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Telomere-proximal DNA in *Saccharomyces cerevisiae* is refractory to methyltransferase activity *in vivo*

(position effect/yeast/Dam DNA methyltransferase/heterochromatin/chromosome structure)

DANIEL E. GOTTSCHLING

Department of Molecular Genetics and Cell Biology, The University of Chicago, 920 East 58th Street, Chicago, IL 60637

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ABSTRACT **Genes located near telomeres in *Saccharomyces cerevisiae* undergo position-effect variegation; their transcription is subject to reversible but mitotically heritable repression. This position effect and the finding that telomeric DNA is late replicating suggest that yeast telomeres exist in a heterochromatin-like state. Mutations in genes that suppress the telomeric position effect suggest that a special chromatin structure exists near chromosomal termini. Thus transcriptional repression may be explained by the inability of DNA binding proteins to access the DNA near telomeres. To test this hypothesis, the *Escherichia coli* Dam DNA methyltransferase, which modifies the sequence GATC, was introduced into *S. cerevisiae* cells. DNA sequences near the telomere were highly refractive to Dam methylation but were modified when located at positions more internal on the chromosome. Telomeric sequences were accessible to methyltransferase activity in strains that contained a mutation that suppressed the telomeric position effect. These data support the model that sequence-specific DNA binding proteins are excluded from telomere-proximal sequences *in vivo* and show that expression of DNA methyltransferase activity may serve as a useful tool for mapping chromosomal structural domains *in vivo*.**

Genes located near telomeres in *Saccharomyces cerevisiae* undergo position-effect variegation; their transcription is subject to reversible but mitotically heritable repression (1). This position effect and the finding that telomeric DNA replicates late in S phase (2) suggest that yeast telomeres exist in a heterochromatin-like state. Heterochromatin is defined cytologically as a region of the chromosome that shows maximal condensation during interphase. Although current methods of microscopy have not identified heterochromatin in *S. cerevisiae*, cytological observations in a number of other eukaryotes indicate that telomeric regions are indeed heterochromatic (3–7).

Recently, it has been shown that mutations in *SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, or *HHF2* completely relieve silencing at the yeast telomere (8). These results suggest that a special chromatin structure is present near chromosomal termini. This special structure, which is the likely cause of transcriptional repression, may preclude most DNA binding proteins from accessing the DNA near telomeres. Hence, sequence-specific factors necessary for transcription (e.g., TFIID) may not be able to interact with promoters of genes located near telomeres.

Unlike the DNA of most eukaryotes, the genomic DNA of wild-type *S. cerevisiae* is not methylated to a detectable level (9, 10). Nonetheless, *in vivo* expression of any of several prokaryotic DNA methyltransferase genes in *S. cerevisiae* results in sequence-specific methylation of yeast nuclear DNA (11–14). For instance, expression of the *Escherichia coli dam* gene results in methylation of GATC sequences in the yeast nucleus; the N-6 position of adenine is methylated and methylation can be detected on isolated DNA by sensitivity to the restriction enzyme *Dpn* I (11, 12). *Dpn* I cleaves GATC sequences only when the adenines on both DNA strands are methylated (15). The expression of Dam methyltransferase in yeast does not affect cell viability and has a very modest effect on mitotic recombination rates (12). The innocuous effect of Dam methylation on yeast cells may be explained by the observation that methylation of adenines does not alter DNA conformation (16). In addition GATC sites in the *S. cerevisiae* genome, which occur ≈1 in 300 base pairs, may not overlap with target sequences of essential DNA binding proteins (17). Nonetheless, the frequency of GATC sites should occur at least once in most genes or chromosomal domains.

It had been suggested that *in vivo* DNA methyltransferase activity might be useful as a probe for chromosome structure (12–14). In these earlier studies only transcriptionally active loci were examined in detail, and although variations in levels of DNA methylation were observed, it was concluded that incomplete methylation of a sequence was dependent on the relative efficacy of yeast DNA excision repair functions, which removed the methylated adenines (e.g., *RAD1* and *RAD3*) (18, 19). Recently, however, Singh and Klar* found greater methyltransferase accessibility *in vivo* within transcriptionally active genes relative to the same transcriptionally inactive genes.

