Yeast telomeric sequences function as chromosomal anchorage points *in vivo*

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Site-specific recombination in *Saccharomyces cerevisiae* was used to generate non-replicative DNA rings containing yeast telomeric sequences. In topoisomerase mutants expressing *Escherichia coli* topoisomerase I, the rings adopted a novel DNA topology consistent with the ability of yeast telomeric DNA to block or retard the axial rotation of DNA. DNA fragments bearing portions of the terminal repeat sequence C₁₋₃A/TG₁₋₃ were both necessary and sufficient to create a barrier to DNA rotation. Synthetic oligonucleotide sequences containing Rap1p binding sites, a well represented motif in naturally occurring C₁₋₃A arrays, also conferred immobilization; mutant Rap1p binding sites and telomeric sequences from other organisms were not sufficient. DNA anchoring was diminished by addition of competing telomeric sequences, implicating a role for an as yet unidentified limiting *trans*-acting factor. Though Rap1p is a likely protein constituent of the DNA anchor, deletion of the non-essential C-terminal domain did not affect the topology of telomeric DNA rings. Similarly, disruption of *SIR2*, *SIR3* and *SIR4*, genes which influence a variety of telomere functions in yeast, also had no effect. We propose that telomeric DNA supports the formation of a *SIR*-independent macromolecular protein–DNA assembly that hinders the motion of DNA because of its linkage to an insoluble nuclear structure. Potential roles for DNA anchoring in telomere biology are discussed.

Keywords: DNA topoisomerases/*RAP1* gene/
R recombinase/*SIR* gene/transcriptional supercoiling

Introduction

Telomeres are specialized nucleoprotein complexes at the ends of linear eukaryotic chromosomes (reviewed in Zakian, 1995). The structures prevent exonucleolytic degradation and end-to-end fusion, and avert the gradual loss of DNA associated with replication of linear molecules by conventional DNA polymerases. Telomeres are also believed to be involved in the nuclear positioning of chromosomal DNA: in a variety of organisms, chromosome ends are clustered together and localized to the nuclear periphery during mitotic growth (Chung *et al.*, 1990; Funabiki *et al.*, 1993; Palladino *et al.*, 1993). The role of localization in the preservation of genomic integrity is unclear; however, examination of yeast telomeres indicates that other functions may correlate with nuclear

position. In *Schizosaccharomyces pombe*, chromosome ends lead pre-meiotic chromosomal movement, suggesting that telomeres are involved in chromosome mobility (Chikashige *et al.*, 1994). In *S.pombe* and *Saccharomyces cerevisiae*, as well as *Drosophila*, placement of genes in the vicinity of telomeres results in the reversible repression of transcription by a phenomenon termed telomere position effect (TPE) (Levis *et al.*, 1985; Gottschling *et al.*, 1990; Nimmo *et al.*, 1994).

Saccharomyces cerevisiae telomeres are composed of ~300 bp of the repetitive unit C₁₋₃A/TG₁₋₃ (reviewed in Louis, 1995). Located immediately adjacent to the terminal C₁₋₃A arrays at most telomeres are either one or both sub-telomeric repeats, X (0.3–3.7 kb) and Y' (6.7 kb). Whereas sub-telomeric regions are organized into nucleosomes, the terminal C₁₋₃A sequences are packaged into a nuclease-resistant structure termed the telosome (Wright *et al.*, 1992). Several lines of evidence indicate that the transcriptional activator and repressor protein Rap1p (reviewed in Shore, 1994), the product of the essential yeast gene *RAP1*, is a major telosome component. The protein binds a loose consensus which appears every 18 bp on average within C₁₋₃A arrays and forms a nucleoprotein complex that, by gel-shift and footprinting criteria, resembles partially purified telosomes (Gilson *et al.*, 1993; Wright and Zakian, 1995). Mutations in *RAP1* have profound effects on TPE and telomere structure, with different alleles yielding either abnormally long or short C₁₋₃A arrays (Conrad *et al.*, 1990; Lustig *et al.*, 1990; Sussel and Shore, 1991; Liu *et al.*, 1994). *RAP1*-interacting factors, such as *SIR3* and *SIR4* (Moretti *et al.*, 1994; Cockell *et al.*, 1995), also modulate chromatin structure near telomeres (Gottschling, 1992). The *SIR* genes were identified originally as negative regulators of gene expression at the silent mating loci, *HMR* and *HML* (Rine and Hershkowitz, 1987). Subsequently, they were shown to be required for repression of genes located near chromosomal ends (Aparicio *et al.*, 1991). Deletion of *SIR3* or *SIR4* results in shortening of telomeric C₁₋₃A and loss of the perinuclear localization of telomere-bound Rap1p (Palladino *et al.*, 1993).

Simple circular ARS plasmids, containing a replication origin but lacking a *cis*-acting partitioning locus, are present in only a small percentage of cells grown on selective media. Plasmid instability results from the failure to distribute newly replicated plasmids out of the mother cell (Murray and Szostak, 1983). Incorporation of telomeric repeats improves the efficiency of symmetric plasmid segregation to levels achieved with centromere sequences (Longtine *et al.*, 1992). Whereas long tracts of C₁₋₃A are sufficient for partitioning, sub-telomeric X repeat sequences amplify the effect in a *SIR*-dependent fashion (Enomoto *et al.*, 1994). An active transport model has been proposed to account for telomere-based

partitioning. According to the model, plasmid-borne $C_{1-3}A$ sequences associate with a nuclear structure which divides equally between mother and daughter cells at mitosis. The available data also support a passive transport model in which plasmids migrate by free diffusion. In this contrasting model, $C_{1-3}A$ tracts improve plasmid segregation because the sequences block the retention of plasmids by mother cells.

