE) and is conserved from budding yeast (Gottschling et al. 1990; Tham and Zakian 2002) to humans (Baur et al. 2001).

Using several independent synchronization procedures, we have demonstrated previously that the terminal *Hin*dIII restriction fragments of fission yeast chromosomes I and II replicate in very late S phase (Kim and Huberman 2001), consistent with the paradigm of late heterochromatin replication. However, we were surprised when the same studies revealed that the outer portions of fission yeast centromeres replicate in early S phase.

Previous results: outer centromeres

Fission yeast centromeres consist of variable numbers of outer repeat (*otr*) sequences arranged, in inverted orientation, around an inner portion consisting of chromosome-specific innermost repeats (*imr*) inverted around a central sequence (*cnt*) that is fully or partially unique for each chromosome (Chikashige et al. 1989; Takahashi et al. 1992; Smith et al. 1995).

The *otr* region consists primarily of *dg* and *dh* repeats. These repeats are heterochromatic, because an indicator gene transplaced into *otr* becomes silenced (Allshire et al. 1995), and *otr* is associated with the heterochromatin protein, Swi6 (Partridge et al. 2000). Our previous results led to the surprising conclusion that the *Hin*dIII fragments from the *dg* repeats (also called K repeats) within the *otr* portions of the centromeres contain active chromosomal replication origins and replicate in very early S phase (Kim and Huberman 2001).

Inner centromeres

In fission yeast, the chromatin within the inner portions of centromeres is heterochromatic in the sense that euchromatic genes introduced into inner centromeres become epigenetically silenced (Allshire et al. 1994, 1995). However, in fission yeast as in all eukaryotic organisms, histone H3 within the inner centromeres is replaced by a histone H3 paralog, frequently called CENP-A (for review, see Smith 2002). Because the N-terminal portion of CENP-A differs substantially from that of histone H3, CENP-A is not subject to K9 methylation, and it does not bind Hp1/Swi6-like proteins. Instead, inner centromeres are associated with the Mis6 protein (Partridge et al. 2000; Takahashi et al. 2000; Kniola et al. 2001).

The surprising early replication of the outer portions of the centromeres raised the following question: Do the inner portions, which have a different heterochromatin

structure, also replicate in early S phase? To answer this question, we used the *cdc10* block and release procedure (Kim and Huberman 2001) to synchronize cells, and we used two-dimensional agarose gel electrophoresis (Brewer and Fangman 1987) to measure the abundance of replication intermediates (RIs). In Figure 1A (bottom panel), the course of S phase is indicated by the position of the flow cytometry peak, which shifts from a DNA content of 1N to 2N primarily between 50 and 90 min after temperature downshift. Examples of complete flow cytometry profiles for this experiment are shown in Figure 3 of Kim and Huberman (2001). We found that RIs (primarily Y arcs) from the *cnt* sequences were most abundant in early S phase—between 50 and 70 min after shift to 25°C. The weaker signals from RIs at earlier and later time points may be due to imperfect synchrony or may be indicative of some heterogeneity of replication times.

We confirmed the early replication of *cnt* sequences by using the independent hydroxyurea (HU) block and release procedure. RIs corresponding to *cnt* sequences accumulate in the presence of HU (Fig. 1B; zero-minute time point). After HU is removed, *cnt* RIs disappear as replication forks run off. This behavior is typical of sequences that replicate in early S phase (Kim and Huberman 2001). Examples of flow cytometry profiles for this experiment are in Figure 6 of Kim and Huberman (2001).

Comparison of the *cdc10* block and release results for *cnt* sequences in Figure 1A with our previously published *cdc10* results for outer centromere (*dg*) sequences (Fig. 4B in Kim and Huberman 2001) suggests that the *dg* sequences replicate in very early S phase, whereas the *cnt* sequences replicate about 10 min later, but still in early S phase. The later replication of the *cnt* sequences is consistent with their having few, if any, active replication origins (note the near absence of bubble arc signals in Fig. 1). Our results suggest that the *cnt* sequences are primarily replicated by replication forks coming from active origins in the *dg* or *dh* sequences, all within early S phase.