It remained possible that DNA near telomeres might be refractory to the Dam methyltransferase activity if telomeric chromatin somehow prevented interaction of this sequence-specific DNA binding protein with its target site. To test this idea, the *URA3* gene, which contains a single GATC site within its coding sequence, was introduced adjacent to a telomere (1, 20). This GATC site was then examined for methylation *in vivo* in yeast cells expressing the *dam* gene. The telomeric GATC site was highly refractory to Dam methylation, but when *URA3* was at its normal chromosomal location the GATC site was methylated in all cells of a culture. Furthermore, the telomeric GATC was completely accessible to methyltransferase activity in strains that contained a mutation that relieved the telomeric position effect (8). These data indicate that sequence-specific DNA binding proteins are excluded from telomere-proximal sequences *in vivo* and provide a basis for understanding the mechanism of transcriptional repression near yeast telomeres. In addition, the data show that *in vivo* expression of DNA methyltransferase activity may serve as a useful tool for mapping chromosomal structural domains.

MATERIALS AND METHODS

***E. coli* Plasmids.** Plasmid pDP6-dam contained the *S. cerevisiae LYS2* gene and the *E. coli dam* gene. It was

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constructed by digesting plasmid pDP6 (21) with *Sma* I and ligating the 1.5-kilobase-pair *Hin*dIII–*Pvu* II DNA fragment from pMFH1 (12) (a gift from R. Malone, University of Iowa, Iowa City) that encoded the *dam* gene, after the ends of the fragment had been made blunt-ended with T4 DNA polymerase treatment. All other plasmids used for strain constructions have been described (1).

Yeast Strains. The two parents for all strains used in this study were YPH250 (*MATa ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) and YPH102 (*MATα ura3-52 lys2-801 ade2-101 his3-Δ200 leu2-Δ1*) (22). UCC1001 had *URA3* at the VII-L telomere and was created by transformation of YPH250 with pVII-L URA3-TEL as described (1). UCC1021 and UCC1023 were made *dam*$^+$ by transformation of UCC1001 and YPH250, respectively, with pDP6-dam that was digested with *Xho* I, selecting for *LYS*$^+$ colonies and screening for Dam methylase activity as determined by sensitivity of isolated genomic DNA to *Dpn* I cleavage. UCC1024 was derived from UCC1021 and was made *rad1*::*LEU2* with plasmid RR46 (a gift from R. Schiestl, University of North Carolina, Chapel Hill) as described (23); these strains were UV-sensitive. UCC1042, UCC1044, and UCC1045 were derivatives of UCC1021 that were *sir2*::*HIS3*, *sir1*::*HIS3*, and *sir4*::*HIS3*, respectively, and were created by transformation as described (24).

Strain UCC1058 was a derivative of YPH102 that had been made *dam*$^+$ and *rad1* as described above. The *ura3-52* locus of YPH250 was converted to *URA3*$^+$ by transformation to create UCC1004, as described (8). UCC1004 was then made *dam*$^+$ to create UCC1059.

Analysis of Dam Methylation Sites. *S. cerevisiae* cells were grown in 5 ml of YEPD to stationary phase (25), and total genomic DNA was isolated by disrupting cells with glass beads as described (26). Six units of *Dpn* I (Boehringer Mannheim) and/or other restriction enzymes were incubated with 10% of the isolated DNA sample in a buffer of 20 mM Tris·HCl (pH 7.5), 70 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 50 μM spermine, 12.5 μM spermidine, 1:10,000 (vol/vol) aprotinin (Sigma), 10 mM dithiothreitol, and 0.05% digitonin (Sigma). Incubation was at 37°C for at least 8 h in a total reaction volume of 30 μl. Gel electrophoresis and Southern blot hybridization were performed as described (8).

RESULTS

The *URA3* gene was placed adjacent to the terminus of the left arm of chromosome VII to serve as a marker of telomere-adjacent sequence (1). The *URA3* gene contains a single GATC site within its coding sequence (20) and is subject to position effects when located near a chromosomal terminus (1). The position effect is manifested as transcriptional repression of *URA3*. The repression is phenotypically analyzed by a cell's resistance to the drug 5-fluoroorotic acid (1, 27). Within experimental error, little or no difference in the level of repression on the telomeric *URA3* gene was observed between cells that did or did not express the *dam* gene (Table 1).

Table 1. Effect of Dam methyltransferase activity *in vivo* on telomeric transcriptional repression in *S. cerevisiae*

Strain	Genotype	Fraction of 5-FOA-resistant colonies
UCC1001	Parent	0.55 (±0.15)
UCC1021	*dam*$^+$	0.26 (±0.17)
UCC1024	*dam*$^+$*rad1*	0.35 (±0.26)

Complete genotype of each strain can be found in *Materials and Methods*. The fraction of 5-fluoroorotic acid (5-FOA)-resistant colonies for each strain was determined as described (1), and the mean of four determinations along with the standard deviation (in parentheses) is presented.