In addition to positioning chromosome ends within the nucleus, telomeres may play a role in packaging DNA termini into the higher order structure of chromosomes. It is generally accepted that chromosomal DNA is organized into discrete structural domains. Direct visualization of bacterial nucleoids and eukaryotic chromosomes by electron microscopy showed that lengths of DNA, of the order of 100 kb, emerged from and reentered a central amorphous core (Delius and Worcel, 1974; Paulson and Laemmli, 1977). Consonant with the images of DNA loops, physicochemical analyses indicated that chromosomes contained discrete topological domains (Worcel and Burgi, 1972; Benyajati and Worcel, 1976; Sinden and Pettijohn, 1981). More recently, *in situ* hybridization of sequence landmarks on mapped regions of human chromosomes revealed a spatial arrangement consistent with looped intervals of DNA, in both interphase nuclei and extracted metaphase chromosomes (Yokota *et al.*, 1995; Bickmore and Oghene, 1996). Biochemical attempts to identify eukaryotic loop attachment sequences have yielded a class of molecules which remain associated with residual nuclear structures, known as nuclear matrices or nuclear scaffolds (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986). The composition and physiological relevance of these structures is not clear and it is not certain whether the DNA sequences, termed matrix association regions (MARs) or scaffold attachment regions (SARs), serve as chromosomal anchorage points *in vivo*. However, the finding that mammalian telomeres bind the nuclear matrix is consistent with their sub-nuclear localization (de Lange, 1992; de Lara *et al.*, 1993). The argument is strengthened further by the observation that rodent telomeres associate exclusively with the ends of the synaptonemal complex, whereas bulk chromatin forms a diffuse halo around the axial core structure (Moens and Pearlman, 1990).

On the basis of the nuclear matrix binding activity, the plasmid stabilization property and the sub-nuclear localization of telomeres, we reasoned that telomeric DNA would be affixed to a structural support within intact cells. To test for telomere immobilization, we used a DNA topology-based assay which identifies DNA sequences and associated proteins that prevent or hinder the rotation of DNA about its helix axis (Gartenberg and Wang, 1993; see Methodology below). We show that the rotational motion of *Saccharomyces* telomeric DNA is blocked *in vivo*. Our data establish that $C_{1-3}A$ tracts and at least one unidentified *trans*-acting factor, possibly Rap1p, are required for immobilization; DNA ends and sub-telomeric sequences are not essential. In accordance with the partitioning properties of telomere-based plasmids, we argue that telomeric DNA anchoring arises from association of $C_{1-3}A$ tracts with a large nuclear structure which distributes equally between dividing cells. Attachment does not require the silencing domain of *RAP1* or the *SIR* genes,

Fig. 1. Use of paired supercoiling domains to detect DNA anchoring. (A) Translocation of RNA polymerase generates domains of positive and negative supercoiling. An arrow shows the direction of polymerase (ellipse) movement. Supercoils of opposite sign cancel rapidly through rotation of the DNA segment connecting the twin supercoiling domains. Other DNA helix-tracking processes could substitute for transcription in the generation of twin domains of supercoiling. (B) Incorporation of a second DNA rotation barrier (circle) prevents the merger of positive and negative supercoiled domains. (C) Relaxation by the negative supercoil-specific *E. coli* topoisomerase I yields positively supercoiled DNA rings. (D) Experimental strategy for detecting DNA anchoring in yeast. In step 1, the excision cassette of a recombination substrate is converted into a small DNA ring by galactose induction of the R site-specific recombinase. The half-filled rectangles represent RS recombination target sites, the filled circle represents a putative DNA anchoring element and the arrow represents a promoter (or site of entry for other helix-tracking proteins). In step 2, transcription (or helix tracking) of the DNA ring generates twin supercoiled domains. In step 3, inactivation of endogenous yeast DNA topoisomerases I and II and expression of the negative supercoil-specific *E. coli* topoisomerase I leads to positive supercoiling of DNA rings containing anchors. DNA rings without anchors remain negatively supercoiled due to nucleosomal wrapping.

indicating that TPE and perinuclear localization of Rap1p are not prerequisites for the immobilization of telomeric sequences.

Results

Methodology

In a DNA circle, negative and positive supercoils generated by transcription will cancel by rotation of the DNA segments that join them (Figure 1A) (Liu and Wang, 1987). Assuming that the transcriptional machinery serves as one barrier to DNA rotation, cloned inserts that provide a second barrier prevent loss of the twin-supercoiled domains (Figure 1B). We refer to DNA 'rotation barriers' as DNA anchors. Selective relaxation of one of the twin domains results in the unbalanced accumulation of the other (see Figure 1C) (Tsao *et al.*, 1989). In yeast, this is accomplished by inactivating DNA topoisomerase I and II, enzymes which relax both positive and negative supercoils, and expressing the negative supercoil-specific *Escherichia*

coli topoisomerase I (Giaever and Wang, 1988; Gartenberg and Wang, 1992). Under these conditions, DNA with anchors becomes positively supercoiled. DNA without anchors remains negatively supercoiled, as a physiological consequence of nucleosomal packaging (Germond *et al.*, 1975); neither the bacterial nor yeast topoisomerases can relax negative supercoils wrapped around histone cores. Earlier use of the supercoiling assay showed that the partitioning system of the ubiquitous yeast 2 μ m plasmid forms a DNA anchor (Gartenberg and Wang, 1993).

Since 2 μ m and CEN-based plasmids already contain DNA anchors (A.Mirabella and M.R.Gartenberg, unpublished results), we used inducible site-specific recombination to generate simple non-replicating DNA rings *in vivo*. Fusion of the R recombinase gene from *Zygosaccharomyces rouxii* to the *GAL1* promoter permitted galactose-inducible formation of DNA rings in *S.cerevisiae* (Matsuzaki *et al.*, 1990). Substrates for the excision reaction were derived from plasmid pKWD50N which contains an excision cassette composed of a 2.5 kb fragment of the yeast *LYS2* internal coding sequence flanked by directly repeated recombination targets, termed *RS* sites (Gartenberg and Wang, 1993). Telomeric fragments to be tested were cloned within the *LYS2* sequence. For clarity, we follow the convention of a prefix 'p' for plasmid and 'r' for ring (e.g. plasmid pKWD130C yields ring rKWD130C).

In earlier work, we found that addition of a promoter to the ring enhanced detection of the 2 μ m-based DNA anchor, but that added transcription units were not essential (Gartenberg and Wang, 1993). We presume that DNA helix-tracking factors, such as those found in bacteria, yeast and frog, substitute for transcription in the generation of twin domains of supercoiling (Zhang *et al.*, 1990; Koo *et al.*, 1991, 1992). Alternatively, generation of paired supercoiling domains could result from transcription of cryptic promoters in the DNA rings. To simplify experimental procedures, exogenous promoters were not incorporated in the excised sequences here. Figure 1D graphically summarizes the sequence of experimental steps. DNA rings were generated in strains in which the yeast DNA topoisomerase I gene was deleted, the yeast DNA topoisomerase II gene was replaced with the temperature sensitive *top2-4* allele and the *E.coli* topoisomerase I gene (*topA*) was expressed by the constitutive *GPD* promoter (Giaever and Wang, 1988). At 35°C, the non-permissive temperature for *top2-4*, the negative supercoil-specific bacterial enzyme provided the only appreciable topoisomerase activity. The entire assay required that cells be perturbed for only 4 h, 2.5 h less than the doubling time for these strains under these conditions.

