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A Methyltransferase Targeting Assay Reveals Silencer-Telomere Interactions in Budding Yeast

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We have designed a modified version of the Dam identification technique and used it to probe higher-order chromatin structure in *Saccharomyces cerevisiae*. We fused the bacterial DNA methyltransferase Dam to the DNA-binding domain of TetR and targeted the resulting chimera to Tet operators inserted in the yeast genome at the repressed locus *HML*. We then monitored the methylation status of *HML* and other sequences by a quantitative technique combining methylation-sensitive restriction and real-time PCR. As expected, we found that TetR-Dam efficiently methylated *HML* in *cis*. More strikingly, when TetR-Dam was present at *HML*, we observed increased methylation in the III-L subtelomeric region but not in intervening sequences. This effect was lost when the *HML* silencers were inactivated by mutations. When the *HM* silencers and the Tet operators were transferred to a plasmid, strong methylation was clearly observed not only in the III-L subtelomeric region but also at other telomeres. These data indicate that *HM* silencers can specifically associate with telomeres, even those located on different chromosomes.

Eukaryotic genomes are divided into functional compartments in which transcription is potentially active (euchromatin) or repressed (heterochromatin), and these compartments correspond to distinct physical domains of the nucleus. Consequently, there is a correlation between the position of a gene in the nucleus and its transcriptional activity. Relocation into a heterochromatic domain usually correlates with transcriptional repression (this has been well described for the *Drosophila* gene *brown*, for instance [10, 13]). In contrast, enhancers, which stimulate gene expression, seem to act in part by excluding genes from heterochromatin (18). What determines the spatial positioning of genes within the nucleus and the functional consequences of heterochromatin proximity are therefore important questions to address.

Saccharomyces cerevisiae has proved to be a powerful tool with which to study the role of heterochromatin in gene repression. Extensive work in *S. cerevisiae* has shown that the mating type loci *HML* and *HMR* are kept transcriptionally repressed by *cis*-acting elements called silencers (24). The silencers act by recruiting a complex of proteins called Sir (silent information regulator) proteins (35). This complex then spreads along the nucleosomal fiber through multiple interactions with histone tails (21, 22).

The telomeres play an important role in silencer activity in *S. cerevisiae* (40). Mutations that disrupt telomere structure and/or function greatly impair silencer function (15, 26, 31–33). Silencers also become less active when moved away from the telomeres along the same chromosome (30). These results, together with microscopy analyses, have led to a "reservoir" model in which telomere clusters are thought to constitute a subnuclear compartment that sequesters limiting silencing factors, including the Sir proteins (19, 30, 36). In accordance with this model, tethering of a weak silencer to the nuclear periphery, in which the telomeres are clustered, facilitates its repression (2). Relocation to the peripheral compartment probably does not cause repression per se (43). Rather, it appears to provide a high local concentration of silencing factors (2) and/or to prevent the switch to an active state (15). One prediction of the model is that silencers may have to associate with the telomeric compartment, at least transiently, to establish silencing. However, direct telomere-silencer interactions have not yet been evidenced.

In this work we asked whether silencers can physically interact with telomeres. This question cannot be addressed by microscopy because the distance between the mating type silencers and their proximal telomeres is below the resolution limit of this technique. We therefore designed an in vivo system in which a DNA methyltransferase is targeted to a silencer and the methylation of telomeric sites is precisely measured. Our data show that *HML* silencers preferentially associate with their proximal telomere, III-L. However, when the same silencers are removed from their natural location and placed on a plasmid, this specificity is lost, and the silencers can associate with any telomere. Therefore, silencers have the inherent capacity to interact in *trans* with telomeres, but intrachromosomal constraints appear to restrain their association mainly to their proximal telomere. We propose that telomeres play an active role in silencer-mediated silencing by addressing silencers into a nuclear repressive compartment.

MATERIALS AND METHODS

Plasmid constructions. Molecular biology manipulations were performed as described previously (39). The *S. cerevisiae* SURE2 strain (Stratagene), grown at 30°C, was used for plasmids containing $TetO_{112}$. All constructs were verified by DNA sequencing.

The TetR open reading frame was PCR amplified off plasmid p6501 (a generous gift of F. Feuerbach and U. Nehrbass). *Not*I and *Eco*RI restriction site were inserted at the end of the upstream and downstream primers TetRa (AA TTC**GCGGCCGC**GCCCTTGCTCACCATGGACC) and TetRb (AGTTG**GAA**

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TABLE 1. Coordinates of the primers used for quantitative PCR

GATC no.	Upstream primer (5′→3′)	Downstream primer (5′→3′)
1	TATGTGATGATTCGCTTGGAAGGG	GCAATTTCATCTACAGGCTTGGAGG
2	CGAGAAATTCGGTGACTCTAAGGC	GCAGAGAACAGTTGCTTATGCG
3	GCTTAAACGAAGAATACCAGAAGCACG	CATAGTCAGGAATCGCGACACTAGC
4	CTTACTTGTTGCTGCTCCCTCTCC	CTTGAAGGGAGAGAGTGATGTCTCCG
5	CTTATCAGAGCATAGTTGGTCAGC	GCATCACAATATACAGTTAATGCCACCTG
6	TATTGTGATGCATCTCATGGAGC	GTATTCTGCCTCAGTAGATGG
7	CCTTGTCTATTAGTTCCGGGTC	CTCATGAAGGTGTTATCGCTGC
8	TCACTGCTCTTTTCTGTGTTCC	GACCAAACTTACGATCTTTGG
9	GCATATATATAATTAAGCGGGAGC	CAATATACTTACAGAGACCTC
10	GAAACCGTCTTCCTCGGATACG	CAGATTGAGCGACTTAGAAGGTGCTGG
11	GTTGTATCCTTGATAGCTCCTTATCCG	GTACGGCTACTGACCTAGATACTCAGG
12	ATGAGGATTGAAATTGTTCTTGG	AACAGAAAGAAAAGGAGCACGAGGC
13	CCAAATCAACCTTTCTAGGC	CTTGCCCATGTTTAAGAAGAGG
14	CTCTGGCTTTCAAAATGATAGCG	GAGTTTAAGGAAGAGGATAACGCATCCG
15	CGAGGAGCTGATCAAGGACCAGG	AATATGCAGTACCATTCCGCTC
16	CTGAGGTGAACACACCCACGCC	GCAGATTAACTTTGCTACGAGAGGG
17	GTAAGGTAGAGAGCCCTTCCG	GCCTTCGATTGAACATCCTGCCAG
18	TCCAATTCCAAATTCTAGGGACG	GTCAATGAGTAGTAGATAGTAAAGCC
19	TCAGTGCCCAACTCAGCTTCCG	GTGGCCTTCCTTCCTTTGGTGGAGC
20	CTATACTCCAGCAGAGGAACCC	CTTTCTGAGCATTTCCTAACACG
Myo5	GAAGGCCACCTACAGCAGGC	CCTGCTCTTCTGATACGCACG
Spt15	GAATCGAGATGGTACAAAACCAGC	CTCTAATACGCATGATGACAGCAGC
Y′	GCGCAGATCTGAAAGTTGGAGTTTTTCAGCG	GCGGGATCCAACCACACCTCCGAAATCTGC
KAN	GGTCAGACTAAACTGGCTGACGG	CCATGAGTGACGACTGAATCCG