Marking the telomere with *URA3* also facilitates the unequivocal examination of a single telomere. Normally, the DNA adjacent to the telomere repeat sequence $(TG_{1-3})_n$ is composed of nonessential middle-repetitive elements such as X and Y′ sequences; up to four tandem repeats of the 6.7-kilobase-pair Y′ sequence have been observed adjacent to a chromosomal end (28–31). In the strains used in this study, these nonessential repetitive elements adjacent to the VII-L telomere have been replaced by *URA3* (1).

The *dam* gene was integrated at the *lys2* locus in a set of congenic yeast strains. These strains contained the *ura3-52* allele, which is a Ty transposon inserted within the coding sequence of *URA3* at its normal chromosomal locus (32), and a wild-type *URA3* gene located adjacent to the VII-L telomere (1). Cells were grown to stationary phase under nonselective conditions, and total genomic DNA was isolated and digested with the restriction enzymes *Bam*HI and *Hin*dIII to resolve a fragment length polymorphism between the *ura3-52* allele and the telomeric *URA3* gene (Fig. 1). The DNA probe indicated in Fig. 1 spans the GATC site at both locations and was used in DNA blot hybridization analysis to detect the unique restriction fragments produced at *ura3-52* and the telomeric *URA3*, both before and after *Dpn* I cleavage (Fig. 1).

When the *dam* gene was expressed in cells that contained only the *ura3-52* allele (UCC1023), the GATC sequence within this locus was cleaved by *Dpn* I in >95% of the cells, indicating that *ura3-52* was methylated in nearly all cells [Fig. 1, lanes 1 and 2, compare the difference in mobility of the bands without (−) and with (+) *Dpn* I]. In addition to being

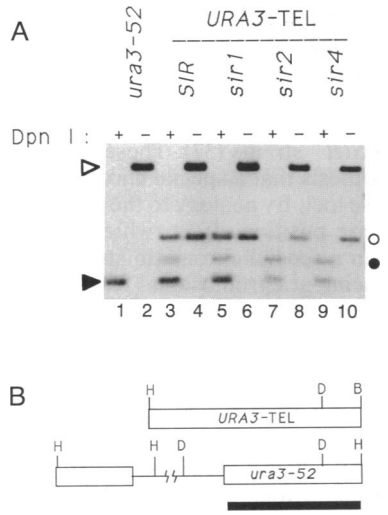

FIG. 1. Telomeric *URA3* gene is refractory to Dam methylation *in vivo*. (*A*) Total yeast DNA was isolated from cultures of UCC1023 (*ura3-52*), UCC1021 (*SIR*), UCC1044 (*sir1*), UCC1042 (*sir2*), and UCC1045 (*sir4*). Each strain expressed the *E. coli dam* methylase; strain UCC1023 contained the *ura3-52* locus, and the other four strains had *ura3-52* in addition to *URA3* at the VII-L telomere (URA3-TEL). Each DNA sample was cleaved with *Bam*HI and *Hin*dIII (lanes −) or *Bam*HI, *Hin*dIII, and *Dpn* I (lanes +) and subjected to gel electrophoresis and DNA blot hybridization analysis using a 0.7-kilobase-pair *Eco*RV–*Sma* I *URA3* DNA probe (represented by the solid box). The resulting autoradiogram is presented. Open arrowhead, *Hin*dIII fragment from *ura3-52*; solid arrowhead, *Dpn* I fragment; open circle, *Hin*dIII–*Bam*HI fragment from URA3-TEL; solid circle, *Hin*dIII–*Dpn* I fragment. The smaller *Dpn* I–*Hin*dIII or *Dpn* I–*Bam*HI fragments are not shown on this autoradiogram. (*B*) The open box in the schematic diagram represents the *URA3* gene; the location of the Ty element at *ura3-52* is indicated by the discontinuous line. In the construction of URA3-TEL, the 3′ *Hin*dIII site was replaced by a *Bam*HI site (1). B, *Bam*HI; H, *Hin*dIII; D, *Dpn* I (GATC site).

