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Telomere-associated chromosome breakage in fission yeast results in variegated expression of adjacent genes

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The sequence requirements for *in vivo* telomere function in the fission yeast, *Schizosaccharomyces pombe*, have been investigated. A 258 bp tract of previously characterized cloned fission yeast terminal repeats adjacent to 800 bp of telomere-associated sequences is sufficient to seed new telomeres onto linearized *ars*-containing plasmids when introduced into cells. The resulting transformants contain unrearranged, acentric, linear episomes. Cloned telomeres, with and without telomere-associated sequences adjacent to the 258 bp terminal repeats, were utilized to introduce chromosome breaks at specific sites in a non-essential minichromosome. Truncated minichromosome derivatives were recovered containing the *ura4* or *ade6* gene adjacent to a newly formed telomere. These telomeres exert reversible position effects on the expression of the adjacent *ura4* or *ade6* genes.

Key words: minichromosome/position effect/*Schizosaccharomyces pombe*/silencing/telomeres

Introduction

The natural ends of linear chromosomes are stabilized by a structure termed the telomere. The telomere has at least two functions: first it enables the end of a chromosome to be replicated completely in each cell cycle, and secondly, the telomere forms a protective cap at a chromosome end so that, unlike a broken end, it is unreactive to degradative processes or in fusions with other free ends. Telomeres of many organisms are composed of short tracts of repetitive sequences. In general these repeats have one strand which is rich in G residues running 5′−3′ towards the end of the chromosome [for reviews see Zakian (1989) and Blackburn (1991)].

In *Saccharomyces cerevisiae* the addition of the telomeric repeat TG_{1-3} to the ends of a linearized plasmid containing an autonomous replication sequence (*ars*) enables it to replicate as a linear episome when reintroduced into cells (Szostak, 1982; Szostak and Blackburn, 1982; Shampay *et al.*, 1984). These terminal repeats seed the formation of new telomeres. Artificial chromosomes can therefore be constructed containing the three essential components, a replication origin, a centromere and telomeres (Murray and Szostak, 1983). This led to the development of the YAC (yeast artificial chromosome) system for cloning large fragments of DNA from heterologous organisms (Burke *et al.*, 1987).

When combined with targeted homologous recombination, tracts of the terminal repeat TG_{1-3} can also be used to break endogenous chromosomes in *S.cerevisiae* (Vollrath *et al.*, 1988). Telomere-induced breakage therefore allows highly accurate manipulation of *S.cerevisiae* chromosomes. This has aided chromosome mapping and allowed novel chromosomes to be constructed which have contributed to the understanding of chromosome structure and function (Hegemann *et al.*, 1988).

The fission yeast, *Schizosaccharomyces pombe*, is a convenient genetically tractable organism in which to study chromosome biology. *S.pombe* telomeres were originally cloned by transformation of *S.pombe* with a linear plasmid terminating with *Tetrahymena* telomeres, $(TTGGGG)_n$, and retrieval of end clones from plasmids healed by the addition of fission yeast telomeres (Sugawara and Szostak, 1986; Sugawara, 1989). The *S.pombe* terminal repeat arrays are ~300 bp in length with the consensus sequence $TTAC_{0-1}A_{0-1}C_{0-1}G_{1-8}$ running towards the end of the chromosome. A similar 300 bp array of repeats was found at the ends of a rescued minichromosome generated by γ-irradiation, suggesting that these sequences were added *de novo* to the broken ends (Matsumoto *et al.*, 1987). Of the six telomeres in fission yeast four share similar sequences, adjacent to the terminal repeats, which extend for at least 19 kb from the ends. The terminal 2.75 kb of one of these telomere clones has been sequenced and terminates with several copies of repeats matching the consensus sequence (Sugawara, 1989). At the remaining two telomeres on chromosome 3 the terminal repeats directly abut ribosomal DNA (Sugawara, 1989; Hoheisel *et al.*, 1993; Mizukami *et al.*, 1993).

Formation of a new telomere next to genes in *S.cerevisiae* can result in reversible repression so that, in a genetically identical population, only a proportion of cells express the gene (Gottschling *et al.*, 1990). This phenomenon is similar to that of position effect variegation (PEV) which was first observed in *Drosophila* (Muller, 1930). The classical example of PEV in *Drosophila* involves the *white* gene which, when placed adjacent to pericentric heterochromatin by a chromosomal inversion, results in variegated eye colour. Variegation is due to mosaic expression of the *white* gene in the facet cells of the eye so that only some cells express the gene (Spofford, 1967). These opposite states of expression are apparently stable for several generations indicating that the gene must be fixed in the 'on' or 'off' states in different cells (Muller, 1941; Tartoff and Bremner, 1990). Variegation is thought to be due to spreading of the adjacent transcriptionally inert heterochromatin into nearby genes, resulting in their inactivation (Demerec, 1940; Hartmann-Goldstein, 1967).

The extent of heterochromatinization varies from cell to cell and therefore the gene is not inactivated in all cells. In the case of a variegating *white* gene such mosaic expression leads to a mottled eye colour.

Recently, Allshire *et al.* (1994) described a phenomenon resembling PEV at *S.pombe* centromeres when either the *ura4* or the *ade6* gene was placed within the central domain of the three centromeres. Here small acentric vectors containing an *ars*, selectable markers and two tracts of *S.pombe* telomere repeats adjacent to telomere associated sequences (TAS) have been constructed and are shown to be maintained as linear episomes under selection in *S.pombe*. DNA constructs with a single tract of telomere repeat adjacent to a selectable gene were used to make targeted breaks in a minichromosome. The resulting strains contain altered minichromosomes which have been reduced to predicted sizes and have acquired new terminally located genes encoding selectable markers. Further analyses demonstrated that the two telomere-adjacent genes are reversibly position-affected and that the strength of the position effect is dependent on the distance from the telomere and the incubation temperature.

Results

Linear episomes in S.pombe

To test directly if cloned *S.pombe* telomeres could seed new telomeres when reintroduced at the ends of a linear plasmid into *S.pombe*, a series of vectors was constructed. Firstly, two copies of a telomere clone containing 258 bp of the terminal repeat repeats and 800 bp of TAS (see Materials and methods) were cloned in a head-to-head arrangement around the *Escherichia coli* kanamycin gene. Selection for resistance to kanamycin prevented deletion and rearrangement of the telomere repeats in *E.coli*. This *S.pombe* 'long' telomere cassette was incorporated into plasmid vectors which, when cleaved with *Sac*I to generate linear molecules and remove the kanamycin gene, resulted in pEN51, containing *S.pombe ars1* and the *ura4* gene, and pEN53 with the *S.pombe ars1* and the *S.cerevisiae LEU2* gene, which complements mutations in the *S.pombe leu1* gene (Figure 1).

The *S.pombe* strain, SP813, was transformed with linearized and circular forms of pEN51 and pEN53. The plasmids were linearized either by cutting with *Sac*I, which releases a molecule with cloned telomere repeats at each end with the G-rich strand running 5′−3′ towards the end or by cutting with *Not*I which cuts the plasmids once but leaves the telomere repeats internally located. Transformants were selected by growth on plates lacking either uracil (pEN51) or leucine (pEN53). The frequency of recovery of transformants from the *Sac*I-digested linear plasmids with liberated telomeres was comparable with

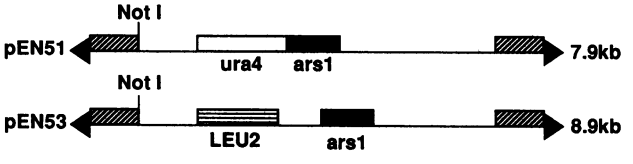

Fig. 1. Schematic representation of linear plasmids. Linear plasmids terminated with *S.pombe* telomere repeats. Digestion of plasmids pEN51 and pEN53 with *Sac*I releases the kanamycin gene (not shown) and results in a linear molecule carrying the *ura4* gene and *ars1* (pEN51) or the *S.cerevisiae LEU2* gene and *ars1* (pEN53). These linear molecules terminate with long telomeres consisting of the telomere-associated sequences (TAS, crosshatched) and the telomere repeats (filled arrowhead). Cleavage of the circular plasmids at the unique *Not*I sites results in linear molecules with the telomere sequences internal.