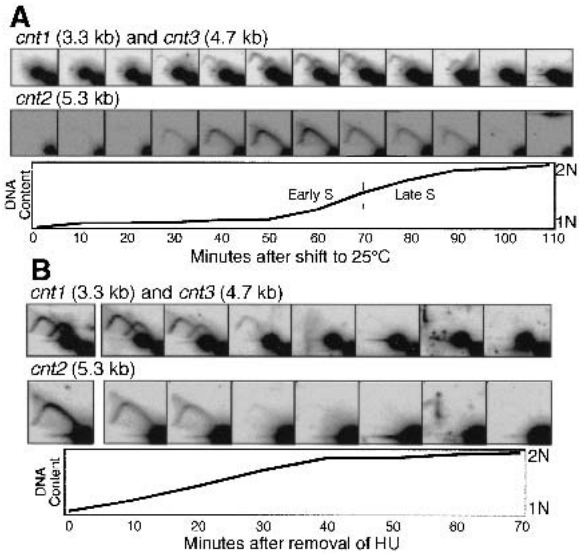

Figure 1. The central portions of the centromeres are replicated in early S phase. (*A*) The *cdc10* temperature block and release procedure (Kim and Huberman 2001) was used to synchronize passage through S phase. This experiment is identical to the one shown in Figure 4 in Kim and Huberman (2001). The Southern membrane used in that experiment was stripped and rehybridized with probes specific for the *cnt* sequences. (*B*) The hydroxyurea (HU) block and release procedure (Kim and Huberman 2001) was used to synchronize passage through S phase. This experiment is identical to the one shown in Figure 7 in Kim and Huberman (2001). The Southern membrane used in that experiment was stripped and rehybridized with probes specific for the *cnt* sequences. Note that the cells in the zero-minute time point had been exposed to HU for 5 h at 25°C.

Silent mating-type cassettes

The remaining well-characterized heterochromatic region in fission yeast is the region containing the silent mating-type cassettes. There are two mating types in fission yeast, plus and minus. Wild-type fission yeast cells have three sets of genes (cassettes) encoding mating-type information, Plus or Minus. The three cassettes—*mat1*, *mat2*, and *mat3*—are located within 30 kb of each other on chromosome II. This wild-type configuration, h^{90}, is diagrammed in Figure 2 (top panel). The region between *mat1* and *mat2* is called the "L region," and that between *mat2* and *mat3* is called the "K region." The genes (Plus or Minus) in *mat1* are expressed (*mat1-P* or *mat1-M*). Those in *mat2* (usually Plus; *mat2-P*) and *mat3* (usually Minus; *mat3-M*) are silenced by heterochromatinization that is partially dependent on Swi6. The silenced state is indicated in the diagram by a gray (rather than black) plus sign or minus sign. The silenced, heterochromatic region is located between two inverted repeats that form boundaries between heterochromatin and euchromatin (IR-L and IR-R; Noma et al. 2001; Singh and Klar 2002; Thon et al. 2002). Halfway between these inverted repeats is a stretch with strong sequence similarity to a portion of the *otr* of *CEN2*. This "*CEN* homology" domain contributes significantly to formation of regional heterochromatin (Hall et al. 2002).

To measure replication timing in the mating-type region, we used fission yeast strains bearing the *cdc10-M17* mutation, which facilitates cell cycle synchronization (Kim and Huberman 2001). These are haploid strains with alterations of their mating-type regions that inhibit mating-type switching. The alterations are called h^{-S} and h^{+N}, and they are diagrammed in Figure 2 (bottom panel). h^{-S} strains arise from *mat1-M* h^{90} cells by recombination between homologous sequences at the borders of *mat2-P* and *mat3-M*, leading to deletion of the K region (Beach and Klar 1984). In h^{-S} strains, the retained silent cassette contains Minus information. In Figure 2 (middle panel, h^{-S}), the silent cassette is designated *mat2:3-M*, to indicate that the sequences to its left are identical to those to the left of *mat2* in h^{90}, and the sequences to its right are identical to those to the right of *mat3* in h^{90}. Similarly, h^{+N} strains arise from *mat1-P* h^{90} (Beach and Klar 1984), but in this case, an extra K region is inserted by recombination into *mat1*. The leftmost cassette (*mat1:2-P*) in the h^{+N} configuration remains transcriptionally active and expresses Plus information. The second cassette (*mat3:1*) is inactive for transcription, but it retains the *cis*-acting sequences necessary for mating-type switching, and it is capable of switching. The switching has no phenotypic consequences, as the second cassette is silenced.