Rings containing telomeric DNA are anchored in yeast

Using excision substrate pKWD130C, we generated a DNA ring which contained a 639 bp fragment of the telomere on the left arm of the *Saccharomyces* chromosome III (Button and Astell, 1986). The sequence is composed of a 369 bp C₁₋₃A repeat array and 270 bp, or ~20%, of a sub-terminal X repeat element. The *ARS* region associated with the X repeat was not included. DNA supercoiling levels were analyzed by two-dimensional agarose gel electrophoresis in buffer containing chloro-

Fig. 2. Two-dimensional agarose gel electrophoresis of a telomeric DNA ring. (A) Topoisomer distributions are resolved into an arc by electrophoresis in two orthogonal dimensions differing only by the concentration of chloroquine in the buffer (Peck and Wang, 1983). Topoisomers of progressively higher linking number align in a clockwise fashion: a, negatively supercoiled rings; b, relaxed rings; c, highly positively supercoiled rings; d, nicked rings. In some samples, negatively supercoiled topoisomers are represented by pairs of spots (see Figure 4B, for example). This phenomenon varied between sample preparations and probably reflects structural transitions in naked C₁₋₃A sequences upon isolation (Lyamichev *et al.*, 1989; Belotserkovskii *et al.*, 1996). (B) Influence of topoisomerase activities on topology of the telomeric DNA ring rKWD130C. Each lane contains the products of an excision reaction performed in a strain with a unique combination of topoisomerase activities. Lane 1, JCW27 [Δ *top1 TOP2*] and *E.coli* topoisomerase I; lane 2, JCW26 [*TOP1 top2-4*] and *E.coli* topoisomerase I; lane 3, JCW28 [Δ *top1 top2-4*] and *E.coli* topoisomerase I, at the permissive temperature for *top2-4*; lane 4, JCW28 [Δ *top1 top2-4*] and *E.coli* topoisomerase I. *GAL1*-linked R recombinase was encoded by plasmid pHM153 (Matsuzaki *et al.*, 1990). *GPD*-linked topoisomerase I was encoded by YEptopA-GPD(TRP) (Giaever and Wang, 1988). The non-permissive temperature for *top2-4* is 35°C; otherwise, cells were grown at 26°C. The circularized excision cassette was visualized selectively by hybridization with a *LYS2*-specific radiolabeled probe.

quine (Peck and Wang, 1983). Under the conditions used, DNA topoisomers were resolved into an arc with negatively supercoiled topoisomers migrating in a broad region at the counter-clockwise end and positively supercoiled topoisomers coalescing predominantly in a spot at the clockwise end (Figure 2A). In each lane, DNA ring topology was ascertained by comparing the relative intensities of these two regions. Figure 2B shows that a DNA ring containing the telomere fragment became highly positively supercoiled in a yeast strain lacking endogenous topoisomerase activities but expressing *E.coli* topoisomerase I (lane 4). Positive supercoiling depended explicitly on the arrangement of topoisomerases. Expression of the negative supercoil-specific bacterial topoisomerase had little effect on the topology of rings in cells containing endogenous topoisomerase activities (Figure 2, lanes 1, 2 and 3) (Giaever and Wang, 1988; Gartenberg and Wang, 1992). If positive supercoils arose from

sequestration of DNA in a static macromolecular complex, such as wrapping right-handed DNA turns about a protein surface, they would have been refractory to changes in topoisomerase activities (see, for example, Lynch and Wang, 1994). Rather, the observed topoisomerase dependence is consistent with a build-up of twin-supercoiled domains on opposite sides of a barrier to DNA rotation. Our interpretation of these results is that telomeric DNA sequences are anchored; DNA supercoils cannot be dissipated by rotation of intervening telomeric $C_{1-3}A$ sequences.

DNA sequence requirements for telomeric DNA anchoring

To identify the sequences responsible for telomeric DNA anchoring, we created deletions in the telomeric DNA insert in pKWD130C. Previously, it was shown that the mitotic loss rates of plasmids containing telomeric sequences increased as the length of the $C_{1-3}A$ tract was shortened (Longtine *et al.*, 1992). pKWD130CΔ2 and pKWD130CΔ1 retain 186 and 49 bp of the original 369 bp $C_{1-3}A$ sequence array, respectively. The partial X repeat region is unperturbed in these clones. Excision experiments were performed in the double yeast topoisomerase mutant MRG6 which contains the *GAL1-R* recombinase fusion integrated at the chromosomal *top1* locus. The *GPD-topA* gene fusion was plasmid-borne. As shown in Figure 3, the extent of positive supercoiling decreased as the length of the $C_{1-3}A$ repeats decreased (lanes 2, 3 and 4). Moreover, a ring which contains 276 bp of $C_{1-3}A$ without associated X repeat sequence, rKWD-ST, also accumulated positive supercoils (lane 5). As in telomere-based plasmid partitioning, the role of the X repeat is not essential (Longtine *et al.*, 1992). The $C_{1-3}A$ arrays in rKWD-ST were derived from a source other than rKWD130C, indicating that $C_{1-3}A$ -based anchoring is a general phenomenon and not clone specific. A ring with no telomeric DNA, rKWD50N, was included as a control in lane 1; rKWD50N remains negatively supercoiled at 35°C. Taken together, these results show that $C_{1-3}A$ repeat sequence is both necessary and sufficient for positive supercoil accumulation in DNA rings. To strengthen this conclusion, time courses of positive supercoil accumulation were performed on selected substrates (Figure 3B). After shifting to non-permissive temperature for *top2-4*, the ring containing the intact $C_{1-3}A$ array, rKWD130C, accumulated positive supercoils more rapidly than the ring containing half of the original sequence, rKWD130CΔ2. The distribution of supercoils in rKWD130C at 45 min is comparable with the distribution in rKWD130CΔ2 at 90 min.

To test whether anchoring of telomeric sequences in *S.cerevisiae* is a property intrinsic to telomeric DNA, we examined a ring which contains ~300 bp of the C_4A_2/T_2G_4 telomeric repeat sequence of *Tetrahymena thermophila*. Lane 6 of Figure 3 shows that the *Tetrahymena* fragment is not sufficient for positive supercoil accumulation. *Tetrahymena* telomeric repeats also do not confer a partitioning phenotype to plasmids lacking other partitioning loci (Longtine *et al.*, 1992). We draw two conclusions from this result. First, immobilization is not caused by DNA structures intrinsic to all guanine-rich sequences. Second, immobilization probably involves *trans*-acting factors in yeast which do not recognize the *Tetrahymena* sequence (see below).