TTCAGATCTCGCTCTAGAACTAGTGGA) (in all sequences, restriction sites designed in oligonucleotides for cloning purposes are shown in boldface) (Table 1). The PCR product was then cloned into *Eco*RI- and *Not*I-cut pCmycDam (45). Then a *Bgl*II-*Sal*I fragment containing the genes for TetR, Myc, and Dam was inserted after the *GAL1/10* promoter at the *Bam*HI and *Xho*I sites of pESC-HIS (Stratagene), resulting in plasmid pTetRDam.

HML derivatives with LexA binding sites were all constructed as follows. A *TRP1* fragment was amplified from pFL39 (4) with primers TRPa (AGTTG**AAGCTT**ACTAGTGGTCGAAAAAAGAAAAGGAGAGGGCC) and TRPb (AATTC**AAGCTT**GGCAAGTGCACAAACAATACTTAAATAAATACTAC). It was inserted downstream of the *HML*-I sequence at the unique *Hin*dIII site. During this cloning, an *Spe*I site was created immediately downstream of *HML*-I. The region flanking *HML*-I to the right between positions 15220 and 15915 was amplified with primers HDa (AGTTG**AGATCT**CGGAACACATTCTTATAAATCTATAGG) and HDb (AATTC**AGATCT**GCGCGCCCTCTAATACTATAAAGGACTTGG). It was inserted at the unique *Bgl*II site to serve as a target during homologous recombination. The downstream primer also contains at its end a *Bss*HII site. This procedure was carried out in parallel on the wild-type *HML* sequence and on the IΔ242 mutant form (29). It yielded plasmids pITRPHD and piTRPHD, respectively.

The *HML*-E silencer and *URA3* reporter gene were amplified from a previously described plasmid (27) with primers E+URAa (AAGCTG**GAGCTC**GCGCGCCGGTTGATGACATGATTTTGTATCGTC) and E+URAb (AATTC**GAGCTC**TCATTACGACCGAGATTCCCG) and inserted upstream of the I sequence at the unique *Sac*I site. The upstream primer also contains a *Bss*HII restriction site. As above, this was done with the wild-type and mutant *HML* to give pEURA3I and pEURA3i, respectively. A PCR fragment containing four binding sites for LexA was obtained by PCR amplification of plasmid pSH18-34 (Stratagene) with primers LexAa (AGTTG**ACTAGT**CCATATCTAATCTTACCTC) and LexAb (AATTC**ACTAGT**CGCATTATCATCCCTC). It was cloned at a unique *Spe*I site, resulting in plasmids pEURA3ILEXA and pEURA3iLEXA.

Plasmids used to introduce $TetO_{112}$-containing constructs at *HML* were derived from plasmids pITRPHD and piTRPHD. First, a plasmid harboring the *kanMX4* gene adjacent to 112 $TetO_2$ operators was built. A 5.6-kb *Sal*I-*Bam*HI $TetO_2$ fragment obtained by excision from p306tetO_2x112 (from the Nasmyth laboratory) was inserted between the *Sal*I and *Bgl*II sites of pFA6a-KanMX4 (47), resulting in plasmid pTETKAN. A 180-bp *Spe*I-*Xba*I fragment from plasmid pITRPHD or piTRPHD removing the ATG of *TRP1* was replaced by the 6.7-kb *Spe*I TETKAN fragment of pTETKAN, resulting in plasmids pITETKAN and piTETKAN, respectively.

pSILTet was derived from pITETKAN by insertion of the *HML*-E silencer and *URA3* at a unique *Kpn*I site upstream of *HML*-I. PCR amplification was performed with primers E+URA-*Kpn*Ia (AAGCTGCCA**GGTACC**TGGCGGTTGATGACATGATTTTGTATCGTC) and E+URA-*Kpn*Ib (AATTCCCA**GGTAC C**TGGTCATTACGACCGAGATTCCCG). These primers each contain a *Bst*XI site (boldface) engineered to create *Kpn*I-compatible ends after *Bst*XI digestion of the PCR products. The 1.5-kb *Not*I *kanMX* fragment of pFA6a-KanMX4 was cloned into the *Not*I site of pEURA3ILexA, resulting in plasmid pSIL.

pCENTet is a *LEU2*-CEN-ARS plasmid containing the 6.7-kb *Spe*I fragment from pTETKAN cloned at the *Spe*I site of pRS315 (41). pCEN is also a pRS315-based plasmid, in which the 1.5-kb *Not*I *kanMX* fragment of pFA6a-KanMX4 was cloned into the *Not*I site of pRS315.

Yeast strains, media, and methods. Manipulations of *S. cerevisiae* were performed as described previously (38). The *S. cerevisiae* strains used in this study are all derivatives of S150-2B (*MAT***a** *leu2-3,112 URA3-52 TRP1-289 his3*Δ *gal2 gal4*::*LEU2*). All gene replacements were confirmed by Southern blot analysis.