The results of our analyses of mating-type region replication timing with cells synchronized by *cdc10* block and release (Kim and Huberman 2001) are shown in Figure 3, and the locations of the tested fragments are shown as horizontal boxes in Figure 2 (middle and bot-

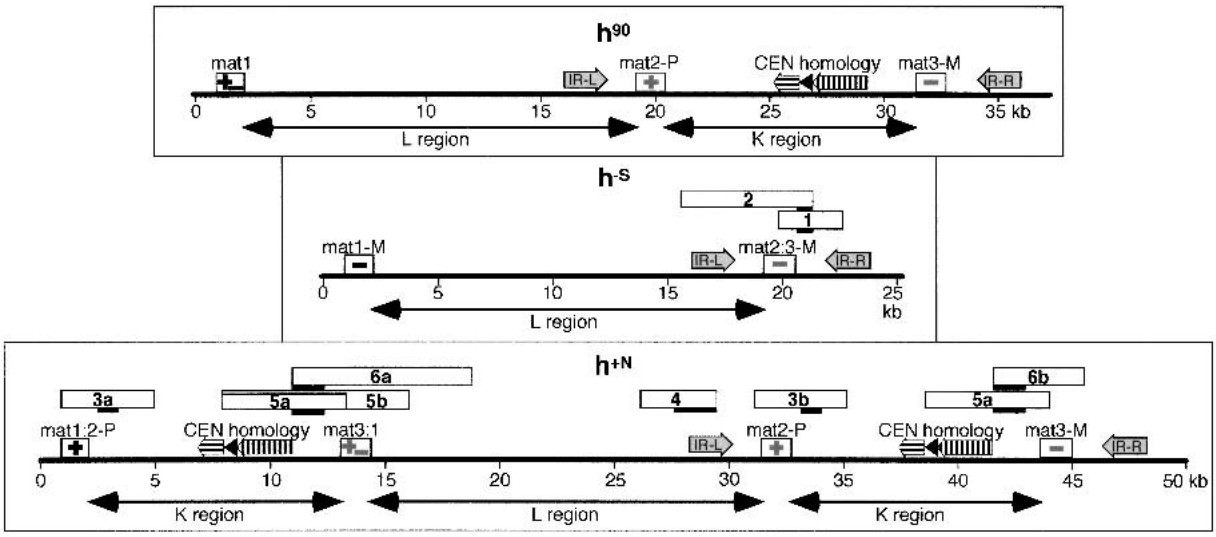

Figure 2. Structures of the major fission yeast mating-type locus configurations. These diagrams are based on nucleotide sequence information (GenBank accession nos. AL035065, AL356712, AL353012, and U57841) and on previous studies describing the derivation of h^{-S} and h^{+N} by recombination from h^{90} (Beach and Klar 1984). All three configurations are shown at the same scale. Restriction fragments studied for replication timing are indicated by horizontal boxes in the upper portions of the diagrams. The position of the hybridization probe used to identify each restriction fragment is shown as a thick black line immediately below the restriction fragment. The horizontally striped arrow in the *CEN* homology region represents *dg* sequences. The vertically striped arrow represents *dh* sequences, and the black arrowhead indicates an *otr* repeat that is frequently located between *dh* and *dg* sequences. See the text for additional description.

tom panels). The probes used to detect the restriction fragments in Southern blotting are indicated by thick black lines immediately below these boxes. It is evident in Figure 3 that all tested fragments replicated in early S phase. The maximum abundance of RIs occurred at 40–60 min after temperature downshift. In contrast, the maximum abundance of RIs from telomeres occurred at 90–100 min after downshift in similar experiments (Kim and Huberman 2001).