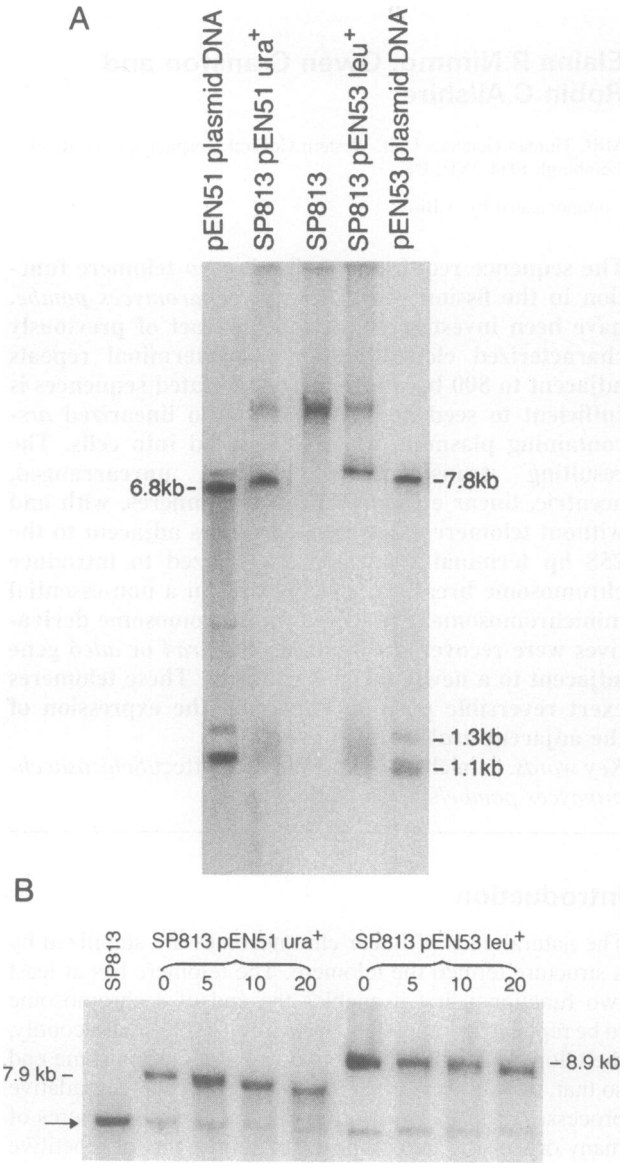

Fig. 2. The plasmids pEN51 and pEN53 replicate as linear molecules in *S.pombe*. (**A**) SP813 was transformed with linearized plasmid vectors, DNA of the transformants prepared and analysed by digestion with *Not*I. pEN51 plasmid DNA was digested with *Sac*I and *Not*I to give bands of 6.8, 1.3 (the kanamycin resistance gene) and 1.1 kb and pEN53 plasmid DNA digested with *Sac*I and *Not*I to give bands of 7.8, 1.3 (the kanamycin resistance gene) and 1.1 kb. Digested DNA was transferred to Genescreen membrane and probed with ^{32}P-labelled plasmid containing telomere repeats. Partial digestion bands can be seen in some lanes. (**B**) DNA from SP813 transformed with *Sac*I-linearized pEN51 and pEN53 was digested with Bal31 for 0, 5, 10 and 20 min and the reaction stopped with EGTA. After ethanol precipitation the DNA was digested with *Bgl*II (which does not cut either plasmid), subjected to gel electrophoresis, transferred to Genescreen membrane and probed with ^{32}P-labelled plasmid containing *ars1*. The arrow indicates the band detected by the *ars1* sequence and this serves as a control for the specificity of Bal31 digestion.

that of circular plasmids, whereas few transformants were obtained with the *Not*I-linearized plasmids. DNA from 15 independent transformants containing the *Sac*I-linearized vectors was analysed. All were found to have the hybridization pattern predicted for unrearranged linear episomes (examples of these are shown in Figure 2A). To confirm that these vectors do replicate as linear episomes in *S.pombe*, DNA from a transformant of each type was treated with the exonuclease Bal31 and then digested with the restriction enzyme *Bgl*II which does not cut in the plasmids. The Bal31 exonuclease attacks free ends so that a small linear molecule will be degraded with time and the size of that molecule will be seen to decrease. Figure 2B shows that the plasmids in these cells are sensitive to digestion with Bal31 and therefore must be linear in structure. The fact that few transformants were recovered with *Not*I-digested vector also indicates that telomeres are required to stabilize linear episomes when transformed into cells.

Chromosome breakage and introduction of a new telomere

The Ch16 minichromosome is a 530 kb radiation-induced derivative of chromosome 3 in which the ends have been healed by the addition of a 300 bp tract of telomeric repeats (Matsumoto *et al.*, 1987). There are no TAS elements associated with these new telomeres; however, this minichromosome is mitotically stable with a loss rate of 1×10^{-3}/cell division (Niwa *et al.*, 1986; Matsumoto *et al.*, 1987). The Ch16-23R minichromosome (Figure 3A), carries the *S.cerevisiae LEU2* gene and plasmid sequences integrated via the sequence m23 into the left arm ~50 kb from the telomere (Niwa *et al.*, 1989). The m23 sequence was duplicated in the process so that it flanks the *LEU2* gene as a direct repeat. The *ade6*-216 allele of the *ade6* gene is carried on the right arm of Ch16-23R. Further breakage or loss of Ch16-23R is not lethal to the cell, and can be followed by loss of the *LEU2* gene. In addition, loss of Ch16-23R, and therefore *ade6*-216, can be monitored by a change in colony colour from white (ade^+) to red (ade^-) on plates containing limiting adenine.

Since, as shown above, *S.pombe* telomere repeats can provide telomere function when reintroduced into fission yeast, we next tested if, as in *S.cerevisiae*, homologous recombination adjacent to arrays of these cloned telomere sequences could be utilized to rescue broken versions of the Ch16-23R minichromosome. Vectors were constructed containing the m23 sequence adjacent to the *ura4* gene which was cloned next to a potential telomere. A series of vectors were constructed with the *ura4* gene in both orientations and with either a 'short' 258 bp telomere repeat array or a 'long' TAS-containing telomere (pEN72, pEN73, pEN76 and pEN77). In each case the entire unit could be released upon digestion with *Sac*I and *Cla*I (Figure 3B).

The purified *Sac*I−*Cla*I fragments from pEN72, 73, 76 and 77 were transformed into the strain FY538 which contains Ch16-23R, and transformants were selected on plates lacking uracil. A homologous recombination event occurring at one of the copies of m23 on CH16-23R should result in the breakage of the minichromosome ~50 kb internal to the existing left arm telomere, insertion of *ura4* gene and the formation of a new telomere. The reciprocal recombination event should not be rescued since it has no telomere at one end. Breakage occurred at equal frequencies in both copies of m23 (data not shown). Transformants were isolated where the putative integration event had occurred at the proximal copy of m23 by screening for colonies which had lost the ability to grow in the absence of leucine and hence lost the *LEU2* gene. In addition, as the strain FY538 requires the minichromosome-borne *ade6*-216 allele for growth in the absence of adenine, only white (ade^+) colonies which had lost the *LEU2* gene were chosen as potential breakage candidates in order to maintain selection for the presence of the minichromosome. Several independent ade^+ ura^+ leu^- transformants obtained with the pEN72, 73, 76 and 77 constructs were analysed by pulsed field gel analyses of high molecular weight DNA (Figure 4, left panel). As predicted in all transformants the minichromosome was found to be ~50 kb smaller than the starting molecule. Further analyses demonstrated that in each case the altered minichromosome had gained the *ura4* gene and lost the *LEU2* gene (Figure 4, middle and right panels). Digestion of DNA from these transformants with Bal31 exonuclease also confirmed that in each case the *ura4* gene was Bal31-sensitive and was therefore adjacent to a telomere (Figure 5). A non-functional allele of the *ura4* gene (*ura4*-DS/E) located at an internal site on chromosome 3 was not degraded by Bal31. These results clearly demonstrate

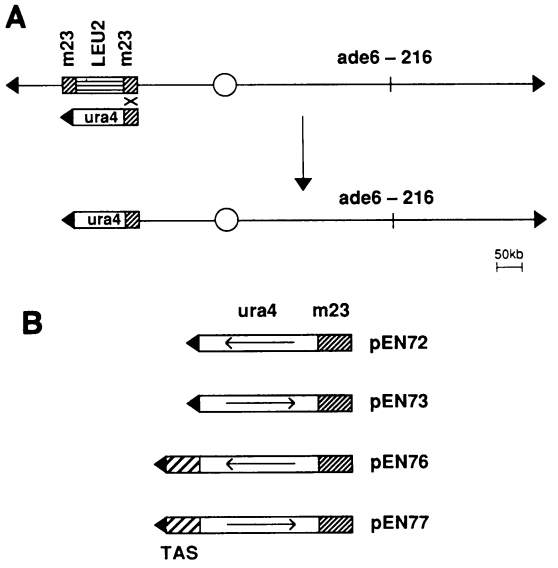

Fig. 3. Insertion of the *ura4* gene next to telomeres by breakage of the Ch16-23R minichromosome. (**A**) Strain FY538 carries the Ch16-23R minichromosome (Niwa *et al.*, 1989). The *LEU2* gene is carried on Ch16-23R at m23 as shown. Ch16-23R can be maintained by selecting for leu^+ or ade^+ cells; *ade6*-216 intragenically complements the endogenous *ade6*-210 mutation on chromosome 3. Linear fragments containing telomere sequences, the *ura4* gene and m23 sequences were transformed into FY538 containing Ch16-23R and cells were selected for their ability to grow on Ura^- plates and screened for the loss of the *LEU2* gene. Homologous recombination between m23 on linear fragments and m23 on Ch16-23R will result in breakage of Ch16-23R at m23. If breakage occurs at the most proximal copy of m23 then the *LEU2* gene will be lost and cells will be unable to grow in the absence of leucine. (**B**) *Cla*I/*Sac*I digests of plasmids pEN72, pEN73, pEN76 and pEN77 release linear vectors containing an m23 sequence, the *ura4* gene and 'short' telomeres (pEN72 and 73) or 'long' TAS-containing telomeres (pEN76 and 77). The orientation of transcription of the *ura4* gene is indicated by the arrows.