To insert LexA binding sites at *HML*, we transformed EG42 (5) with *Bss*HII-digested, LexA4-containing *HML* constructs. We then screened for loss of the *LEU2′-′lacZ* marker by a filter-based β-galactosidase color assay. This resulted in strains EL20 and EL21 (see Fig. 1A). To integrate the $TetO_{112}$ array at *HML*, we transformed the *Pvu*II-linearized plasmids pITETKAN and piTETKAN into strains EL20 and EL21. This gave rise to strains EL22 and EL23, respectively (see Fig. 1A).

Analysis of *URA3* expression. The expression of *URA3* was monitored essentially as described by Fourel et al. (17) by spotting 10 μl of serial dilutions of overnight culture onto appropriate selective synthetic medium with or without 5-fluoroorotic acid (1 g/liter).

Quantitative PCR. Cells carrying the TetR-Dam plasmid were grown overnight in selective medium with 2% raffinose. Genomic DNA was prepared, and equal amounts of DNA were incubated for 16 h at 37°C with *Dpn*II. The enzyme was heat inactivated for 20 min at 65°C, and the mixture was ethanol precipitated. Samples were then assayed by quantitative PCR with a LightCycler (Roche Molecular Biochemicals) according to the manufacturer's recommendations. A standard dilution series of nondigested genomic DNA was included in every experiment to allow relative quantification of each sample. Each sample was assayed in triplicate, revealing a standard error of about 5% for the quantitation of the DNA sample. Figures 3 to 5 only show the results of one set of experiments for better clarity. Each of the experiments was repeated at least twice, with independent DNA preparations. Identical patterns of methylation profile were obtained, although some variation in the absolute quantity of methylated GATC sequences was observed, perhaps as the result of varying expression of the methyltransferase (data not shown).

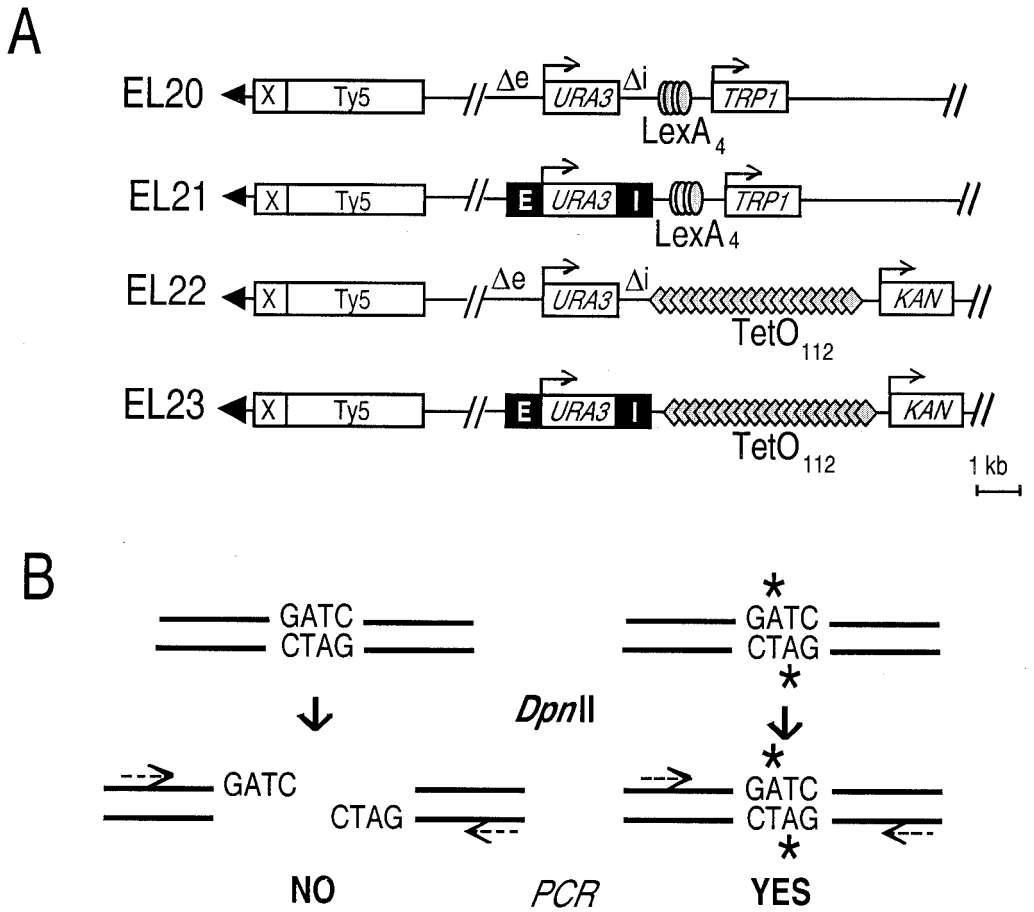


FIG. 1. Outline of the experimental system. (A) Schematic representation of the left end of chromosome III in the test strains. The *HML*-E and *HML*-I silencers are drawn as solid boxes (E and I). A fragment containing either four LexA sites or a cluster of 112 TetO sites was inserted next to *HML*-I, together with the adjacent *TRP1* or *KAN* resistance gene, respectively, used for selection purposes. The subtelomeric III-L region was left unaltered and carries an X subtelomeric element and a Ty5 retrotransposon known to be subject to telomere-driven silencing. Arrowheads, telomeric repeats. (B) Principle of the PCR-based quantification assay. Primer pairs are designed so that each one brackets a single GATC. Unmethylated sites are cut by *Dpn*II and fail to be amplified. In contrast, sites that have been methylated by Dam (stars) become resistant to *Dpn*II and can yield a PCR fragment. This permits quantification of the methylation level for a given GATC.

RESULTS

System to target and detect methylation in *S. cerevisiae*. The Dam identification technique is based on the fusion between the bacterial methyltransferase Dam and a DNA-binding protein (45). One can then identify the binding sites for the protein of interest by virtue of their *cis* methylation. Our goal was to examine physical interactions between loci in the yeast *S. cerevisiae*. In particular, we wanted to address whether the silenced mating type locus *HML* interacts with telomeres in a repressive nuclear compartment. We reasoned that we could adapt the technique to suit our purposes. Our rationale was that if Dam was tethered to *HML*, then loci that interacted with *HML* would become preferentially methylated. One important aspect of this system is that, methylation being a covalent modification, a trace of the interaction would remain even if the interaction was transient.