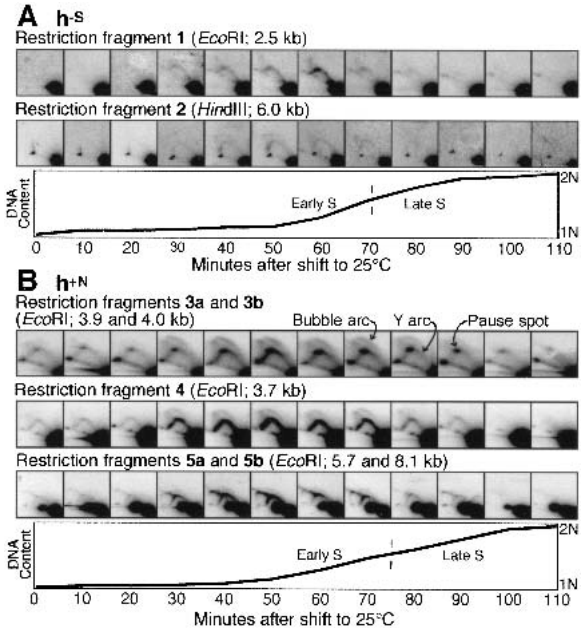

Figure 3. The mating-type region is replicated in early S phase. Locations of the studied restriction fragments are shown in Figure 2. (*A*) The *cdc10* block and release procedure was used to synchronize the h^{-S} strain. This experiment is identical to the one shown in Kim and Huberman (2001). The membrane was stripped and rehybridized with probes for the indicated restriction fragments. (*B*) The same procedure was used to synchronize the h^{+N} strain. The experiment is the same as in Figure 5B in Kim and Huberman (2001). The membrane was stripped and rehybridized with probes for the indicated restriction fragments.

Two features of the results in Figure 3 merit comment. First, the probe used to detect restriction fragment 3b (*Eco*RI, 4.0 kb) also detects restriction fragment 3a (*Eco*RI, 3.9 kb; Figs. 2, bottom panel, 3B, top panel). These two fragments are too close in size to be resolved. Therefore, we cannot determine whether the bubble arc detected by this probe arises from fragment 3a, fragment 3b, or both. It is interesting that this probe also detects a spot near the apex of the Y arc (Fig. 3B, top panel). This spot is probably due to replication fork pausing in one or both of the detected restriction fragments.

Another result that deserves comment is the behavior of the two h^{+N} *Eco*RI fragments (5a and 5b) detected by the probe used in the bottom two-dimensional gel panel of Figure 3B. Generation of fragment 5a requires an *Eco*RI site that is located within Minus—but not within Plus—sequences. It can be calculated from the information in Figure 2 (bottom panel) that the overall signal strength from fragment 5a should be three times greater than from fragment 5b. This ratio is consistent with our observations.

We have used two additional synchronization procedures to confirm the early replication timing of some of the restriction fragments from the mating-type region. Use of HU block and release showed that restriction fragment 2 (Fig. 2) accumulated RIs in the presence of HU, and the abundance of RIs decreased after HU was removed (Fig. 4A). Thus, this restriction fragment is also replicated in early S phase (Kim and Huberman 2001).

We found previously that sufficient synchronization of passage through S phase could be obtained by centrifugal