dam$^+$, strain UCC1021 contained both *ura3-52* and the telomeric *URA3* (on chromosome VII-L). Although the site in *ura3-52* was methylated in virtually all the cells, only ≈25% of the telomeric sites were methylated. This is demonstrated in lanes 3 and 4. The *Dpn* I(−) lane (lane 4) contained two bands; the band with greater mobility represented the telomeric *URA3* (indicated by the open circle), and the band with slower mobility was indicative of *ura3-52* (arrowhead). In the *Dpn* I(+) lane (lane 3), the band with the fastest mobility was the result of cleavage of the *ura3-52* locus by *Dpn* I (solid arrowhead); note that there was no trace of an uncleaved *ura3-52* band. However, there were two bands representative of the telomeric *URA3* in the *Dpn* I(+) lane (lane 3); the band with slower mobility was the same as that observed in the *Dpn* I(−) lane (lane 4, open circle) and the new intermediate band was the result of *Dpn* I cleavage (solid circle). Thus, in most cells, the telomeric *URA3* DNA was refractory to Dam methylation. The partial Dam accessibility was consistent with phenotypic variegation, a result of a heterogeneous population of cells that were either in a repressed or active transcriptional state of the telomeric *URA3* (1); presumably, the repressed cells were inaccessible to the methyltransferase, whereas the active ones were methylated. However, at this point it is impossible to exclude a model in which the methylase had partial accessibility to the telomeric *URA3* in some repressed cells.

A number of modifiers of the telomeric position effect have been identified. They include *SIR2, SIR3, SIR4, NAT1, ARD1*, and *HHF2* (histone H4), all of which have previously been shown to be required for transcriptional silencing at the mating-type loci, *HML* and *HMR* (8, 33–36). A mutation in any of these genes completely relieves transcriptional repression caused by proximity to the telomere. However, *SIR1*, which acts in silencing at the *HM* loci, plays no role in the telomeric position effect (8).

It has previously been shown that the *in vitro* nuclease sensitivity of *HML* and *HMR* is altered in *sir*$^-$ strains compared to *SIR*$^+$ strains (37). These results are consistent with the hypothesis that a special chromatin structure exists to silence these loci. By analogy to the *HM* loci, the modifiers of the telomeric position effect, which include histone H4, likely maintain a special chromatin structure that is responsible for silencing at chromosomal termini (8).

Sir$^-$ mutations were tested for their effect on Dam methylation at the telomere. In strains with a *sir2* or *sir4* mutation, which completely relieved the telomeric position effect, the telomeric site was now methylated in all cells (Fig. 1, lanes 7–10). Yet in a *sir1* strain, the telomeric *URA3* gene was protected from methylation as in the *SIR*$^+$ strain (Fig. 1, lanes 3–6), consistent with the result that mutations in *SIR1* do not affect the telomere-specific silencing (8). Thus methylation within the telomeric *URA3* gene correlated directly with the ability of the gene to be expressed. Apparently, in cells in which the telomeric *URA3* is repressed, the telomere-proximal DNA cannot be modified by the methyltransferase activity.

The yeast DNA excision repair pathway can affect the level of Dam methylation detected in some yeast strains (18). Cells that carry mutations in *RAD1* are defective in DNA excision repair (38). To examine whether the methylation patterns observed in the present study were a consequence of the *RAD1*-dependent DNA excision repair, experiments were also performed in congenic strains that were *rad1*. In the haploid strains used in these experiments, there was no discernible difference in methylation between *RAD*$^+$ and *rad1* cells at the *ura3-52* or telomeric *URA3* locus (data not shown), nor did *RAD1* have an effect on repression of the telomeric *URA3* gene (Table 1).

As an additional control experiment, the wild-type *URA3* gene located at its normal chromosomal locus was examined for sensitivity to methyltransferase activity in a *SIR*$^+$ strain. To include an internal reference for complete cleavage, a diploid strain was made that was heterozygous at the normal chromosomal location of *URA3:ura3-52/URA3*.† As shown in Fig. 2, complete cleavage of both the *URA3* and *ura3-52* alleles was accomplished by either *Sau*3A (*Sau*3A is insensitive to the methylation state of DNA) or *Dpn* I. Thus the GATC sites within the wild-type *URA3* locus and within *ura3-52* were equally accessible to methylation. Thus it can be concluded that the *SIR*-dependent inaccessibility of *URA3* was a function of its proximity to a telomere.