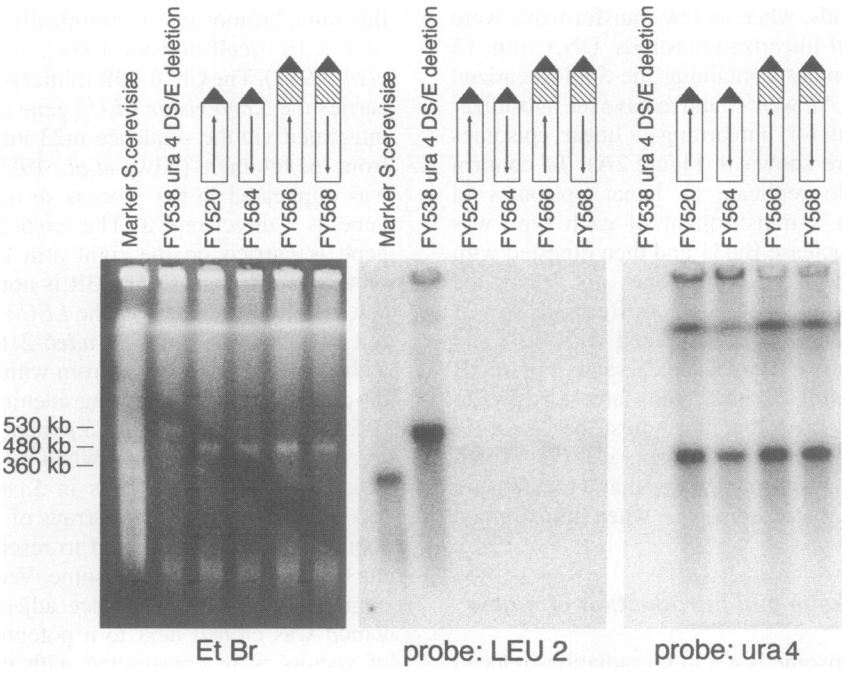

Fig. 4. Pulsed field gel analyses of transformants with Ch16-23R broken at m23. Ethidium bromide stained pulsed field gel of high molecular weight DNA from FY538 containing Ch16-23R before breakage and from ura$^+$/leu$^-$ transformants after breakage with pEN72 to give FY520, pEN73 to give FY564, pEN76 to give FY566 and pEN77 to give FY568. *S.cerevisiae* chromosomes are markers (left panel). After transfer to Genescreen the filter was hybridized with ^{32}P-labelled *ura4* DNA (right panel), stripped and rehybridized with ^{32}P-labelled *LEU2* DNA (middle panel). Hybridization of the *ura4* probe to the *ura4*-DS/E minigene on chromosome 3 can be seen at the limiting mobility. Orientation of the *ura4* gene and type of telomere are as indicated above each track.

that chromosome fragmentation events can be targeted to specific regions of the fission yeast genome and that new telomeres can be seeded at that site, thereby rescuing truncated versions of an endogenous chromosome.

The ura4 gene is subject to reversible position effect at telomeric locations

It has previously been shown in *S.cerevisiae* that genes placed adjacent to telomeres are subjected to reversible repression (Gottschling *et al.*, 1990). In *S.pombe* the *ura4* gene provides a convenient selectable marker to test if a proportion of cells in a population are repressed for expression. Cells expressing the *ura4* gene grow on plates lacking uracil but not in the presence of 5-fluoro-orotic acid (FOA), while cells with the *ura4* gene deleted grow on plates containing FOA but not on those lacking uracil. Four strains (FY520, 564, 566 and 568) carrying one version of each broken minichromosome generated above were tested for their ability to grow both on Ura$^-$ plates and on plates containing FOA. In order to quantify their relative growth, cultures pregrown in non-selective (NS) liquid medium were plated onto NS, Ura$^-$ and FOA plates. After growth for 3–4 days at 32°C the fraction of cells capable of forming colonies in the absence of uracil or presence of FOA was determined (Table I). In addition serial dilutions were performed and aliquots of cells were spotted onto plates of each type (Figure 6).

Strains FY520 and 564 bearing minichromosomes where the break was mediated by pEN72 and pEN73 (short telomeres) grew poorly in the absence of uracil, producing mainly small colonies with a few large colonies visible. In contrast on FOA plates these cells formed colonies which were uniform in size. Strains FY566 and

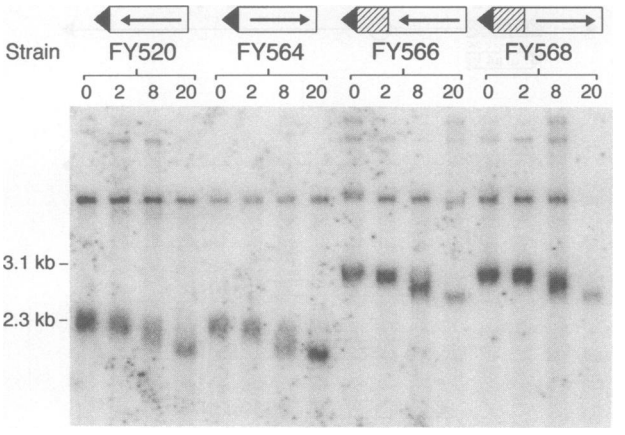

Fig. 5. Breakage at m23 renders the *ura4* gene sensitive to Bal31 digestion. DNA from strains containing a broken minichromosome was digested with Bal31 for 0, 2, 8 or 20 min and the reaction stopped with EGTA. The DNA was digested with *Bam*HI, subjected to gel electrophoresis, transferred to Genescreen membrane and probed with ^{32}P-labelled *ura4*-DS/E. The band across the top is the *ura4*-DS/E allele at the *ura4* locus. FY520 carries Ch16-23R broken with pEN72, FY564 carries Ch16-23R broken with pEN73, FY566 carries Ch16-23R broken with pEN76 and FY568 carries Ch16-23R broken with pEN77. The orientation of the *ura4* gene and type of telomere are as indicated above the tracks.

568, containing minichromosomes broken with pEN76 and pEN77 (long TAS telomeres), could grow relatively well on Ura$^-$ plates. However, fewer colonies developed on FOA plates than in those strains with only a short telomere adjacent to the *ura4* gene. A control strain (FY521), where the *ura4* gene had been inserted at m23

but retained the original telomeres 50 kb from m23, grew in the same manner as a completely wild-type strain on plates lacking uracil but could not grow in the presence of FOA. Colonies from strains bearing the broken minichromosomes which grew on FOA plates were able to grow when replica plated to Ura$^-$ plates, indicating that the *ura4* gene was reversibly position-affected. Those strains which have the additional TAS element located between the *ura4* gene and the terminal repeat are apparently less severely position-affected since most cells can grow well on Ura$^-$ plates but form very few colonies on FOA plates. This decreased position effect correlates with increased distance of the *ura4* gene from the telomere; however, it is possible that the TAS element exerts a specific effect such as acting as a barrier to telomere position effects (see Discussion).

Table I. Effect of chromosome location on resistance to FOA at 32°C

Strain	Location of *ura4*	% FOA^R colonies	% ura$^+$ colonies
FY521[a]	Ch16m23::*ura4*	0	100
FY521	Ch16m23::*ura4*	0.5	95
FY538	*ura4*-DS/E	100	0.0
FY520	Ch16m23::*ura4*-Tel (pEN72)	98	(90)[b]
FY564	Ch16m23::*ura4*-Tel (pEN73)	97	(84)[b]
FY566	Ch16m23::*ura4*-TAS-Tel (pEN76)	10	83
FY568	Ch16m23::*ura4*-TAS-Tel (pEN77)	4	90

Cells of each strain were grown to 1.5–2 × 10^7 cells/ml in NS medium at 32°C, plated on NS, Ura$^-$ or FOA plates and incubated at 32°C for 3 days. Numbers result from at least three different cultures of each strain.

[a]When FY521 was grown in medium lacking adenine in order to select for retention of the minichromosome no growth was detected on plates containing FOA. Without this selection a low frequency of cells grew on FOA plates from FY521 due to loss of the minichromosome.

[b]The cells on these plates formed very small colonies compared with controls.