A

B

Fig. 3. Sequence requirements for positive supercoil accumulation in telomeric DNA rings. Experiments were performed in strain MRG6 [$\Delta top1::GAL1::R top2-4$] with *E.coli* topoisomerase I expressed from YEtopA-GPD(LEU). (A) DNA rings containing different telomeric inserts were examined with two-dimensional agarose gel electrophoresis. The 'rKWD' prefix is omitted in the figure. Lane 1, rKWD50N, contains no telomeric DNA; lane 2, rKWD130C, contains a 639 bp *Saccharomyces* telomeric insert composed of 369 bp of $C_{1-3}A$ and 270 bp of adjacent X repeat; lane 3, rKWD130CΔ2, retains 186 bp of $C_{1-3}A$ and 270 bp of adjacent X repeat; lane 4, rKWD130CΔ1, retains 49 bp of $C_{1-3}A$ and 270 bp of adjacent X repeat; lane 5, rKWD-ST, contains a 276 bp $C_{1-3}A$ tract; lane 6, rKWD C4A2, contains an ~300 bp *Tetrahymena* C_4A_2 telomeric insert. See Materials and methods for more detailed descriptions of the excision plasmids. (B) Time courses of positive supercoil accumulation. Top panel generated with ring rKWD130C; bottom panel generated with ring rKWD130CΔ2. The elapsed time after shifting from 26 to 35°C is posted above each lane.

DNA anchoring requires a limiting trans-acting factor

In principle, the inability of telomeric DNA to rotate axially could result from tertiary folding of the DNA or from direct association with other telomeric DNAs. If anchoring requires telomere-telomere associations, elevating the number of interaction sites should increase immobilization. Alternatively, anchoring of the $C_{1-3}A$ element could result from the binding of protein or RNA *trans*-acting factors which, in turn, attach to a large nuclear structure. If anchoring involves a limiting *trans*-acting factor, it might be possible to titrate it away from the assayable DNA ring. To test this possibility, we employed a series of dual selection plasmids as competitor DNAs (Stavenhagen and Zakian, 1994). These 2 μ m-based plasmids are maintained at high copy, 20–50 per cell, in media lacking histidine. Cells which carry the plasmid at a copy number 5- to 10-fold higher are selected when grown in media lacking leucine due to reduced expression of a plasmid-borne *leu2-d* allele (Erhart and Hollenberg,

1983). The four derivatives we used contain different amounts of $C_{1-3}A$ repeat sequence as competitor: FatRS303'b'-ST and FatRS303'b'-TT contain one and three copies of a 276 bp fragment of $C_{1-3}A$, respectively; FatRS303'b' contains no telomeric DNA; and a linear form of FatRS303'b', titled FatRS303'b'-lin which, by virtue of its linearity, contains two terminal $C_{1-3}A$ telomeric DNA tracts of ~400 bp each (see Materials and methods).

Experiments with dual selection plasmids were performed in a double topoisomerase mutant, MRG3, which contains both the *GAL1-R* recombinase and *GPD-topA* gene fusions integrated at chromosomal locations. Under histidine selection, the competitors had little effect on the topology of rKWD130CA2, regardless of the $C_{1-3}A$ content of the competitor (Figure 4A); the extent of positive supercoiling was comparable with cells which did not contain a dual selection plasmid (data not shown). When selection for leucine was enforced, alterations in DNA ring topology were observed (Figure 4B). In cultures bearing competitor plasmids with $C_{1-3}A$ segments, FatRS303'b'-ST, FatRS303'b'-TT or FatRS303'b'-lin, the topoisomer distribution of rKWD130CA2 shifted such that both negative and positive supercoiled rings were present. Time courses with these transformants revealed that the rate of positive supercoil accumulation was significantly reduced (data not shown). The dual selection plasmid without a $C_{1-3}A$ insert, FatRS303'b', had no effect on topology, even at amplified copy number. These data indicate that anchoring of telomeric DNA rings appears to require a factor (or factors) which can be titrated away by ultra-high copy plasmids containing $C_{1-3}A$ arrays.

The specificity of the competitor plasmids was examined by testing their effects on the topology of rKWD73C, a ring which contains a synthetic *REP3* locus. Anchoring of the *REP3* sequence occurs in cells that bear *REP1* and *REP2*, genes encoded by the resident 2 μ m plasmid which is present in all of the strains used here (Gartenberg and Wang, 1993). Figure 4C shows that rKWD73C attained the same level of positive supercoiling, albeit low, whether FatRS303'b' or FatRS303'b'-TT was present as competitor (lanes 3 and 4). For comparison, lanes 1 and 2 reiterate that FatRS303'b'-TT greatly reduces the level of positive supercoiling of rKWD130CA2 whereas FatRS303'b' has no effect. Clearly, $C_{1-3}A$ -containing competitor plasmids titrate out a limiting factor involved in anchoring telomeric DNA rings but not *REP3*-based rings.

Rap1p binding sites are sufficient for DNA anchoring

We created artificial telomeric sequences with a family of related synthetic oligonucleotides to further refine the DNA requirements for telomeric DNA anchoring. The prevalence of Rap1p binding sites in telomeric DNA suggested that these sequence elements should be examined directly. pKWD1CA13 and pKWD3CA13 contain one and three copies, respectively, of a sequence used originally to characterize the DNA binding activity of Rap1p in crude extracts (Buchman *et al.*, 1988). The oligonucleotide contains three potential overlapping Rap1p binding sites (Table I). Positive supercoils accumulate in both the rKWD1CA13 and rKWD3CA13 rings, albeit less efficiently in the ring with only one copy of the

Fig. 4. Ultra-high copy number competitors diminish the accumulation of positive supercoils in DNA rings containing telomeric DNA. (A) rKWD130CA2, which contains 186 bp of $C_{1-3}A$ repeats, was used as the telomeric ring. Experiments were performed with strain MRG3 [Δ lys2::GPD-topA Δ top1::GAL1::R top2-4] transformed with the dual-selection plasmid indicated above each lane. The FatRS303 prefix is omitted in the figure. Cultures were grown in SD -ura,his. (B) Same as (A) except that cultures were grown in SD -ura,his,leu. (C) Competitor plasmids reduce positive supercoil accumulation in telomeric rings, specifically. Topoisomer distributions of rKWD130CA2 in the presence of competitors FatRS303'b' and FatRS303'b'-TT are shown in the first two lanes. Topoisomer distributions of a ring containing a synthetic *REP3* variant, rKWD73C, are shown in the second two lanes. Cultures were grown in SD -ura,his,leu. Chromosomal integration of *GPD-topA* accounts for the low level of positive supercoiling in rKWD73C; when *GPD-topA* is on a multi-copy plasmid, rKWD73C becomes highly positively supercoiled (Gartenberg and Wang, 1993; A.Mirabella, unpublished results).