For this method to work satisfactorily, two preliminary conditions had to be met. First, the recruitment of Dam to *HML* and the *cis*-methylation of this locus had to be efficient. Second, the amount of unbound Dam free to diffuse in the nucleus and cause background methylation had to be minimal. In order to find these optimal conditions, we tried two different targeting systems. In a first series of experiments, we expressed a LexA-Dam hybrid protein in strains containing four LexA sites inserted into *HML* loci with or without silencers (Fig. 1A, EL20 and EL21). Methylation was examined by Southern blotting after restriction of genomic DNA with the methylation-sensitive enzymes *Dpn*I and *Dpn*II. We found methylation of the *HML* region to be no greater than that of a control locus on another chromosome, indicating inefficient targeting or high background (data not shown).

We therefore turned to another system in which Dam was fused to TetR and an array of 112 TetO sites (hereafter called $TetO_{112}$) was inserted at *HML* loci with and without functional silencers (Fig. 1A, EL22 and EL23). By Southern analysis (see above), we observed increased methylation of the *HML* region in comparison to other loci (data not shown). The effect was maximal when the expression of TetR-Dam driven by the *GAL1* promoter was kept low by growing the cells in the presence of raffinose, not galactose (data not shown). In all the

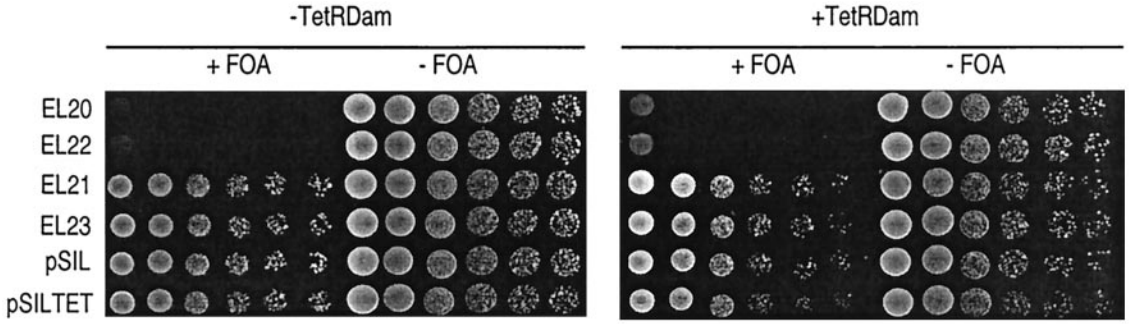


FIG. 2. *HML* silencing is not affected by insertion of TetO sites and expression of TetR-Dam. Silencing is measured by the ability of cells to grow in the presence of 5-fluoroorotic acid (FOA), a drug that kills cells expressing *URA3*. A representative experiment is shown, and identical results were obtained with independent isolates from each strain. From left to right: nondiluted culture, three successive 10-fold dilutions, then two threefold dilutions. Growth on 5-fluoroorotic acid, and therefore silencing, depended on the presence of the E and I silencers flanking the *URA3* reporter gene (EL21, EL23, pSIL, and pSILTet). It was not affected by the presence of flanking insertions (LexA or TetO sites) or the expression of TetR-Dam (− or + TetR-Dam). It was also properly silenced when placed on a plasmid (EL21 and EL23 versus pSIL and pSILTet).

experiments presented hereafter, the cells were transformed with TetR-Dam and raffinose was used as the carbon source.

We then devised a system to quantify the amount of methylation at individual GATC sites accurately and sensitively. Its principle, outlined in Fig. 1B, relies on quantitative PCR. Genomic DNA is overdigested with *Dpn*II, which only cuts nonmethylated GATC sequences. There is no endogenous GATC methylation in *S. cerevisiae*, so in the absence of Dam methylation, no GATC is methylated and no amplification product can be formed. In contrast, a fragment encompassing a GATC site that has been methylated by Dam can be amplified by PCR. Therefore, the amount of PCR product directly reflects the methylation level of the GATC site examined.