The incubation temperature was found to affect the ability of all strains with *ura4* adjacent to a telomere to grow under selective conditions (Figure 6). This effect was particularly clear on FOA plates, where fewer colonies were formed by the strains with the long telomeres (FY566 and FY568) at 35°C than at 25°C. This is consistent with decreased expression of the *ura4* gene at lower temperatures. Strains with a more strongly position-affected *ura4* gene (FY520 and 564) showed more subtle differences in their response to temperature on Ura$^-$ and FOA plates. The *m23::ura4* integrant control grew equivalently at all temperatures on Ura$^-$ plates and likewise the strain bearing a deletion in the endogenous *ura4* gene grew equivalently at all temperatures on FOA plates. Similar effects of temperature on position-affected genes have been described in *Drosophila* (Spofford, 1967).

To compare further the relative severity of telomere-mediated position effects in the four strains, the amount of mRNA produced from each telomere-adjacent *ura4* gene was quantified and compared with that in control strains (Figure 7). A convenient internal control in all

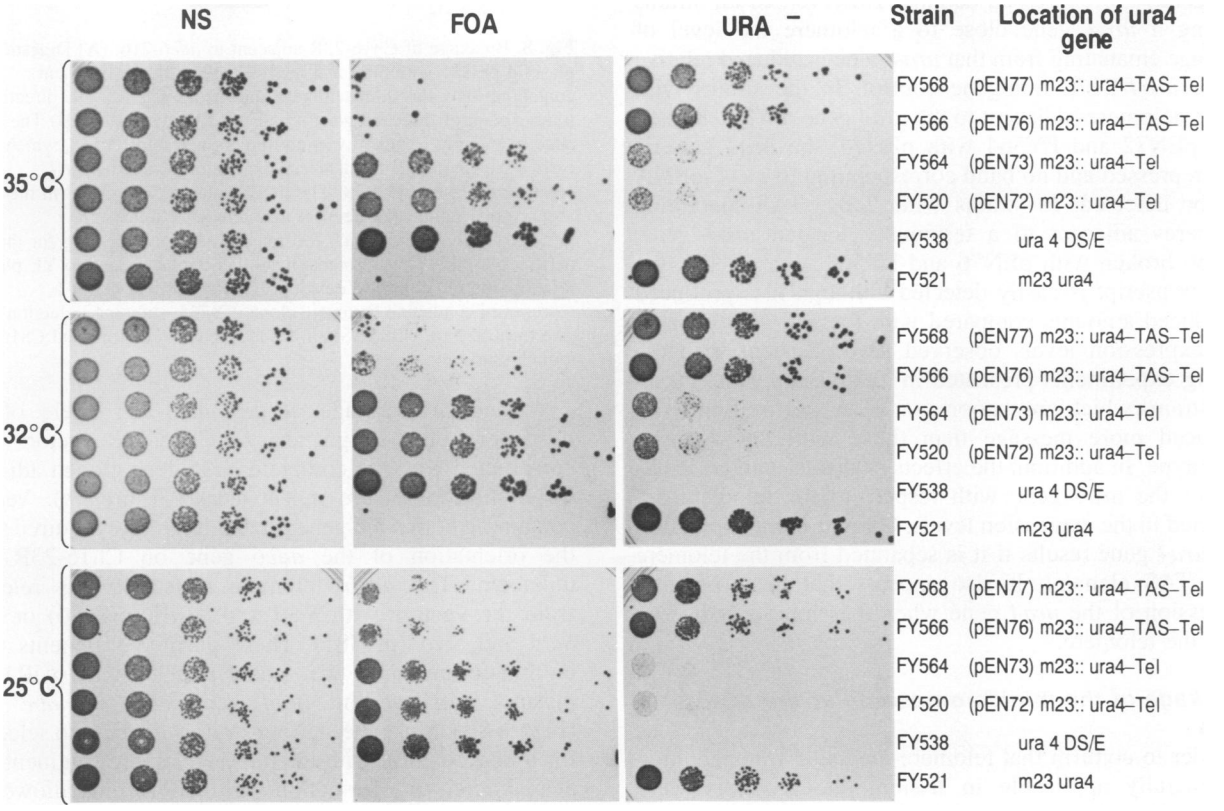

Fig. 6. Effect of temperature on FOA resistance and uracil prototrophy in strains with *ura4*$^+$ located adjacent to telomeres. Strains with the *ura4* gene adjacent to telomere sequences were grown overnight in non-selective medium at 32°C. Serial 1:5 dilutions were made, colonies plated onto selective and non-selective plates and grown at 25°C, 32°C or 35°C. FY538 has only the *ura4*-DS/E allele and provides the ura$^-$ control while FY521 has *ura4* integrated on CH16-23R at the m23 locus 50 kb from the telomere and provides the ura$^+$ control.

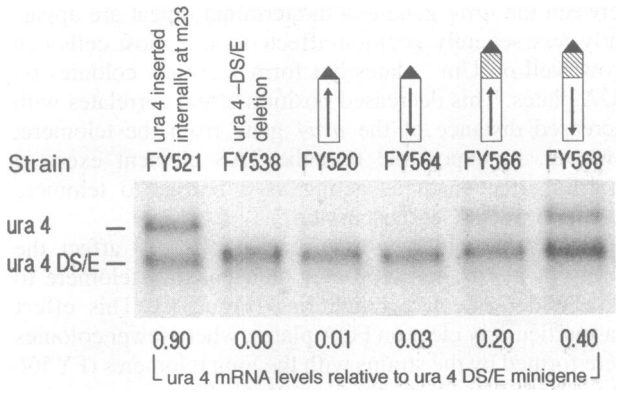

Fig. 7. Expression of *ura4* is repressed adjacent to telomeres. Northern blot of strains with the *ura4* gene adjacent to telomere sequences was probed with ^{32}P-labelled *ura4*-DS/E DNA. All strains have the *ura4*-DS/E allele at the *ura4* locus which serves as an internal control, while the upper band is the message produced from the *ura4* gene. The amount of *ura4* message relative to the *ura4*-DS/E control is shown beneath each lane. The orientation of the *ura4* gene and type of telomere are as indicated above each track.

strains was provided by the presence of the *ura4*-DS/E allele at the *ura4* locus which produces a truncated, non-functional, *ura4* mRNA from the normal *ura4* promoter (Allshire *et al.*, 1994). The strain FY521 provided a positive control having a copy of the *ura4* gene inserted at the m23 locus on Ch16 but 50 kb from the telomere. As can be seen in this strain, the m23::*ura4* and *ura4*-DS/E bands have a similar intensity, indicating that the two genes are expressed equally. However, in all strains bearing a *ura4* gene close to a telomere the level of message emanating from that *ura4* gene is reduced relative to the *ura4*-DS/E minigene control. In the strains with 'short' telomeres adjacent to the *ura4* gene, FY520 broken with pEN72 and FY564 with pEN73, the *ura4* gene is very repressed and no band corresponding to *ura4* mRNA can be detected. In strains with 'long' TAS-containing telomeres adjacent to a terminally located *ura4* gene, FY566 broken with pEN76 and FY568 with pEN77, the *ura4* transcript is easily detected although it is produced at reduced amounts compared with the *ura4*-DS/E band. The expression levels observed are consistent with the plating experiments presented in Table I and Figure 6, in that strains which grew poorly on FOA and well on Ura$^-$ produced more message than those with the opposite phenotype. In addition, the effects of distance and orientation of the *ura4* gene with respect to the telomere are reflected in the expression levels. Clearly, more expression of a *ura4* gene results if it is separated from the telomere by a TAS element. It also appears that there is more expression of the *ura4* gene when it is transcribed away from the telomere.

Breakage of the minichromosome at the ade6 gene

In order to confirm that telomere-mediated fragmentation is generally applicable in fission yeast, vectors were constructed which would allow the minichromosome to be broken at *ade6*. Recovery of such a broken minichromosome would simultaneously test if expression of a second, polII-transcribed gene is also repressed when positioned adjacent to a telomere.