oligonucleotide (Figure 5, lanes 3 and 4). The specificity of oligonucleotide-based anchors was tested by constructing excision substrates with mutations in critical residues in the Rap1p binding sites. pKWD3CA3 contains three copies of an oligonucleotide with a C→A change in the central base pair of the 5'-CCC-3' core of each Rap1p binding site. The change abolishes Rap1p binding *in vitro* (Buchman *et al.*, 1988). Lane 2 of Figure 5 shows that positive supercoil accumulation in rKWD3CA3 is negligible. The structural difference between the wild-type and mutant rings (rKWD3CA13 and rKWD3CA3, respectively) is striking since both rings have similar nucleotide composition, varying in sequence at only nine positions (three C→A changes per oligonucleotide unit). The absence of anchoring is comparable with rKWD50N

Table I. Oligonucleotide inserts in excision substrates

Plasmid	Oligo name	Sequence	No. of copies
pKWD1CA13	CA13	5'-CCGGC <u>ACACCCACACACC</u> ACACACCCACACACCCAC-3'	1
	TG13	3'-GTGTGGGTGTGTGGGTGTGTGGGTGTGGGTGCCGG-5'	1
pKWD3CA13	CA13	5'-CCGGC <u>ACACCCACACACC</u> ACACACCCACACACCCAC-3'	3
	TG13	3'-GTGTGGGTGTGTGGGTGTGTGGGTGTGGGTGCCGG-5'	3
pKWD3CA3	CA3	5'-CCGGC <u>ACACACACACACACACACACACACACCC</u> AC-3'	3
	TG3	3'-GTGTGTGTGTGTGTGTGTGTGTGTGTGTGGGTGCCGG-5'	3
pKWD1CA2	CA2	5'-CCGGC <u>ACACACACACACACACACCCACACACCC</u> AC-3'	1
	TG2	3'-GTGTGTGTGTGTGTGTGTGTGTGTGTGGGTGCCGG-5'	1

Locations of potential RAP1 binding site sequences are underlined and boldface base pairs represent locations of sequence changes between oligonucleotides.

Fig. 5. Two-dimensional agarose gel electrophoresis with DNA rings containing Rap1p binding sites. Experiments were performed in MRG6 [$\Delta top1::GAL1::R top2-4$] with *E.coli* topoisomerase I expressed from YEtopA-GPD(LEU). The 'rKWD' prefix is omitted from ring designations in the figure. Lane 1, rKWD50N, contains no insert; lane 2, rKWD3CA3, contains three copies of the CA3-TG3 oligonucleotide pair; lane 3, rKWD3CA13, contains three copies of the CA13-TG13 oligonucleotide pair; lane 4, rKWD1CA13, contains one copy of the CA13-TG13 oligonucleotide pair; lane 5, rKWD1CA2, contains one copy of the CA2-TG2 oligonucleotide pair. A small portion of an omitted lane partially obscures the relaxed topoisomer region of this arc. See Table I or the text for description of the oligonucleotides.

which contains no cloned inserts (Figure 5, lane 1). DNA anchoring is not a trivial consequence of protein-DNA interactions involving sequence-specific binding proteins. We have tested this possibility using full-length *lexA* and *lexA* operators. The topology of rings containing *lexA* operators is unaffected by *lexA*, even when six high affinity sites are present (A.Ansari and M.R.Gartenberg, unpublished data).

Varying the number of Rap1p binding site oligonucleotides established that anchoring is detected more readily in rings with greater numbers of binding sites. To extend this observation, we examined a ring with a single Rap1p binding site. rKWD1CA2 contains a single oligonucleotide insert, in which two of the three Rap1p binding sites are mutated. The remaining Rap1p site was not sufficient for positive supercoil accumulation (Figure 5, lane 5). Taken together, these data infer that multiple Rap1p binding site sequences are important for telomeric DNA anchoring. It is tempting to speculate that multiple Rap1p molecules are a primary component of the anchoring complex.

Telomere position effect and peripheral nuclear localization of Rap1p are not prerequisites for telomeric DNA anchoring

The experiments with competitor plasmids indicate that a limiting *trans*-acting factor is required for telomeric DNA

anchoring, and the experiments with synthetic $C_{1-3}A$ sequences implicate Rap1p as a primary candidate. To test the importance of *RAP1*, we deleted the chromosomal *RAP1* locus in MRG15 and provided either the wild-type gene or the *rap1-10* allele on a CEN plasmid (Moretti *et al.*, 1994). *rap1-10* is a C-terminal truncation which retains the essential DNA binding and transcriptional activation domains of the gene but lacks the coding region beyond amino acid 695 required for transcriptional silencing. Cells bearing this allele neither repress modified *HMR* loci nor silence genes located near telomeres. Telomere lengths also elongate dramatically (data not shown). Moreover, similar though non-identical truncation alleles (*rap1'* alleles) result in the appearance of non-perinuclear Rap1p clusters in a subset of cells (Cockell *et al.*, 1995). Anchoring of rKWD130CA2 was tested in either *RAP1* or *rap1-10* strains. As shown in Figure 6, exchanging the *rap1* alleles had no effect on the topology of a ring containing telomeric DNA. Ring rKWD50N was included as a negative control. Two simple explanations may account for this result. Anchoring via Rap1p may involve domains other than the C-terminal 132 amino acids. Alternatively, unidentified factors other than Rap1p may bind to and immobilize telomeric DNA *in vivo*.