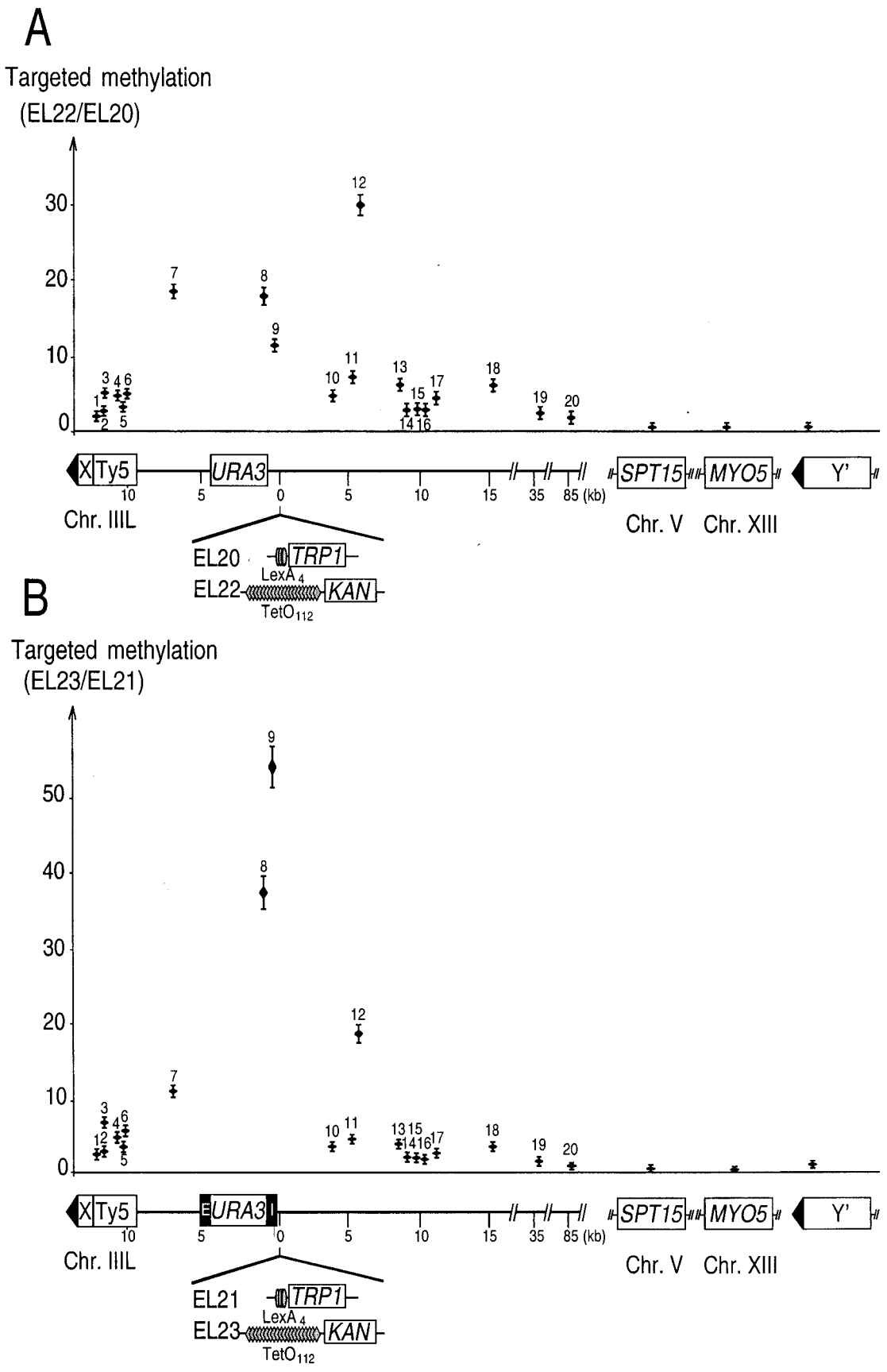
To evaluate this assay, we sought conditions in which binding of TetR-Dam should be high and easily detectable. We therefore carried out our pilot experiments in strain EL22, in which *HML* is tagged with TetO sites and bears mutations that prevent silencing (Fig. 1A). We verified that these mutations incapacitated *HML* silencing, as indeed EL22 did not grow on 5-fluoroorotic acid-containing medium (Fig. 2). In these conditions, the chromatin at and around *HML* should be open and permissive for TetR-Dam binding. We extracted genomic DNA from strain EL22 expressing TetR-Dam, digested it with *Dpn*II, and then performed quantitative PCR measurements with primer pairs that spanned chromosome III. Primer pairs within *SPT15* on chromosome V and *MYO5* on chromosome XIII and in Y′ subtelomeric repeats (present at several chromosome ends, but not III-L in our strain) were used as controls. Each primer pair brackets only one GATC site.

We were concerned that different sites of the genome may display inherently different susceptibility to methylation because of their sequence or the local DNA structure. To take this variability into account, we normalized our data as follows. We first measured the methylation in strain EL20, which is identical to EL22 except that it contains LexA binding sites instead of $TetO_{112}$ at *HML* (Fig. 1A). Consequently, TetR-Dam is not bound to *HML* in this strain but diffuses within the nucleus. This first methylation measurement therefore quantifies differences in accessibility and/or methylation efficiency between different sequences. We then measured methylation in strain EL22 (Fig. 1A). For each primer pair, this second value was divided by the value obtained in strain EL20, and the ratio was named targeted methylation. The results of this experiment are shown in Fig. 3A.

The most salient finding was that the targeted methylation was highest, up to a value of 30, for GATC sites located in the *HML* region (sites 7 to 12). In other words, sites around *HML* were methylated up to 30-fold more efficiently when TetR-Dam was recruited to *HML* than when it diffused freely. In contrast, for sites outside of chromosome III, the values of targeted methylation were not significantly different from 1. These two results reflect efficient targeting of TetR-Dam to $TetO_{112}$ and demonstrate the feasibility of our approach.

The highly methylated region around *HML* spanned about 15 kb. It displayed local variations in methylation (compare site 12 to 10 and 11), which might be explained by local differences in chromatin structure. The extent of methylation dropped off sharply for sites further than about 7.5 kb from the $TetO_{112}$ sites. This distance is similar to that observed in *Drosophila melanogaster*, in which targeted methylation extended about 5 kb in either direction from the binding site. Importantly, we noticed that all the sites on the left arm of chromosome III, even those most distant from $TetO_{112}$, were more methylated than any of the sites on other chromosomes. Sites 19 and 20, for instance, 38 and 88 kb away from $TetO_{112}$, respectively, displayed about 50% more targeted methylation than *SPT15*, *MYO5*, and Y′ (Fig. 3A). A possible explanation is that chromosome III is folded into a given domain of the yeast nucleus and that *HML* is more likely to interact with sequences on III than on other chromosomes, which localize to other nuclear regions. Previous work in *S. cerevisiae* (8, 12), as well as findings reported in mammalian cells (6, 9), seem to support this idea.

We were also concerned that silencing may limit access of TetR-Dam to $TetO_{112}$. We therefore examined the targeted methylation pattern obtained with TetR-Dam targeted to a wild-type silenced *HML* locus (Fig. 1A, strain EL23). We first tested whether the presence of $TetO_{112}$ or the expression of TetR-Dam would interfere with *HML* silencing. As shown in Fig. 2, we observed that neither modification altered the capacity of *HML* silencers to silence a *URA3* reporter gene. These results are consistent with previous studies showing that DNA methylation does not interfere with silencing (20) and


A
Targeted methylation
(EL22/EL20)
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
X Ty5
URA3
SPT15
MYO5
Y'
10 5 0 5 10 15 35 85 (kb)
Chr. IIIL
Chr. V
Chr. XIII
EL20
TRP1
LexA4
EL22
KAN
TetO112
B
Targeted methylation
(EL23/EL21)
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
X Ty5
E URA3 I
SPT15
MYO5
Y'
10 5 0 5 10 15 35 85 (kb)
Chr. IIIL
Chr. V
Chr. XIII
EL21
TRP1
LexA4
EL23
KAN
TetO112

that binding of TetR does not modify the higher-order organization of chromatin (3, 15, 34).