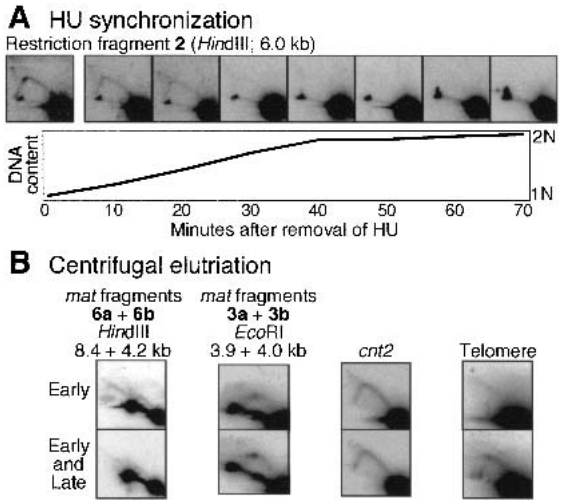

Figure 4. Early replication of the mating-type region. (*A*) The HU synchronization procedure was used with the h^{-S} strain. This experiment is identical to the one shown in Figure 7 in Kim and Huberman (2001). The membrane was stripped and rehybridized with a probe for restriction fragment 2 (Fig. 2). (*B*) The centrifugal elutriation procedure was used with the h^{+N} strain. This experiment is the same as in Figures 1 and 2 in Kim and Huberman (2001). The membrane was stripped and rehybridized with probes for the indicated regions. See the text and Materials and Methods for further details on the probes used.

elutriation to allow distinction between early- and late-replicating restriction fragments (Kim and Huberman 2001). In this procedure, the smallest cells in an unsynchronized population growing in rich medium are separated from the other cells by centrifugation and permitted to continue growing in rich medium. The small cells, which are initially in early G2 phase, continue through G2 and mitosis and then through G1 and S phase of the following cell cycle. Although the quality of the S-phase synchrony is not as good as with the other procedures described here, and only low numbers of cells are obtained, this procedure has the advantage that no cell cycle blocks are required. Consequently, this procedure should be free of potential artifacts that might be produced by temporarily blocking the cell cycle.

For the experiment in Figure 4B, two samples were collected from the synchronized culture for analysis of RIs. One sample, called "Early" in Figure 4B, contained mostly cells in early S phase with very few cells in late S phase. The other sample, called "Early and Late," contained cells in both early and late S phase (Kim and Huberman 2001). The proportions of cells in early and late S phase in these two samples can be estimated from the quantitative fluorescence microscopy data in Figure 1 of Kim and Huberman (2001). The RIs detected by the 6a+6b and 3a+3b probes appear stronger in the "Early" sample, but the spot detected by the 3a+3b probe that is probably due to replication fork pausing appears stronger in the "Early and Late" sample, consistent with the relative apparent strengths of the Y arc and probable pause spot at different time points in the top panel of Figure 3B. Thus, both of the mating-type probes used in this experiment detect restriction fragments that are replicated predominantly in early S phase. For comparison, Figure 4B also shows results obtained with a probe specific for *cnt2* (roughly equal signal strengths in the "Early" and "Early and Late" samples, consistent with *cnt2* replication in mid-early S phase) and with a telomere probe (much stronger Y arc signals in the "Early and Late" sample, consistent with late telomere replication).

Occasional early heterochromatin replication in other organisms

Heterochromatin sometimes replicates early in other organisms, but examples in other organisms have been sufficiently rare that they have not called into question the paradigm of late heterochromatin replication. These examples include (1) early initiation of replication within bovine satellite I, which comprises 5% of bovine genomic DNA and is located in the centromeric heterochromatin of most chromosomes (Matsumoto and Gerbi 1982); (2) early initiation of replication in the pericentric heterochromatin and centromeres of mouse chromosomes (Holló et al. 1996); (3) early replication of some human telomeres (Wright et al. 1999), despite the fact that human telomeres exhibit TPE (Baur et al. 2001); (4) occasional early replication of autosomal genes inactivated by spreading of heterochromatin from a translocated human X chromosome (Sharp et al. 2001); (5) early replication of inner centromeres in *Drosophila* (Ahmad and Henikoff 2001, 2002; Sullivan and Karpen 2001); and (6) early replication of *Drosophila* β-heterochromatin (the form of heterochromatin containing occasional expressed genes and located immediately distal to pericentric α-heterochromatin; Schübeler et al. 2002).