The observation that anchoring occurs in the absence of the Rap1p silencing domain suggests that transcriptional silencing is not a prerequisite for telomere immobilization. To develop this notion further, we tested DNA anchoring in strains with wild-type *RAP1* but mutant alleles of *SIR2*, *SIR3* and *SIR4*, genes involved in transcriptional repression at telomeres and the perinuclear localization of telomere-bound Rap1p (Palladino *et al.*, 1993). An expanding field of evidence suggests that the *SIR* gene products are chromatin-associated proteins which are targeted to telomeres by interacting with other DNA-bound factors. Sir3p interacts directly with Rap1p, co-immunoprecipitates Sir4p, Rap1p and histones from cell extracts, and cross-links to telomeric DNA *in vivo* (Moretti *et al.*, 1994; Hecht *et al.*, 1996). Sir4p co-immunoprecipitates Rap1p and forms a complex with Sir2p (Cockell *et al.*, 1995; Moazed and Johnson, 1996). In addition, *SIR2* plays an undetermined role in histone deacetylation, which may account for the reduced acetylation state of histones at the silent mating-type loci and sub-telomeric regions (Braunstein *et al.*, 1993). Single disruptions of *SIR2*, *SIR3* and *SIR4*, as well as a pair-wise disruption $\Delta sir3/\Delta sir4$, were made in strain MRG6. Though the deletions do not remove all of the coding sequence (see Materials and

Fig. 6. The *RAP1* C-terminal domain is not required for positive supercoil accumulation in telomeric DNA rings. Experiments were performed in MRG15 [$\Delta top1::GAL1::R top2-4 \Delta rap1::LEU2$] transformed with YEtopA-PGPD(TRP). The strain also contained a CEN plasmid with either the *rap1-10* or *RAP1* alleles. *rap1-10* lacks the sequence encoding the C-terminal 132 amino acids. Lanes 1 and 3, rKWD130CΔ2, which contains 186 bp of C₁₋₃A repeats. Lanes 2 and 4, rKWD50N which contains no insert. The *rap1-10* strains grew more slowly and acquired abnormally long telomeres (data not shown).

methods), the alleles abolished the mating pheromone response in MRG6 and led to a subtle shortening of telomere length, as reported previously (Marshall *et al.*, 1987; Palladino *et al.*, 1993). None of these mutations had a significant effect on topology of rKWD130CΔ2 in the anchoring assay (data not shown). These results indicate that telomeric DNA is immobile even in the absence of the *SIR* genes. The *SIR* independence of telomeric DNA anchoring parallels the lack of a requirement for the *SIR* genes in the partitioning of plasmids containing C₁₋₃A arrays (Enomoto *et al.*, 1994). Consistent with the *rap1-10* experiment, these data show that the components for transcriptional silencing are not prerequisites for DNA anchoring.

Discussion

DNA anchors: barriers to DNA rotation

Association of DNA segments with nuclear matrices or nuclear scaffolds has, to date, been the experimental hallmark of loop attachment sites in spite of the fact that the residual nuclear structures are the products of disruptive biochemical procedures. In order to identify sites which demarcate DNA domains *in vivo*, we used the functional criterion that sequences of interest block the propagation of supercoils. This definition is significantly more broad than the notion of a loop attachment site, since it includes all immobile DNA sequences; anchoring may result from association of DNA with the intracellular counterpart to the nuclear matrix or to any other nuclear assembly too large to rotate about DNA (e.g. the nuclear lamina). In our experimental system, anchors prevent the cancellation of supercoiled regions of opposite sign by restricting the rotational freedom of DNA. We show here that *Saccharomyces* telomeric DNA is rotationally immobile, anchored, *in vivo*. We anticipate that telomeres are members of a diverse class of DNA sequences and associated proteins that are unified by the ability to create topological boundaries in DNA. In native chromosomes, DNA supercoiling near chromosome ends might be stabilized

by telomere immobilization; without DNA anchors, supercoils would dissipate out of the terminal regions through the swivel action of DNA ends. A rigorous search of contiguous chromosomal sequences for DNA anchors will establish the frequency with which independent supercoiling domains occur in the *Saccharomyces* genome. Some anchors may form reversibly, under specific conditions or at specific times during the cell cycle. Conditional anchors might be used as regulatory switches to attenuate DNA-templated processes by preserving localized changes in DNA supercoiling. For example, DNA rotation barriers which prevent the dissipation of negative supercoils would favor DNA strand separation in adjacent promoters. Conversely, the accumulation of positive supercoils would antagonize local transcription (Gartenberg and Wang, 1992).

Telomeric DNA anchoring and plasmid partitioning

Berman and co-workers have shown that tracts of the telomeric C₁₋₃A repeat improve the segregation of circular ARS plasmids in dividing cells (Longtine *et al.*, 1992, 1993; Enomoto *et al.*, 1994). Here we establish that C₁₋₃A tracts are also necessary and sufficient for telomeric DNA anchoring. Whether X repeats augment C₁₋₃A-based anchoring, as they do C₁₋₃A-based partitioning, was not determined. However, in the absence of the X repeat, neither process is influenced by the *SIR* genes. These correlations help delineate between active and passive transport mechanisms for partitioning telomere-based plasmids. It seems reasonable that both plasmid segregation and DNA anchoring arise from the interaction of C₁₋₃A repeats with a nuclear component large enough to prevent the axial rotation of DNA. Under this constraint, plasmid transport would be achieved by active distribution of the plasmid attachment sites between dividing cells. Models in which plasmids segregate passively by diffusion are not consistent with DNA immobilization. The *REP* partitioning components of the 2 μm plasmid form a DNA anchor, indicating that segregation of the endogenous extrachromosomal element also involves an active transport process (Gartenberg and Wang, 1993).

Earlier, it was shown that human telomeres bind the nuclear matrix but that short internal telomeric tracts do not (de Lange, 1992). It was argued that proximity to a DNA end was critical for matrix association. In contrast, internal tracts of yeast telomeric sequence suffice for DNA anchoring and telomere-based plasmid partitioning. Both processes are dependent on telomere tract length, with longer sequences yielding enhanced DNA immobilization and symmetric plasmid segregation. Binding of human telomere tracts to the nuclear matrix could be length dependent as well; differences in the sizes of the telomere sequences examined previously could account for the observed binding specificity (telomeres averaged 6 kb whereas internal tracts were 800 bp).

On the role of DNA structure in telomeric DNA anchoring

G-rich telomeric strands from a variety of organisms, including *S.cerevisiae*, adopt four-stranded structures, quadruplexes, which utilize the G-quartet base pairing scheme (Venczel and Sen, 1993; Williamson, 1994). Though quadruplexes have not been detected *in vivo*, the

novel nucleic acid conformation has been implicated in the dimerization of retroviral RNA genomes and pairing chromosomes for recombination at meiosis, as well as telomere–telomere associations (Sen and Gilbert, 1988; Sundquist and Heaphy, 1993). Telomeric anchoring detected here is not likely to result from intramolecular or intermolecular folding of telomeric sequences. We have shown that the telomere repeat of *T.thermophila* is not anchored in *Saccharomyces* even though the sequence forms quadruplexes readily *in vitro* (Williamson *et al.*, 1989). In addition, we have shown that excess *Saccharomyces* telomeric DNA in the form of extrachromosomal competitors reduces the effectiveness of telomeric DNA anchoring. If immobilization were the result of direct DNA–DNA contact, we would have expected the competitor to increase the efficiency of the phenomenon by providing more opportunities for pairing. Telomeric anchoring most likely results from the association of a sequence-specific DNA binding factor, and not from the formation of an intrinsic telomeric DNA structure.