As in the pilot experiment above, we used a reference strain to normalize our measurements. In this case it was EL21, a strain related to EL23 that contains LexA binding sites in the place of $TetO_{112}$. The targeted methylation value was determined by dividing the methylation obtained in strain EL23 by that obtained in EL21. The pattern that we observed was overall similar to that obtained in the absence of functional *HML* silencers (compare Fig. 3B and Fig. 3A). Methylation was elevated in a 15-kb region centered on $TetO_{112}$ and declined progressively beyond this limit. For sites outside of chromosome III, the methylation levels were again very low (Fig. 3B, *SPT15*, *MYO5*, and Y′). Thus, silencing at *HML* did not prevent targeting of TetR-Dam to $TetO_{112}$ and ensuing DNA methylation.

Finally, we were concerned that the presence of silencing at *HML* may have an effect on the pattern of methylation at other genomic sites independent of targeting. To evaluate this possibility, we now plotted methylation values obtained with EL21 divided by those obtained with EL20. None harbor $TetO_{112}$ sites, and EL20 lacks silencers at *HML*, whereas they are present in EL21. The resulting value was referred to as the silencer effect on *trans* methylation (Fig. 4A). For any given site, a silencer effect greater than 1 means that the site is more methylated when silencers are present at *HML* than in their absence. A silencer effect smaller than 1 means the opposite. Three major phenomena became apparent when we did this calculation (Fig. 4A).

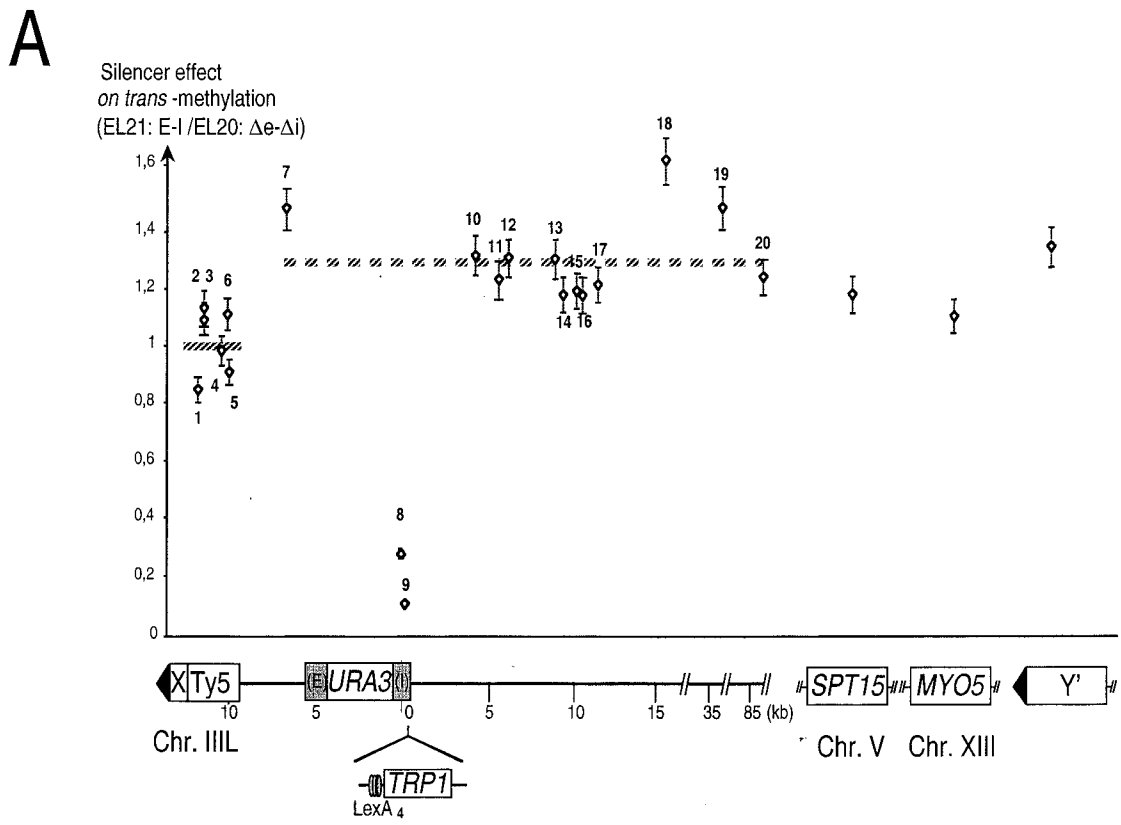
The strongest effect was observed for sites 8 and 9, which were about fivefold less accessible to the enzyme in the presence of active silencers. This agrees with previous reports showing that silenced regions are less accessible to the modifying enzymes (20). Second, sites 7 and 10 to 20 as well as *SPT15*, *MYO5*, and Y′ sites were slightly but significantly more methylated by the enzyme in the presence of silencers at *HML* than in their absence (Student's *t* test: $P < 0.0009$). Although we have no definitive explanation for this observation, it may result from variations in the level of expression of TetR-Dam, which might be slightly higher in EL21 than in EL20. Finally, sites 1 to 6 were clearly less accessible to the enzyme than the latter set of sites (compare solid and broken lines, which indicate the average silencer effect for each group). This shows that the mere presence of a silenced *HML* locus influences chromatin structure in the III-L subtelomeric region. Although it was not investigated further, we presume that this decreased accessibility to TetR-Dam in fact reflects an increase in III-L telomeric silencing dependent on the *HML*-E and -I silencers. This unanticipated result therefore strongly suggests that the *HML*-E and -I silencers reinforce silencing emanating from telomere III-L.