No essential connection between heterochromatin and late replication

These examples from other organisms suggest that formation and maintenance of heterochromatin do not necessarily require late replication, but the surprising early replication of *most* heterochromatin in fission yeast forces us to recognize that the current paradigm of late heterochromatin replication requires revision. We do not make this statement lightly, because the association between heterochromatin and late replication is a strong one in most eukaryotic organisms. Yet the early-replicating heterochromatin in fission yeast appears to resemble heterochromatin in other organisms in all essential respects except replication timing. The best-studied fission yeast heterochromatin (in the outer centromeres and in the mating-type region) is physically associated with the Hp1-like Swi6 protein and with hypoacetylated histones. Its histone H3 is undermethylated on Lys 4 and overmethylated on K9. Normally active genes introduced into this heterochromatin become epigenetically inactivated (for review, see Grewal and Elgin 2002). The RNA interference machinery (RNAi) contributes to establishment and (in the case of centromeres) maintenance of this heterochromatin (Hall et al. 2002; Volpe et al. 2002). During interphase, the centromeres are condensed and associated with the spindle pole body at the nuclear periphery (Funabiki et al. 1993; Ekwall et al. 1995; Kniola et al. 2001). The mating-type region is also condensed and frequently associated with centromeres and/or telomeres at the nuclear periphery (Ekwall et al. 1995). Nevertheless, despite possessing all of these properties of classic heterochromatin, the fission yeast centromeres and mating-type region replicate in very early S phase. Thus, if there is a link between late-replication

timing and heterochromatin, that link must involve a nonessential feature that is commonly, but not universally, associated with heterochromatin.

In many cell types, heterochromatin is located predominantly at the nuclear periphery, where it replicates in late S phase. For these reasons, the hypothesis that late replication is determined by peripheral nuclear positioning is currently popular (for review, see Gilbert 2002). As indicated above, however, peripheral location is not a guarantee of late replication timing in fission yeast. It is particularly interesting that in some fission yeast cells early-replicating mating-type regions appear to colocalize with late-replicating telomeres in regions that contain abundant Swi6 protein (Ekwall et al. 1995). This observation argues that *cis*-acting sequences (which may be involved in specifying type of heterochromatin) are likely to be more important than nuclear position as primary determinants of replication timing in fission yeast. Results from other laboratories suggest that the same may be true in budding yeast (Zappulla et al. 2002) and in mammals (Simon et al. 2001).

Materials and methods

Strains

Easily synchronized *cdc10-M17 ade6-704 leu1-32 ura4-D18* strains [kindly sent to us by Tony Carr (Sussex University, Falmer, Brighton, UK)] were used for all experiments. The mating-type configuration was h^{-S} unless indicated otherwise in the text and figure legends.

Cell cycle synchronization and flow cytometry

Synchronization by centrifugal elutriation, *cdc10* block and release, and HU block and release was carried out as described previously (Kim and Huberman 2001). Evaluations of cell cycle position by flow cytometry or by quantitative fluorescence microscopy were as described (Kim and Huberman 2001).

Two-dimensional agarose gel analyses

Our procedures for purification of DNA from synchronized cell populations and for detection of RIs by two-dimensional gel electrophoresis have been described previously (Kim and Huberman 2001).

Hybridization probes

To detect telomeres, we used a previously described (Nimmo et al. 1994; Kim and Huberman 2001) 750-bp TAS probe. *Cnt1* and *cnt3* were detected using a 3.4-kb *Eco*RI–*Cla*I fragment from *cnt1*, whereas *cnt2* was detected using a 2.15-kb *Eco*RI–*Pvu*II fragment. The *cnt* probes were kindly sent to us by Mary Baum (University of California at Santa Barbara) and Louise Clarke (University of California at Santa Barbara). Probes for the mating-type region were obtained by appropriate restriction digestion of plasmids kindly sent to us by Amar Klar (Frederick Cancer Research and Development Center, Frederick, MD). In h^{-S} cells, the 2.5-kb *Eco*RI and 6.0-kb *Hin*dIII fragments were detected by a 700-bp *Eco*RV–*Hin*dIII probe. In h^{+N} cells, the 3.9- and 4.0-kb *Eco*RI fragments were detected by an 890-bp *Xba*I–*Hin*dIII probe. The 3.7-kb *Eco*RI fragment was detected by a 2.1-kb *Hin*dIII–*Eco*RI probe. The 5.7- and 8.1-kb *Eco*RI fragments, and the 4.2- and 8.4-kb *Hin*dIII fragments were detected with a 1.4-kb *Hin*dIII–*Nsi*I probe.

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