In *Saccharomyces*, either the lengthening of the TG_{1–3} strand and/or the shortening of the C_{1–3}A strand occurs late in S phase to yield single-stranded tails spanning at least 30 bp (Wellinger *et al.*, 1993, 1996). The single-stranded regions promote circularization of linear plasmids via non-covalent interactions. That telomeric anchoring was detected in DNA rings indicates that DNA ends are not essential for immobilization. This point is recapitulated by the ability of internal C_{1–3}A arrays in circular plasmids to function effectively as competitors. We have not ruled out the remote possibility that the telomeric rings contain significant single-stranded regions *in vivo*. However, overwinding DNA by the accumulation of positive supercoils would be expected to antagonize any structure requiring strand separation.

On the role of Rap1p in telomeric DNA anchoring

Excess telomeric sequences were shown previously to increase the steady-state length of C_{1–3}A repeats at the ends of chromosomes (Runge and Zakian, 1989). Here we show that extra C_{1–3}A sequences, in the form of telomeres on linear plasmids or as inserts in circular plasmids, diminish anchoring of telomeric DNA rings specifically. These results indicate that telomeric anchoring and length control are mediated by at least one limiting *trans*-acting factor. Rap1p is a strong candidate, due to the preponderance of Rap1p binding sites in telomeric C_{1–3}A arrays. Using oligonucleotides, we demonstrated that short multimers of synthetic Rap1p binding sites were sufficient for immobilization of DNA rings. Alteration of bases critical for Rap1p binding *in vitro* abolished intracellular anchoring. We anticipate that telomeric anchoring is limited to telomeres and other chromosomal locations where Rap1p binding sites occur multiple times in close proximity.

Rap1p associates with a large number of other proteins to either activate or repress transcription, depending on the context of the Rap1p binding site (Shore, 1994). Two *RAP1*-interacting factors involved in transcriptional repression at telomeres, *SIR3* and *SIR4*, were identified by the two-hybrid assay and confirmed by biochemical methods (Moretti *et al.*, 1994; Cockell *et al.*, 1995). Given the scope of telomere functions mediated by the *SIR*

genes, we were surprised to find that telomeric DNA was immobilized equally well in strains with intact and defective *sir* alleles. The demonstration that telomeric anchoring persisted in the absence of the Rap1p C-terminal domain reinforced these results; the C-terminal domain is required for transcriptional silencing and contains the relevant contacts for association with *SIR3* and *SIR4*. These data suggest that transcriptional silencing is not a prerequisite for DNA anchoring. Other factors which interact with *RAP1* through the C-terminal domain, such as *RIF1* (Hardy *et al.*, 1992), are thus equally unlikely to serve as mediators of a Rap1p-based DNA anchor.

Recently, Wiley and Zakian (1995) showed that transcriptional silencing at telomeres was abolished by linear competitor plasmids but not circular competitors with C_{1–3}A inserts. The results suggest that a DNA end binding factor is involved in transcriptional repression. It seems unlikely that the same factor is involved in DNA anchoring, since both circular and linear C_{1–3}A-containing competitor plasmids reduce positive supercoil accumulation. Though the linear competitor appears to be slightly more effective in time course analyses (data not shown), quantitation of the differences between competitors would require an exact measure of the changes in supercoil distribution, and the total length of competing episomal and chromosomal C_{1–3}A tracts. We doubt that a sufficiently rigorous numerical analysis can be performed with our current data.

DNA anchoring and other functions of telomeres

The role of telomeric DNA anchoring in chromosome function is not clear at present; however, we envisage the following possibility. Aspects of telomere metabolism, such as protection of chromosome ends from degradation, might occur only on a specialized macromolecular assembly. Targeting telomeres to available sites on the structure would result in immobilization of C_{1–3}A sequences. A critical feature of the model is that sites on the structure are not created by other telomeric sequences alone; otherwise, telomeric DNA anchoring would have been enhanced by the addition of ultra-high copy competitors, not diminished as was observed. The competitor plasmids most likely saturate sites on the structure or titrate out a protein critical for linkage, such as Rap1p, a Rap1p interacting factor or an unidentified cellular factor which also binds Rap1p sites. The telomere-containing assembly may be part of an immobile nuclear network, or may simply be too large to rotate freely in the intracellular milieu. Evidence of DNA anchoring alone cannot discern between these two possibilities. However, *in situ* hybridization data indicate that telomeres are held together in clusters in the interphase nucleus and the combined use of immunofluorescence and *in situ* hybridization showed that Rap1p, Sir3p and Sir4p are localized to these telomere clusters (Gotta *et al.*, 1996). If our anchoring results accurately reflect telomere positioning, then telomere clusters should persist despite loss of the Sir proteins. Indeed, deletion of *SIR3* or *SIR4* results in the dispersion of Rap1p but not the underlying clusters of telomere sequences (Gotta *et al.*, 1996). Further work will be required to identify the structural components to which telomeres are attached.

Materials and methods

Plasmid constructions

Plasmids were generously provided by the following investigators: J.Berman (University of Minnesota), D.Shore (Columbia University),

Table II. Yeast strains

Strain	Genotype	Source
FY251	<i>MATa his3-Δ200 leu2-Δ1 trp1-Δ63(GAL3) ura3-52</i>	F.Winston
JCW26	FY251 <i>top2-4</i>	J.C.Wang
JCW27	FY251 <i>Δtop1-h&r</i>	J.C.Wang
JCW28	JCW26 <i>Δtop1-h&r</i>	J.C.Wang
MRG6	JCW26 <i>Δtop1::GAL1::R</i>	this study
MRG6α	MRG6 <i>MATα</i>	this study
MRG3	MRG6 <i>Δlys2::GPD-topA</i>	this study
MRG12	FY251×MRG6α	this study
MRG12R	MRG12 <i>Δrap1::LEU2</i>	this study
MRG15	<i>MAT(a) his3-Δ200 leu2-Δ1 trp1-Δ63(GAL3) ura3-52 Δtop1::GAL1::R top2-4Δrap1::LEU2</i> with pCEN-HIS3-RAP1 or pCEN-HIS3-rap1-10	this study
MRG231	MRG6 <i>sir2::TRP1</i>	this study
MRG331	MRG6 <i>Δsir3::HIS3</i>	this study
MRG431	MRG6 <i>Δsir4::HIS3</i>	this study
MRG341	MRG331 <i>Δsir4::TRP1</i>	this study