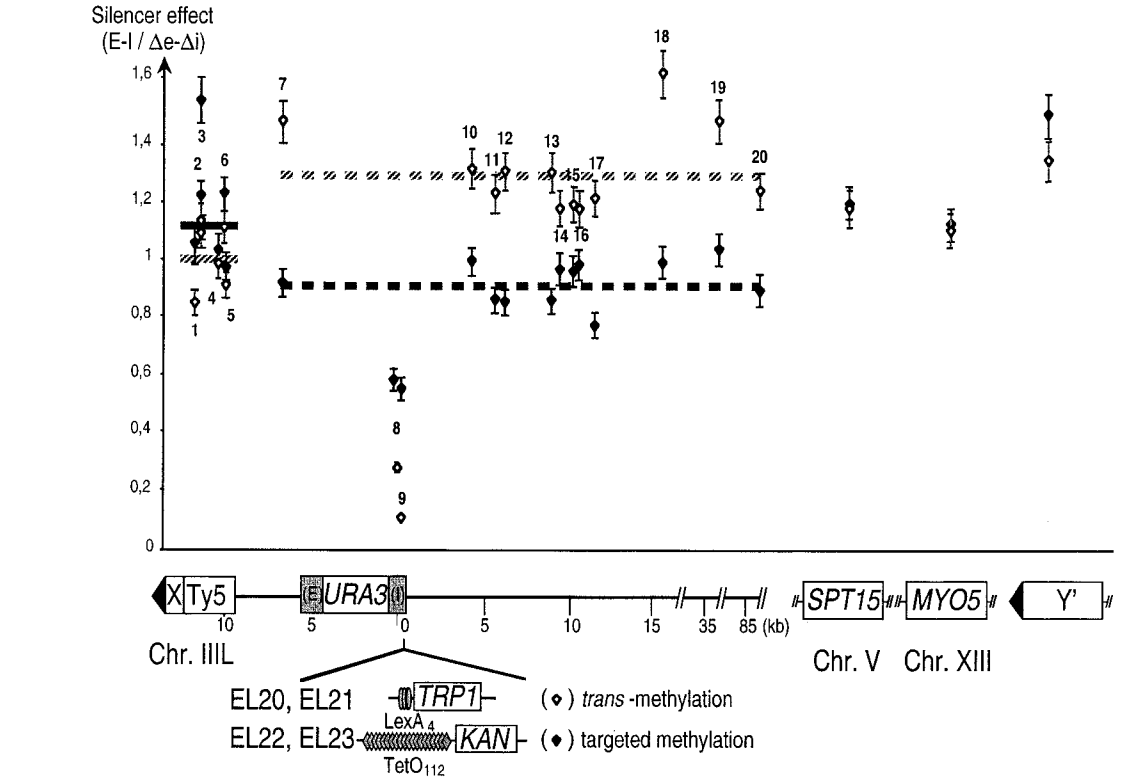
Silencing of *HML* permits interaction with telomere III-L. To visualize the specific contribution of the silencers to the targeted methylation profile, we plotted the ratio of targeted methylation in the silenced versus the nonsilenced context (silencer effect on targeted methylation) (Fig. 4B, solid diamonds). The silencer effect on *trans* methylation is represented again to facilitate comparison (open diamonds). The methylation of sites closest to *HML* (sites 8 and 9) was not as deeply affected in the silenced context upon targeting of TetR-Dam compared to *trans*-methylation, suggesting that targeting of TetR-Dam at *HML* compensates in part for the loss of accessibility due to the presence of silenced chromatin. Second, methylation of reference sites along chromosome III (sites 7 and 10 to 20) was reduced upon TetR-Dam targeting to *HML* (compare hatched and dark broken lines), which was not the case for sites located internally on other chromosomes (*SPT15* and *MYO5*). This phenomenon might be accounted for by a locally reduced availability of TetR-Dam within chromosome III nuclear territory upon its trapping to multiple sites at a single locus of this chromosome. TetR-Dam is indeed known to be expressed in limiting amounts in our system.

By contrast, and most importantly, the methylation of the subtelomeric element Ty5 (sites 1 to 6) increased in the silenced context compared to reference sites of chromosome III (compare solid and broken dark lines). The differences between the cluster formed by sites 7 and 10 to 20 and the one formed by sites 1 to 6 are highly significant (Student's *t* test: $P < 0.000001$). This finding is all the more striking because this increased methylation at sites 1 to 6 contrasts with the reduced accessibility of sites 1 to 6 to *trans* methylation upon silencing at *HML* (see Fig. 4A). One model which is consistent with these results is that the presence of silencers at *HML* increases *HML*-telomere III-L interactions, thereby allowing specific methylation of Ty5 sequences upon targeting of TetR-Dam to *HML*, which, as at *HML*, compensates for the reduced accessibility to a *trans*-acting enzyme associated with reinforced silencing.

Finally, the presence of silencers at *HML* also seemed to increase methylation in the subtelomeric regions of other chromosomes upon targeting of TetR-Dam at *HML* (Fig. 4B, Y′ site, compare solid and open diamonds). Although this result is a first hint that *HML* silencers may be endowed with the capacity to interact with all telomeres, the effect was moderate (Student's *t* test: $0.01 < P < 0.05$), and this hypothesis therefore required independent confirmation. Altogether, these data strongly suggest that *HML* silencers preferentially associate with their proximal telomere.

FIG. 3. Targeted methylation profiles of silenced and nonsilenced *HML* regions and other loci. (A) TetR-Dam targeted to *HML* via $TetO_{112}$ specifically methylates a broad region of the left arm of chromosome III. Targeted methylation is the ratio of the methylation frequency for a given GATC in a $TetO_{112}$-containing strain to that in a $LexA_4$-containing strain. The number above each point refers to the relative position of the probed GATC along the chromosome. A schematic representation of the left arm of chromosome III is depicted below the graph, together with the right-side insertions at *HML* that differed between the strains compared. Other GATCs located on chromosomes other than chromosome III were also assayed. Two are located in the coding sequence of *MYO5* and *SPT15* on chromosomes XIII and V, respectively, and one is present in the Y′ subtelomeric repeats, which are found next to approximately half of the *S. cerevisiae* telomeres. The latter GATC is located 400 bp away from the telomeric TG1-3 repeats, in a conserved region. (B) Targeted methylation profile of strains carrying functional *HML* silencers. Legend is otherwise the same as for Fig. 1.