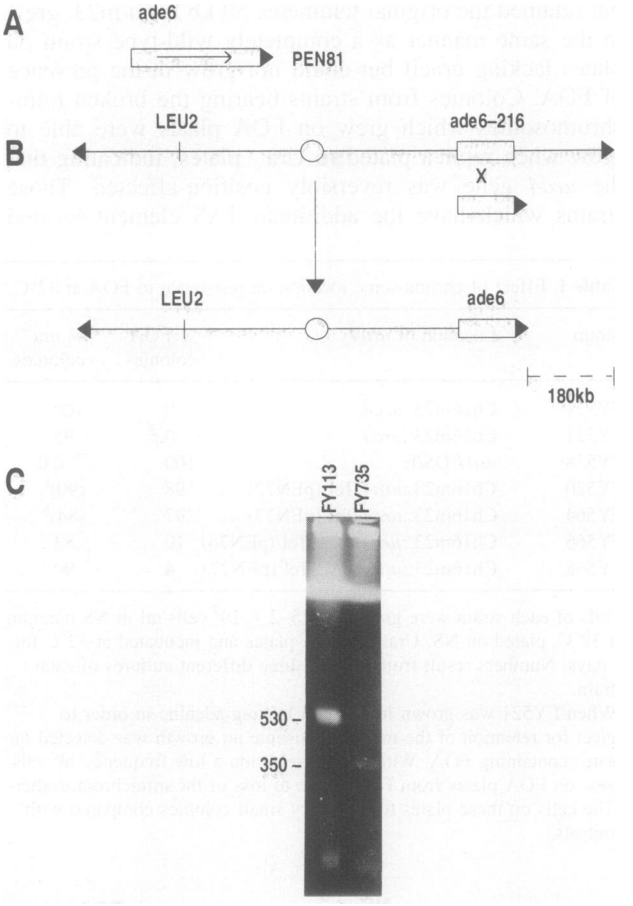

Fig. 8. Breakage of Ch16-23R adjacent to *ade6*-216. (**A**) Digestion of plasmid pEN81 with *Sac*I and *Sca*I releases a linear fragment containing only the short telomere and the *ade6* gene. The direction of transcription of the *ade6* gene is indicated by the arrow. (**B**) The linear *ade6*-containing fragment was co-transformed into FY538, which carries the Ch16-23R minichromosome, along with the *ura4*-containing plasmid pUR19. The transformation was plated in the absence of leucine and uracil to maintain selection for the minichromosome and enrich for transformants by selecting for the pUR19 plasmid. Transformants were then replica plated to YE plates to allow the red colour to develop. (**C**) Pulsed field gel of a representitive adenine breakage clone, FY735, showing reduction in size from 530 to 350 kb. Strain FY113 contains the original Ch16-23R minichromosome.

To induce breakage at the *ade6*-216 allele of the *ade6* gene on the right arm of Ch16-23R vectors were constructed with the complete *ade6* gene cloned adjacent to a 'short' telomere repeat array (Figure 8A). Vectors containing both *ade6* gene orientations were required since the orientation of the *ade6* gene on Ch16-23R was unknown. The *ade6*–telomere construct was released from the vectors with *Sac*I and *Ava*II (pEN80) or with *Sac*I and *Sca*I (pEN81). These purified fragments were co-transformed into the strain FY538 with pUR19, a plasmid carrying the *ura4* gene and *S.pombe ars1* (Figure 8B). Since the starting strain was ade$^+$ no selection for transformation with the *ade6*–telomere fragment was available; co-transformation with pUR19 thus allowed an enrichment for transformants, some of which should also have taken up the *ade6*–telomere fragment. It was assumed that if the *ade6*–telomere fragment induced breakage within the *ade6*-216 allele on Ch16-23R then a new telomere would be formed adjacent to *ade6* on the

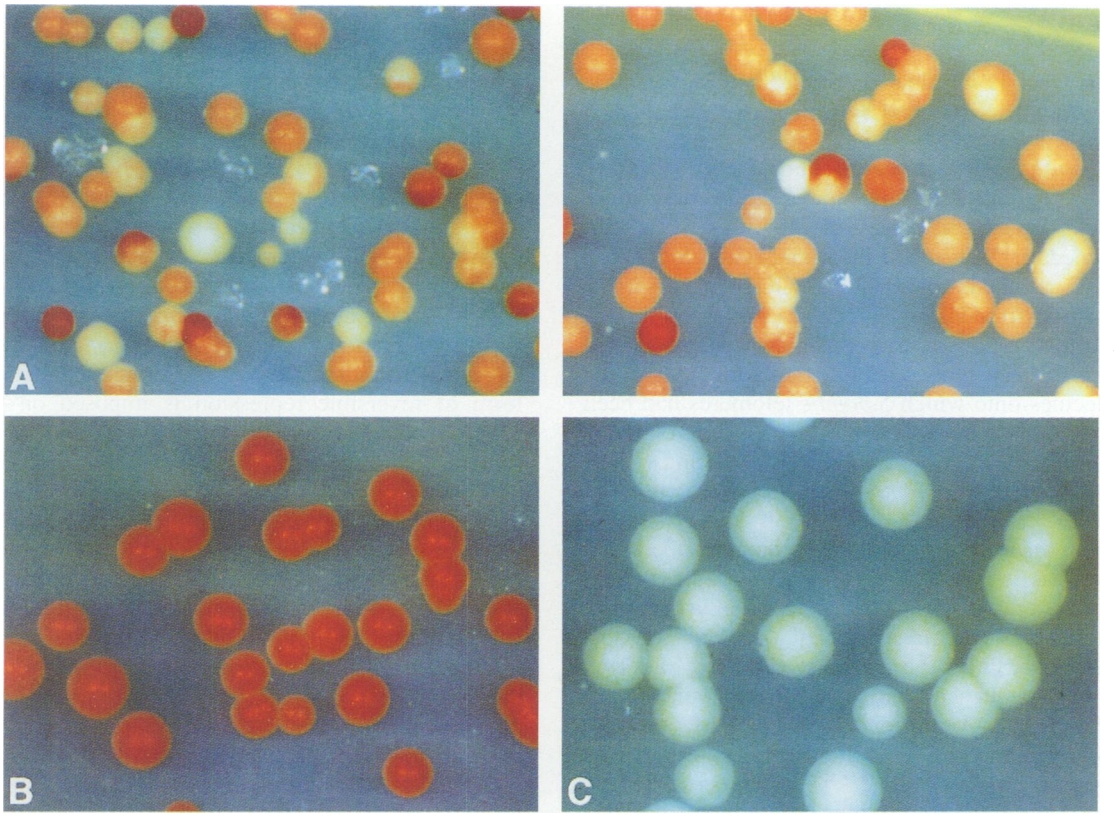

Fig. 9. Colonies with *ade6* adjacent to telomeres display variegated colour. Colonies from plates lacking both leucine and adenine, to select for the minichromosome and expression of *ade6*, were picked and resuspended. Dilutions of these cells were distributed on plates lacking leucine, in order to select for retention of the minichromosome, and containing 12 mg/l of adenine, to allow the red colour to develop. Plates were incubated for 6 days at 25°C. (**A**) The strain FY738 with the *ade6* gene adjacent to a telomere forms colonies which are variegated and range in colour from red to white. (**B**) The strain FY489 which carries only the *ade6*-210 allele and forms only uniformly red colonies. (**C**) The strain FY538 which bears the ade6 gene (*ade6*-216 allele) at an internal site on the minichromosome and forms white colonies.

minichromosome and that expression of this *ade6* gene would be subjected to position effect variegation. It is known that *S.pombe* cells with a variegating *ade6* gene can exhibit red/pink/white sectored colony colour (Allshire *et al.*, 1994), therefore the transformed cells were plated in the presence of only limited adenine to allow the development of colony colour. Red and variegating colonies appeared which were subsequently streaked on Leu^- plates to select for colonies retaining the minichromosome. Several variegating leu^+ colonies were obtained by transformation with the pEN81 construct. High molecular weight DNA from a number of these was prepared and analysed by pulsed field gel electrophoresis (Figure 8C). In strains where breakage at *ade6* on Ch16-23R had occurred the minichromosome had been reduced in size by ~180 kb from 530 to 350 kb. Incubation of DNA from these strains with Bal31 revealed that the *ade6* gene was indeed sensitive to the exonuclease and now located adjacent to a chromosome end (data not shown). Further Southern analyses showed that cosmids known to map distal to the *ade6* gene (Maier *et al.*, 1992; Hoheisel *et al.*, 1993) had been lost from the broken minichromosome (data not shown). The fact that successful breaks at *ade6*-216 on Ch16-23R were only obtained with pEN81 indicates that this construct had the correct orientation of the *ade6* gene with respect to the telomere and that the *ade6* gene at its normal locus is transcribed towards the telomere. It has recently been reported that this is indeed the correct orientation of transcription of the *ade6* gene (Grimm *et al.*, 1994).

Cells from the strain FY735 with the *ade6* gene adjacent to a telomere when grown on plates which select for the retention of the minichromosome (Leu^-) and contain limiting amounts of adenine gave rise to colonies with various degrees of sectoring and also to colonies which were uniformly red, pink or white (Figure 9). At 25°C only 0.5% of colonies formed were uniformly white while 3.5% were entirely red. Of the red colonies tested, on replating at least 99% gave rise to ade^+ colonies indicating that although red colonies were formed this telomere adjacent *ade6* gene was still functional. White colonies when plated under the same conditions as above were always found to generate some red, pink and variegated colonies. In the strain with only the *ade6*-210 allele (FY489) white colonies arise at a low frequency ($<1 \times 10^{-5}$) while red colonies were never detected in a strain (FY538) with the *ade6* gene at an internal site on the minichromosome ($<1 \times 10^{-5}$). This indicates that the telomere adjacent *ade6* gene, although functional, is subject to some form of epigenetic regulation. It is possible that the range of colony colours obtained reflects the establishment of intermediate levels of expression/repression and the maintenance of these states over a number of generations.