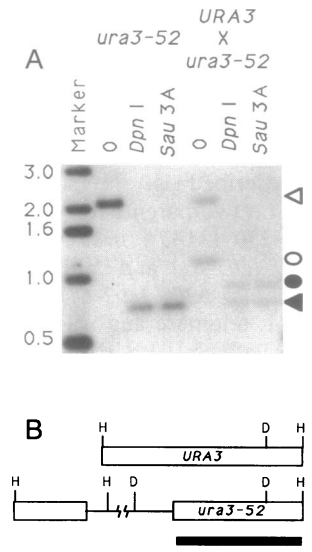

FIG. 2. *URA3* gene at its normal chromosomal locus is sensitive to Dam methylation *in vivo*. (*A*) Total yeast DNA was isolated from cultures of the haploid strain UCC1058 (*ura3-52*) or the diploid cells of a cross between UCC1059 and UCC1058 (*URA3* × *ura3-52*). Each DNA sample was cleaved with *Hin*dIII alone (lanes 0) or in combination with *Dpn* I or *Sau*3A (as indicated) and subjected to gel electrophoresis and DNA blot hybridization analysis using the same DNA probe described in Fig. 1. The resulting autoradiogram is presented. Open arrowhead, *Hin*dIII fragment from *ura3-52*; solid arrowhead, *Dpn* I fragment; open circle, *Hin*dIII fragment from *URA3* at its normal chromosomal locus; solid circle, larger *Hin*dIII–*Dpn* I fragment. The smaller *Hin*dIII–*Dpn* I fragment is not shown on the autoradiogram. (*B*) The schematic diagram is as in Fig. 1, except that *URA3* is represented at its normal locus.

DISCUSSION

When the *E. coli dam* gene is expressed in *S. cerevisiae* cells, the resulting *in vivo* DNA methyltransferase activity modifies GATC sequences in the yeast genome (11, 12). In this study it has been shown that a GATC site located near a telomere is refractory to this methylation in a *SIR2*- and *SIR4*-dependent fashion. Thus these results parallel the *SIR*-dependent resistance of the *HM* loci to cleavage by HO endonuclease *in vivo* (39, 40).

The Dam methyltransferase is likely to prove to be generally useful as a probe for *in vivo* chromatin structure. The methyltransferase leaves an imprint of its interaction with a DNA sequence (the methylated adenine) that does not significantly affect the status of the chromosome yet can be monitored by endonuclease digestion of purified DNA (for review, see ref. 41). This is in contrast to using *in vivo* endonuclease cleavage as a probe for chromatin structure; DNA cleavage may alter chromosome topology or reduce

†To get complete cleavage at *ura3-52* with *Dpn* I, these diploid cells required two copies of the *dam*$^+$ gene and that at least one of the *RAD1* loci be deleted.

chromosome stability (42). In addition, the 4-base recognition sequence of Dam methyltransferase occurs rather frequently (≈1 in 300 base pairs) in the yeast genome. In fact, the *in vivo* assay with Dam methyltransferase described here has also been used to detect differences in DNA methyltransferase accessibility as a function of gene expression at several nontelomeric loci including *HML* and *HMR* (43). Thus the assay may prove useful for examining additional chromosomal domains in yeast and may be extended to other organisms that do not normally contain a GATC-specific adenine methyltransferase activity. (However, the level of endogenous DNA excision repair activity in a cell must be considered.)

The inaccessibility of the Dam methyltransferase to telomeric regions correlates with transcriptional repression of genes located near telomeres. This suggests that transcriptional repression near telomeres is due to the inability of transcription factors to gain access to the DNA for transcription. The molecular basis for the inhibition of Dam methylation at the telomeric site is not known but is probably due to a special chromatin structure. In support of this idea, histone H4 mutations abrogate the telomeric position effect, directly implicating chromatin structure in the silencing mechanism (8). However, in considering how the methyltransferase is prevented from gaining access to the DNA, it is interesting to note that the *URA3* DNA fragment used in this study encodes information necessary for precise positioning of nucleosomes along the gene *in vivo*. The same nucleosome positioning detected along the *URA3* gene at its normal chromosomal locus is also observed when the gene is located on an episome or other chromosomal loci, such as the VII-L telomere (44, 45). The GATC site of *URA3* is located within one of these positioned nucleosomes. Thus the resistance to Dam methyltransferase activity is not due simply to a difference in nucleosome placement. Rather it may be the result of a higher-order chromatin structure near yeast telomeres that may be analogous to heterochromatin in other organisms (for review, see refs. 46–48). The investigation of position effect at yeast telomeres should yield additional insights into fundamental aspects of chromatin organization. These studies are likely to be facilitated by using Dam DNA methyltransferase as an *in vivo* probe.

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