J.Broach and V.Zakian (Princeton University), Y.Oshima (Osaka University) and J.Wang (Harvard University). The pKWD plasmid series described here is based on the recombination substrate vector pKWD50N which was derived from pKWD50 (Gartenberg and Wang, 1993) by the replacement of the *XhoI*-*SacII* fragment with a decameric *NotI* linker. The pKWD derivatives differ by the insertion of a DNA fragment in the *NotI* site. In pKWD plasmids which contain telomere-derived or telomere-like sequences, the TG-rich strand coincides with the coding strand of *URA3*. pYet1 contains the telomere from the left arm of chromosome III (Longtine *et al.*, 1992). To make pKWD130C, a 639 bp *BamHI*-*AccI* fragment from pYET1 was transferred to pKWD50N. Long tracts of *C*₁₋₃A are unstable in *E.coli* (Longtine *et al.*, 1992). After serial passaging of pKWD130C in DH5α, two spontaneous deletion derivatives, pKWD130Δ1 and pKWD130Δ2, were obtained which retained 49 and 186 bp of *C*₁₋₃A, respectively, as well as the adjacent sub-telomeric DNA. Sequences were determined by the chain termination method and are available upon request. FatRS303'b'-ST (Stavenghagen and Zakian, 1994) contains a 276 bp tract of *C*₁₋₃A in a polylinker. To make pKWD-ST, an ~400 bp *SmaI*-*NotI* fragment containing the *C*₁₋₃A sequence was transferred to pKWD50N. pTTCA-2 (Runge and Zakian, 1989) contains ~300 bp of the *Tetrahymena* telomeric T₂G₄/C₄A₂ repeat in a polylinker. To make pKWD4A2, an ~350 bp *HindIII*-*NotI* fragment containing the C₄A₂ sequence was transferred to pKWD50N. The ligation mixture was transformed directly into yeast strain MRG6, and candidate clones were identified by restriction digestion and Southern hybridization. Subsequently, candidates were transferred to DH5α and confirmed by partial sequencing. pKWD1CA13, pKWD3CA13, pKWD3CA3 and pKWD1CA2 were constructed by cloning oligonucleotides into the *NotI* site of pKWD50N (see Table I). Oligonucleotides CA13 and TG13 were ligated to *NotI*-linearized FatRS303'b' and transformed into yeast to generate FatRS303'b'-lin, as described in published procedures (Runge and Zakian, 1989). Monomeric linear versions of the plasmid with ~400 bp of *C*₁₋₃A repeat on each terminal end were identified by restriction digestion and Southern blotting. To make pΔlys2::GPD-topA, the *BglII*-*XhoI* fragment of *LYS2* in pUC18-*LYS2* was replaced with a 4.3 kb *EagI* fragment from YEtopA-PGPD(TRP) (Giaever and Wang, 1988), which contains the coding region of the *E.coli topA* gene linked to the yeast *GPD* promoter. In the resulting construct, the *LYS2* and *GPD* promoters are oriented in opposite directions.

Strain constructions

The yeast strains used in this study are listed in Table II. All strains were derived from FY251 (F.Winston, personal communication). JCW26 was derived directly by two-step gene replacement, and was kindly provided by R.Kim and J.C.Wang (Harvard University). JCW27 and JCW28 were derived from FY251 and JCW26, respectively, by the hit-and-run method of gene replacement (Roca *et al.*, 1992), and were kindly provided by J.Roca and J.C.Wang (Harvard University). MRG6 was derived from JCW26 by two sequential rounds of single-step gene replacement (Gartenberg and Wang, 1993). MRG3 was derived from MRG6 by single-step gene replacement of *LYS2* with a *GPD* promoter-

linked *E.coli topA* gene using plasmid pΔlys2::GPD-topA. *sir2::TRP1*, *Δsir3::HIS3* and *Δsir4::HIS3* derivatives of MRG6 were made by single-step replacement using plasmids C369 (Shore *et al.*, 1984), psir3::HIS3 and pMM10.7 (Marshall *et al.*, 1987), respectively. The *SIR3* disruption plasmid contains a *HIS3* insertion between *BglII* and *XhoI* sites which removes the coding sequence from amino acid 108 to 946 (D.Shore laboratory, personal communication). MRG12 was generated by crossing FY251 with the *MATα* version of MRG6. A *Δrap1::LEU2* derivative of MRG12, MRG12R, was made with a *rap1* disruption plasmid (Moretti *et al.*, 1994). MRG15 was obtained by tetrad dissection of MRG12R transformed with *RAP1* on a CEN vector. All gene disruptions were confirmed by Southern hybridization.

Formation of intracellular DNA rings, inactivation of yeast DNA topoisomerase II and competition experiments

Procedures for the creation of DNA rings by site-specific recombination and inactivation of yeast DNA topoisomerase II are essentially the same as described previously (Gartenberg and Wang, 1993) with the following exception. Galactose inductions in MRG6 were performed for 2.5 h to yield nearly complete excision. The length of time at 35°C, a non-permissive temperature for *top2-4*, was 90 min unless specified otherwise. For competition experiments, transformants of MRG3 containing pKWD130Δ2 and one of the competitor plasmids were obtained on SD -ura,his. To select for cells with the competitor plasmid at high copy number, transformants were restreaked on SD -ura,leu. Cells were passaged on this medium for ~100 generations to allow chromosomal telomeres to reach new steady-state lengths (Runge and Zakian, 1989). Effects on telomere length were confirmed by Southern hybridization. To perform the anchoring experiment, liquid cultures were grown in SD -ura,his for high copy number competitor and SD -ura,leu,his for ultra-high copy number.

DNA isolation and two-dimensional agarose gel electrophoresis

Procedures are similar to those used by Gartenberg and Wang (1993) with the following exceptions. After RNase treatment, samples were phenol extracted twice, ethanol precipitated and resuspended in 40 μl of 10 mM Tris-HCl, pH 8.0/1 mM EDTA. 25% of the sample was used for loading a single lane of a two-dimensional gel. Autoradiograms were cropped to only show DNA rings. Autoradiograms were scanned with a Microtek 600G scanner to generate figures for publication.

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