Discussion

Small linear vectors terminated with 258 bp of *S.pombe* telomere terminal repeats and 800 bp TAS element have been successfully transformed into *S.pombe* at frequencies comparable with that of circular plasmids. Under selection, these vectors replicate and remain unrearranged, indicating that acentric, linear molecules as small as 8 kb can be maintained in *S.pombe*. Linear plasmids containing *Tetrahymena* telomere repeats, $(TTGGGG)_n$, had previously been transformed into *S.pombe* (Sugawara, 1989), but transformants with linear episomes were recovered with a low efficiency. Only one of 11 transformants formed linear molecules onto which *S.pombe* telomeres had been seeded. YACs from *S.cerevisiae* with $(TG_{1-3})_n$ telomere repeats have also been transferred into *S.pombe* and similar low rates of seeding of *S.pombe* telomeres were observed (Hahnenberger *et al.*, 1989).

Restriction digests to confirm the structure of the linear molecules in fission yeast showed that the repeat length remained the same at ~300–400 bp. Naturally occurring *S.pombe* telomeres contain repeats of ~300 bp and also contain TAS internal to these repeats. Some of the TAS DNA has been sequenced and found to be composed of tandem arrays of 86–89 bp repeats. These repeats have a high degree of homology to each other and are part of a larger repeating unit of 0.9–1.2 kb. Differences between the TAS on different chromosomes are thought to be due to variable numbers and arrangements of the repeats. Other repeat units have also been detected at a more internal location (Sugawara, 1989). The function of the TAS elements is unknown but they are perhaps analogous to the X and Y′ repeats found in *S.cerevisiae*. The Y′ repeats are highly conserved, usually 6.7 kb in length and are present as between one and four tandemly arranged copies at the ends of many chromosomes immediately internal to the telomere repeat; while the X repeats are smaller and found in single copies internal to the Y′ repeats (Chan and Tye, 1983). Chromosomes lacking both the X and the Y′ repeats have been found and artificial chromosomes can be constructed which lack these repeats but function perfectly well. Therefore, in the smaller chromosomes at least, these repeats are not necessary for stable maintenance (Murray and Szostak, 1986; Zakian and Blanton, 1988). In *S.pombe* the TAS elements also appear not to be essential since of the six telomeres in fission yeast only four have adjacent TAS repeats. The two remaining telomeres are joined directly to repetitive ribosomal DNA sequences (Sugawara, 1989; Hoheisel *et al.*, 1993; Mizukami *et al.*, 1993).

Telomere-associated chromosome breakage was first used to fragment *S.cerevisiae* chromosomes in order to map genes (Vollrath *et al.*, 1988) and more recently has been applied to mammalian cells (Farr *et al.*, 1992; Itzhaki *et al.*, 1992). Here, vectors with a reporter gene (*ura4* or *ade6*) next to cloned telomeres allowed breakage of the *S.pombe* minichromosome Ch16-23R at two sites by homologous recombination resulting in the insertion of new telomeres adjacent to the selectable markers. As shown in *S.cerevisiae*, chromosome breakage is a powerful tool for mapping genes and can be used to distinguish the order of two adjacent loci. The orientation of a gene on a chromosome with respect to a telomere can be determined. Indeed it has been demonstrated here that the *S.pombe ade6* gene is normally transcribed towards the telomere. Since we have shown that Ch16-23R can be broken at the m23 and *ade6* loci, this may provide a useful method for mapping genes in the fission yeast. In addition, strains with novel linear minichromosomes containing centromeres should now be more easily constructed using telomere-associated breakage allowing targeted breaks to be made rather than relying on random breakage and healing events induced by irradiation (Niwa *et al.*, 1989).

It has been demonstrated here that strains of fission yeast with the *ura4* gene adjacent to a telomere are able to grow on both Ura$^-$ and FOA plates and therefore a form of reversible repression is imposed on the gene. This phenomenon has only previously been seen in the following cases: where the *URA3* gene was placed next to telomeres in *S.cerevisiae* (Gottschling *et al.*, 1990); the *ura4* gene was inserted next to the silent mating type locus in *S.pombe* (Thon and Klar, 1992); or within the central domain of all three *S.pombe* centromeres (Allshire *et al.*, 1994). Repression was greatest when the *ura4* gene was adjacent to the 'short' telomere repeat array with low levels of *ura4* message seen. In this case, almost all of the cells (97%) formed normal sized colonies on FOA plates and very few (<5%) developed normal sized colonies on Ura$^-$ plates. When the 800 bp TAS element was between the terminal telomere repeats and the *ura4* gene, the severity of the position effect was much weaker, 10–30 times fewer colonies grew on FOA plates and 83% of cells formed colonies on Ura$^-$ plates. These results were confirmed by Northern analyses as more *ura4* message was detected in the cells containing the TAS, although still at less than wild-type levels. This reduced repression seen at 'long' TAS-containing telomere constructs could be due to either a specific effect of the TAS or perhaps simply to the increased distance between the *ura4* gene and the end of the telomere. In *S.cerevisiae* it has been noted (Renauld *et al.*, 1993) that the presence of Y′ sequences between the telomeres and the gene causes a greater frequency of silencing than an equal length of unique DNA. This could be due to the absence of elements which could suppress the spreading of the position effect or to the presence of sequences in the Y′ region which enhance the strength of the position effect. It will be of interest to see if an equal length (800 bp) of unique non-TAS DNA has the same effect on the strength of the position effect when placed between the terminal repeat array and the *ura4* gene.

This telomere-mediated reversible repression in *S.pombe* is not specific to the *ura4* gene; a similar breakage experiment was performed using the *ade6* gene on the right arm of Ch16-23R resulting in the formation of a new telomere adjacent to this gene. These strains exhibit a variegated phenotype which can be visualized in individual colonies as white, pink and red sectors. At 25°C most colonies were pink or red, suggesting that low levels of transcription of the *ade6* gene occur at this temperature. Since pure red or white colonies can be obtained, containing ~2×10^6 cells, the state of expression of the gene can be stable for at least 20 generations. The uniformly pink coloured colonies seen may result from the establishment and maintenance of a low level of expression of the *ade6* gene rather than a rapid switching between 'on' and

'off' states to produce a mixed population made up of red and white cells. It is likely that repression of telomere-adjacent genes is due to the assembly of a specialized structure at telomeres. By analogy with PEV in *Drosophila*, variegation may result from encroachment of this structure into the adjacent gene in some cells but not in others. Intermediate levels of expression or repression may result when only a portion of the gene is assembled into this structure. In *Drosophila*, genes placed close to telomeres also variegate (Levis *et al.*, 1985); however, in this case the predominant eye colour was yellow rather than white or red. Perhaps this yellow eye colour in *Drosophila* also reflects intermediate levels of expression of a variegating gene. The fact that strains with the *ura4* gene close to a telomere form colonies with a range of sizes also suggests that expression of the *ura4* gene can be maintained at intermediate levels. The degree of repression and the phenotypes seen are similar to those obtained with the *ura4* and *ade6* genes at the centromeres where they are also subject to variegating position effects (Allshire *et al.*, 1994). In *S.cerevisiae*, cells with the *ADE2* gene placed next to telomeres form mainly red (repressed) colonies but with white sectors. The degree of gene repression is likely to depend on promoter strength versus the potency of repressive complexes formed at telomeres in different organisms (see Renauld *et al.*, 1993).

Telomere position-affected strains showed less growth on FOA plates at higher temperatures. This may be due to increased levels of *ura4* transcription at higher temperatures because of increased accessibility of the *ura4* gene due to a more open chromatin structure. Alternatively, it may just reflect enhanced activity of the *ura4* gene product at elevated temperature. Wild-type *ura4* levels of expression may produce a large excess of the *ura4* product so that this temperature effect is only revealed when the amount of *ura4* produced in cells is limited, as seen in position-affected strains.

The orientation of the gene had little effect on transcription levels when adjacent to the short, TAS-less, telomere but was more noticeable in the long, TAS-containing, telomere strains. In these there were higher mRNA levels and less growth on FOA plates when the gene was transcribed away from the telomere. As the distance between the gene and the telomere had more effect on growth than the orientation of the gene, it is possible that spreading of specialized telomeric chromatin into any part of the gene is enough to prevent expression of that gene and it is not necessary for such chromatin to spread as far as the promoter sequences. It is unclear why genes transcribed towards the telomere should produce less message than those transcribed away from the telomere. However, it has been suggested (Lee and Garrard, 1991) that positive DNA supercoiling is required to promote efficient transcription. It is possible that at telomeres the movement of RNA polymerase along the DNA towards the telomere is inhibited due to torsional constraints imposed by specialized chromatin structure anchoring the telomere to the nuclear periphery (see below). When transcription is away from the telomere this problem does not occur.

Fission yeast telomeres share several characteristics with heterochromatin in *Drosophila* and other higher eukaryotes. Heterochromatin is often found to be associated with the nuclear envelope. Recently it has been shown that, outside of mitosis, fission yeast telomeres are clustered and associated with the nuclear envelope (Funabiki *et al.*, 1993). Heterochromatin in multicellular eukaryotes is known to replicate in the later stages of S phase. Although the replication timing of *S.pombe* telomeres is unknown it is interesting to note that telomeres in *S.cerevisiae* are late replicating and staining with anti-Rap1 antibodies suggests that they may be associated with the nuclear envelope (Klein *et al.*, 1992). Transcriptional silencing occurs at two other known locations, apart from telomeres, in the *S.pombe* genome: within centromeres and at the silent mating type region covering the *mat2*-P and *mat3*-M loci. *S.pombe* centromeres are also known to lie at the nuclear periphery for most of the cell cycle (Funabiki *et al.*, 1993). It is likely that this location plays an important role in silencing mechanisms. In light of this it is interesting to note that localization of the product of the *S.cerevisiae RAP1* gene (and perhaps telomeres) to the nuclear periphery requires a functional *SIR3* product (Palladino *et al.*, 1993). Both *RAP1* and *SIR3* gene products are required for silencing at the silent mating type loci, *HML* and *HMR*, and at telomeres (Kyrion *et al.*, 1993; Palladino *et al.*, 1993).

In *S.cerevisiae* a non-nucleosomal chromatin pattern exists at the telomeres (Wright *et al.*, 1992) and genes inserted close to the telomere are subject to position effects (Gottschling *et al.*, 1990). The severity of this effect is inversely related to the distance between the gene and the telomere: as the distance increases the level of repression decreases (Renauld *et al.*, 1993). Many of the same factors are involved in silencing at the telomeres and at the mating loci, while others factors, such as *SIR1*, are not normally required at the telomere (Aparicio *et al.*, 1991). In *S.pombe* a number of mutations which derepress silencing at *mat2*-P and *mat3*-M have been identified (Egel *et al.*, 1989; Lorentz *et al.*, 1992; Thon and Klar, 1992; Ekwall and Ruusala, 1994). Unusual chromatin occurs within *S.pombe* centromeres (Polizzi and Clarke, 1991; Takahashi *et al.*, 1992) in the same region where genes have been shown to be subject to position effects (Allshire *et al.*, 1994) and a non-nucleosomal structure has also been observed at fission yeast telomeres (Chikashige *et al.*, 1989). It is possible that mutations which affect silencing at the mating loci may also alleviate gene repression at centromeres and telomeres in fission yeast. If so, these mutations may also interfere with telomere and/or centromere function and affect the association of these structures with the nuclear periphery.

Materials and methods

Plasmid constructions

pEN51 and pEN53. Two 1.1 kb *Eco*RI fragments, each containing 258 bp of *S.pombe* telomere repeat sequence and 800 bp of TAS from pNSU70 (Sugawara, 1989), were cloned in a head-to-head orientation around the *E.coli* kanamycin resistance gene on a 1.3 kb *Eco*RI fragment. This 3.5 kb fragment, containing two 'long' telomere sequences and the kanamycin resistance gene, was cloned into the *Eco*RI site in the polylinker of pBluescript II to give pEN42 and the *Sac*I site in this polylinker was subsequently destroyed by filling in and ligating to give pEN47. The 'long' telomere-containing cassette in pEN47 could be removed on a 3.6 kb *Pvu*II fragment and used to replace the *Pvu*II polylinker in pUR19 to give pEN51 or the *Pvu*II polylinker in pRS305SpArsLEU to give pEN53.

pEN72, pEN73, pEN76 and pEN77. These consist of the plasmid pBS with cloned *S.pombe* telomeres, the *ura4* gene and m23. They were constructed as follows, pEN72 and pEN73: m23 was cloned on a 2.6 kb *Hin*dIII fragment into the *Hin*dIII site in the polylinker of pBluescript II (pEN65). The *ura4* gene was isolated as a 1.7 kb *Pvu*II–*Cla*I fragment from pUR19, filled in and cloned into the *Eco*RV site of pEN65, adjacent to the m23 sequence in both orientations (pEN68 and pEN69). The telomere sequences were isolated as a 1.8 kb *Apa*I fragment from pEN42, filled in and blunt-end cloned into the *Sma*I site of pEN68 and pEN69, adjacent to the *ura4* gene to give pEN72 and pEN73. pEN76: the short telomere cassette was removed from pEN72 by digestion with *Eco*RI and replaced with the 1.1 kb *Eco*RI telomere fragment from pNSU70 to give pEN76. pEN77: pEN74, containing the long telomere, *ura4* and m23 in the wrong orientation, was digested with *Hin*dIII to release the m23 sequence. This was recloned into this plasmid in the opposite orientation to give pEN77. In each case, to prepare linear fragments containing only telomere sequences, the *ura4* gene and m23 sequence, the plasmid was digested with *Sac*I and *Cla*I.

pEN80 and pEN81. The 1.8 kb 'short' telomere cassette from pEN42 was cloned into the *Apa*I site of pBS to give pEN56. The 3 kb *Sal*I–*Sma*I fragment of the *ade6* gene was filled in and cloned into the *Eco*RV site of pEN56 in both orientations to give pEN80 and pEN81. Linear fragments of pEN80 and pEN81 containing only the telomere sequences and the *ade6* gene were released by cutting with *Sac*I/*Ava*II and *Sac*I/*Sca*I respectively.

S.pombe media

S.pombe was grown in YEA medium for transformations and on EMMG plates or liquid media (Moreno *et al.*, 1991) with the required supplements for plating experiments and growth of cells for RNA extractions. YE plates were used to develop red colony colour. Non-selective (NS) refers to EMMG containing adenine, leucine and uracil at 75 mg/litre each. FOA plates are the same as NS but with the addition of FOA (5-fluoro-orotic acid; PCR Inc., Gainsville, FL). Ura^- is the same as NS but without the addition of uracil.

Transformations

All transformations were done using a modified version of the lithium acetate method of Moreno *et al.* (1991).

Strain construction

The genotype of all strains used is shown in Table II.

FY538 contains the Ch16-23R minichromosome, a 530 kb radiation-induced deletion derivative of chromosome 3 carrying the *LEU2* gene on the left arm and the *ade6*-216 allele on the right arm.

FY520, FY564, FY566 and FY568 were constructed by transforming linear DNA fragments containing telomere sequences, the *ura4* gene and m23 from pEN72, pEN73, pEN76 and pEN77 respectively into FY538. Ura^+ leu^- transformants were picked and screened for the ability to grow on two rounds of FOA plates and again on Ura^- plates. Confirmation of breakage of Ch16-23R was made using pulsed field gel electrophoresis.

FY735 was made by co-transforming FY538 with linear pEN81 and with the plasmid pUR19 as there was no selection for the pEN81 plasmid. Transformants were selected on Ura^- plates and replica plated to YE plates at 25°C until the red colour developed. Breakage of Ch16-23R was confirmed by pulsed field gel analysis.

Serial dilution spot assays

Single colonies were picked from fresh plates and placed in 5 ml EMMG medium with uracil, leucine and adenine at 75 mg/litre each and grown overnight at 32°C to a density of $0.5–2.0 \times 10^7$ cells/ml. Cells were diluted to 1×10^6/ml in water and an aliquot of each was placed in a 96 microwell plate. Five 1:5 serial dilutions were made and 10 µl aliquots of each were spotted onto NS, Ura^- or FOA plates and plates incubated at 25, 32 or 35°C. This gave 10^4 cells in the first spot and three cells in the last spot. After 3–5 days' growth the plates were removed from the incubator and photographed.

For a quantitative measure of the ability of cells to grow on selective plates, cells were diluted in water and ~1000 cells/plate plated. These were incubated at 32°C for 3–4 days and counted.

DNA preparation

DNA was extracted as described in Moreno *et al.* (1991). Digestion of DNA with restriction enzymes was done according to the manufacturer's instructions (Boehringer Mannheim) and digested DNA electrophoresed on 0.8% agarose gels in 0.5 × TAE buffer. DNA was transferred to Genescreen (Dupont) and filters crosslinked in a Stratalinker (Stratagene) followed by baking for 1–2 hours at 80°C. [α-^{32}P]dCTP (>3000 Ci/mM, Amersham) random prime labelled probes were prepared according to the manufacturer's instructions (BCL) and hybridizations were performed in 0.25 M Na_2HPO_4 pH 7, 5 mM EDTA, 7% SDS, 100 mg/ml denatured sonicated salmon sperm DNA at 68°C, as described by Church and Gilbert (1984). Probes were all gel purified using Geneclean (Bio 101) or B-agarose (Biolabs). The fragments used were a 1.5 kb fragment containing the *ura4*-DS/E gene and a 2.1 kb fragment containing the *LEU2* gene. Loss of sequences distal to *ade6* was confirmed by hybridization with cosmid 25F2 (Maier *et al.*, 1992; Hoheisel *et al.*, 1993).

Bal31 digestion of DNA

0.5 µg DNA was incubated with 0.5 U of Bal31 in a total of 30 µl. Aliquots were removed and the reaction stopped by the addition of EGTA at various time points. Samples were then run on a 1% agarose gel and blotted and probed as described.

Preparation of DNA for pulsed field gels

Cultures were grown overnight to a density of $0.5–1.5 \times 10^7$/ml, spun down and washed three times in 50 mM EDTA pH 8. Cells were resuspended in SP1 (1.3 M sorbitol, 50 mM sodium citrate, 50 mM sodium phosphate, 40 mM EDTA, pH 5.6) to a density of 2×10^9/ml and Zymolyase T100 (ICN Biochemicals Inc.) was added to a final concentration of 0.5 mg/ml. After incubation at 37°C for 1–2 h, an equal volume of 1% LMP agarose (in 125 mM EDTA pH 8.0) at 42°C was added. This DNA suspension was aliquoted into 100 µl moulds and left to set on ice. Once set, the plugs of DNA were removed into NDS solution (1% *N*-lauryl sarcosine, 10 mM Tris, 0.5 M EDTA, pH 9) with Proteinase K at a final concentration of 0.5 mg/ml and left overnight at 55°C. The plugs were removed into fresh NDS solution with Proteinase K the next day, incubated at 55°C for a further 24 h and washed several times in 1 × TE.

Pulsed field gel electrophoresis

Pulsed field gels were run on a Bio-Rad Chef DR-II. Gels were run at 150 V, 70 s, 0.5 × TAE, 1% agarose for 25 h.

Table II. *S.pombe* strains used in this study

Strain No.	Position of *ura4* on Ch16	Background genotype
SP813	No minichromosome	h^+ *leu1*-32, *ade6*-210, *ura4*-D18
FY498	No minichromosome	h^- *leu1*-32, *ade6*-210, *ura4*-DS/E
FY521	*Ch16 ade6*-216 *m23::ura4*	h^- *leu1*-32, *ade6*-210, *ura4*-DS/E
FY538	*Ch16 ade6*-216, *m23::LEU2*	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E
FY520	*Ch16 ade6*-216, *m23::ura4*-Tel[72]	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E
FY564	*Ch16 ade6*-216, *m23::ura4*-Tel[73]	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E
FY566	*Ch16 ade6*-216, *m23::ura4*-Tel[76]	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E
FY568	*Ch16 ade6*-216, *m23::ura4*-Tel[77]	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E
	Position of *ade6* on Ch16	
FY735	(*Ch16 LEU2*, *ade6::*Tel[81])	h^+ *leu1*-32, *ade6*-210, *ura4*-DS/E

RNA preparation and analysis

All RNAs were prepared from cells grown in EMMG medium containing uracil and leucine at 75 mg/l, as described by Moreno *et al.* (1991). 10 μg of total RNA was electrophoresed on a 1% agarose–formaldehyde–MOPS gel as described in Sambrook *et al.* (1989). After electrophoresis for 3 h at 100 V, RNA was transferred to Genescreen according to the manufacturer's instructions (Dupont). Hybridizations were performed as for Southern blots above.

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References

Allshire,R.C., Javerzat,J.-P., Redhead,N. and Cranston,G. (1994) *Cell*, **76**, 157–169.

Aparicio,O.M., Billington,B.L. and Gottschling,D.E. (1991) *Cell*, **66**, 1279–1287.

Blackburn,E.H. (1991) *Nature*, **350**, 569–573.

Burke,D.T., Carle,G.F. and Olson,M.V. (1987) *Science*, **236**, 806–812.

Chan,C.S.M. and Tye,B.-K. (1983) *Cell*, **33**, 536–573.

Chikashige,Y., Kinoshita,N., Nakaseko,Y., Matsumoto,T., Murakami,S., Niwa,O. and Yanagida,M. (1989) *Cell*, **57**, 739–751.

Church,G.M. and Gilbert,W. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Demerec,M. (1940) *Genetics*, **25**, 618–627.

Egel,R., Willer,M. and Nielsen,O. (1989) *Curr. Genet.*, **15**, 407–410.

Ekwall,K. and Ruusala,T. (1994) *Genetics*, **136**, 53–64.

Farr,C.J., Stevanovic,M., Thomson,E.J., Goodfellow,P.N. and Cooke,H.J. (1992) *Nature Genet.*, **2**, 275–282.

Funabiki,H., Hagan,I., Uzawa,S. and Yanagida,M. (1993) *J. Cell Biol.*, **121**, 961–976.

Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) *Cell*, **63**, 751–762.

Grimm,C., Bahler,J. and Kohll,J. (1994) *Genetics*, **135**, 41–51.

Hahnenberger,K.M., Baum,M.P., Polizzi,C.M., Carbon,J. and Clarke,L. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 577–581.

Hartmann-Goldstein,I.J. (1967) *Genet. Res.*, **10**, 143–159.

Hegemann,J., Shero,J., Cottarel,G., Philipsen,P. and Hieter,P. (1988) *Mol. Cell. Biol.*, **8**, 2523–2535.

Hoheisel,J.D., Maier,E., Mott,R., McCarthy,L., Grigoriev,A.V., Schalknyk,L.C., Nizetic,D., Francis,F. and Lehrach,H. (1993) *Cell*, **73**, 109–120.

Itzhaki,J.E., Barnett,M.A., MacCarthy,A.B., Buckle,V.J., Brown,W.R.A. and Porter,A.C.G. (1992) *Nature Genet.*, **2**, 283–287.

Klein,F., Laroche,T., Cardenas,M.E., Hofmann,J.F.X., Schweizer,D. and Gasser,S. (1992) *J. Cell Biol.*, **117**, 935–948.

Kyrion,G., Lui,K., Lui,C. and Lustig,A.J. (1993) *Genes Dev.*, **7**, 1146–1159.

Lee,M.-S. and Garrard,W.T. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 9675–9679.

Levis,R., Hazelrigg,T. and Rubin,G.M. (1985) *Science*, **229**, 558–561.

Lorentz,A., Heim,L. and Schmidt,H. (1992) *Mol. Gen. Genet.*, **233**, 436–442.

Maier,E., Hoheisel,J.D., McCarthy,L., Mott,R., Grigoriev,A.V., Monaco,A.P., Larin,Z. and Lehrach,H. (1992) *Nature Genet.*, **1**, 273–277.

Matsumoto,T., Fukui,K., Niwa,O., Sugawara,N., Szostak,J.W. and Yanagida,M. (1987) *Mol. Cell. Biol.*, **7**, 4424–4430.

Mizukami,T., Chang,W.I., Garkavtser,I., Kaplan,N., Lombardi,D., Matsumoto,T., Niwa,O., Kounosu,A., Yanagida,M., Marr,T.G. and Beach,D. (1993) *Cell*, **73**, 121–132.

Moreno,S., Klar,A. and Nurse,P. (1991) *Methods Enzymol.*, **194**, 795–823.

Muller,H.J. (1930) *J. Genet.*, **22**, 299–334.

Muller,H.J. (1941) *Cold Spring Harbor Symp. Quant. Biol.*, **9**, 151–167.

Murray,A.W. and Szostak,J.W. (1983) *Nature*, **305**, 189–193.

Murray,A.W. and Szostak,J.W. (1986) *Mol. Cell. Biol.*, **6**, 3166–3172.

Niwa,O., Matsumoto,T. and Yanagida,M. (1986) *Mol. Gen. Genet.*, **203**, 397–405.

Niwa,O., Matsumoto,T., Chikashige,Y. and Yanagida,M. (1989) *EMBO J.*, **8**, 3045–3052.

Palladino,F., Laroche,T., Gilson,E., Axelrod,A., Pillus,L. and Gasser,S.M. (1993) *Cell*, **75**, 543–555.

Polizzi,L.M. and Clarke,L. (1991) *J. Cell Biol.*, **112**, 191–201.

Renauld,H., Aparicio,O.M., Zierath,P.D., Billington,B.L., Chhablani,S.K. and Gottschling,D.E. (1993) *Genes Dev.*, **7**, 1133–1145.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shampay,J., Szostak,J.W. and Blackburn,E.H. (1984) *Nature*, **310**, 154–157.

Spofford,J.B. (1967) *Genetics*, **57**, 751–766.

Sugawara,N. (1989) *DNA Sequences at the Telomeres of the Fission Yeast S.pombe*. PhD Thesis, Harvard University.

Sugawara,N. and Szostak,J.W. (1986) *Yeast*, **2** (Suppl.), 373 (Abstract).

Szostak,J. (1982) *Cell*, **29**, 245–255.

Szostak,J. and Blackburn,E.H. (1982) *Cell*, **29**, 245–255.

Takahashi,K., Murakami,S., Chikashige,Y., Funabiki,H., Niwa,O. and Yanagida,M. (1992) *Mol. Biol. Cell*, **3**, 819–835.

Tartoff,K.D. and Bremner, M. (1990) *Development*, Suppl., 35–45.

Thon,G. and Klar,A.J.S. (1992) *Genetics*, **131**, 287–296.

Vollrath,D., Davis,R.W., Connelly,C. and Heiter,P. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 6027–6031.

Wright,J.H., Gottschling,D.E. and Zakian,V.A. (1992) *Genes Dev.*, **6**, 197–210.

Zakian,V.A. (1989) *Annu. Rev. Genet.*, **23**, 579–604.

Zakian,V.A. and Blanton,H.M. (1988) *Mol. Cell. Biol.*, **8**, 2257–2260.