A
Silencer effect
on trans -methylation
(EL21: E-I /EL20: Δe-Δi)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
X Ty5
URA3
SPT15
MYO5
Y'
10 5 0 5 10 15 35 85 (kb)
Chr. IIIL
Chr. V
Chr. XIII
TRP1
LexA 4


B
Silencer effect
(E-I / Δe-Δi)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
X Ty5
URA3
SPT15
MYO5
Y'
10 5 0 5 10 15 35 85 (kb)
Chr. IIIL
Chr. V
Chr. XIII
EL20, EL21
TRP1
LexA 4
(◇) trans -methylation
EL22, EL23
KAN
TetO112
(◆) targeted methylation

Plasmid-borne silencers interact with the III-L Ty5 subtelomeric region and also with Y′ telomeres. The above results suggested a physical contact between the silencers at *HML* and Ty5 at telomere III-L as well as, to a lesser extent, with subtelomeric regions of other chromosomes. However, the magnitude of the observed effects was rather limited, and interpretation was further complicated by a *cis* effect of *HML* silencers on TetR-Dam-mediated methylation independent of its targeting to *HML* (see Fig. 4A). We therefore decided to investigate this model further by asking whether plasmid-borne silencers can interact with telomeric regions.

We targeted TetR-Dam to a plasmid and assessed whether the presence of plasmid-borne silencers would lead to increased methylation of telomeric regions. For this purpose, we created a series of plasmids (Fig. 5A). pSILTet and pSIL both contain the *HML* silencers (SIL). The first plasmid contains $TetO_{112}$, but the second does not. *HML* provides the ARS and CEN functions in these plasmids (1, 25, 28). Plasmid pCENTet contains $TetO_{112}$ but no silencer, while plasmid pCEN has neither. We determined that the plasmid copy number in cells grown under selective conditions was sixfold higher for pSIL than for pSILTet and for pCEN than for pCENTet and fivefold higher for the SIL compared to the corresponding CEN plasmids (data not shown). As expected, the flanking silencers repressed the expression of the *URA3* gene carried by the plasmids whether or not TetR-Dam was expressed (Fig. 2). Importantly, the presence of pSIL, pCEN, pSILTet, or pCENTet did not interfere with chromosomal silencing (data not shown). The *KAN* gene present on pSILTet and pCENTet was strongly methylated upon TetR-Dam expression (data not shown). Altogether, these controls validate the use of the plasmid system to study the effects of targeted methylation.

We measured methylation in the presence of TetR-Dam and of plasmid pSILTet. Again, for normalization purposes, these values were divided by those obtained in the presence of pCENTet, which does not contain a silencer. This ratio is plotted in Fig. 5B (solid diamonds). The value obtained for the control sites *MYO5* and *SPT15* was about 1.5. This means that even the control sites received about 50% more methylation when the cells contained pSILTet than when they contained pCENTet. The most likely explanation for this fact is that TetR-Dam is slightly more expressed in the former situation, for an unknown reason. We found that sites 9, 14, 15, 17, and 20 behaved like the control loci, with ratios between 1.45 and 1.65. In contrast, two regions clearly behaved differently: first, sites within and around Ty5, and second, the site within Y′. In both cases, the ratio was about 3, a value significantly different from that of the controls (Student's *t* test: $P < 0.0000006$). This shows that Ty5 and the Y′ elements are more methylated in the presence of pSILTet. This could have one of two causes. First, pSILTet, and its tethered TetR-Dam, could interact with these regions more than with other genomic loci. Alternatively, these regions could become inherently more accessible to background methylation just because of the presence of the silencers on a plasmid.

We tested this by using plasmids pSIL, which contains the silencers but not $TetO_{112}$, and pCEN, which contains neither. We measured methylation in the presence of pSIL and divided that value by that obtained with pCEN. This ratio is plotted in Fig. 5B (open diamonds). Its value was about 0.4 for the control sites. Again, why the ratio deviates from 1 could be due to slight variations in TetR-Dam expression between strains. At any rate, the relevant result is that all sites behave like the control sites. In other words, no site becomes more or less accessible to TetR-Dam in the presence of the plasmid-borne silencers. This rules out our second hypothesis, and we conclude that *HML*, when present on a plasmid, can interact with the Ty5 element present at telomere III-L and also with the Y′ sequences present on other telomeres.

DISCUSSION

Many genetic arguments have suggested functional interactions between silencers and telomeres (5, 16, 30), but until now direct proof for physical contacts was lacking. We have adapted a DNA methyltransferase targeting assay to *S. cerevisiae* and coupled it to a quantitative method of methylation detection. This allowed us to investigate the interactions of the *HML* silencers with other loci. We show that, in its natural context, *HML* interacts with the proximal subtelomeric element Ty5 and to a lesser extent with Y′ elements present at other telomeres. This interaction specifically depends on the presence of functional silencers at *HML*. Furthermore, although not addressed here, it is presumably transient and may occur at specific stages of the cell cycle.

Our results further suggest that the physical interaction between *HML* silencers and the III-L subtelomeric region contribute to efficient silencing of the Ty5 retrotransposon (46). Given that a GATC site located between telomere III-L and *HML* is not affected by the presence of *HML* silencers (Fig. 4A, site 7), it seems unlikely that the putative cooperation between Ty5 silencing and *HML* silencers would derive from a continuous propagation of silent chromatin emanating from *HML*. This is consistent with previous results demonstrating

FIG. 4. Silencer effects on *trans* methylation and on targeted methylation. The specific influence of silencers on TetR-Dam-mediated methylation can be inferred from processing the same raw methylation data obtained for strains EL20 to EL23 through calculating a different type of ratio from that presented in Fig. 3. Thus, silencer effect is the ratio of the methylation frequency for a given GATC in a strain carrying intact E and I silencers at *HML* to one in which these have been mutated, and this in either of two settings: upon targeting of TetR-Dam to *HML* via $TetO_{112}$ (targeted methylation, solid diamonds), or upon expression of TetR-Dam without any Tet operators to which it may bind (*trans* methylation, open diamonds). For the sake of clarity, *trans* methylation is presented by itself in panel A and targeted methylation is presented together with *trans* methylation in panel B to facilitate comparisons. Sites along chromosome III can be grouped into three classes displaying distinctive behaviors. Sites 8 and 9, within *HML*-I, are in a region where both *trans* and targeted methylation are hampered due to the presence of silenced chromatin. Sites 7 and 10 to 20 yield an average reference level of silencer effect along chromosome III (broken line) that differs between *trans* and targeted methylation settings (hatched and dark lines, respectively). Sites 1 to 6 all appear similarly influenced by their proximity to telomere III-L with regard to both *trans*- and targeted TetR-Dam-mediated methylation and yield a subtelomeric average silencer effect level (solid lines).

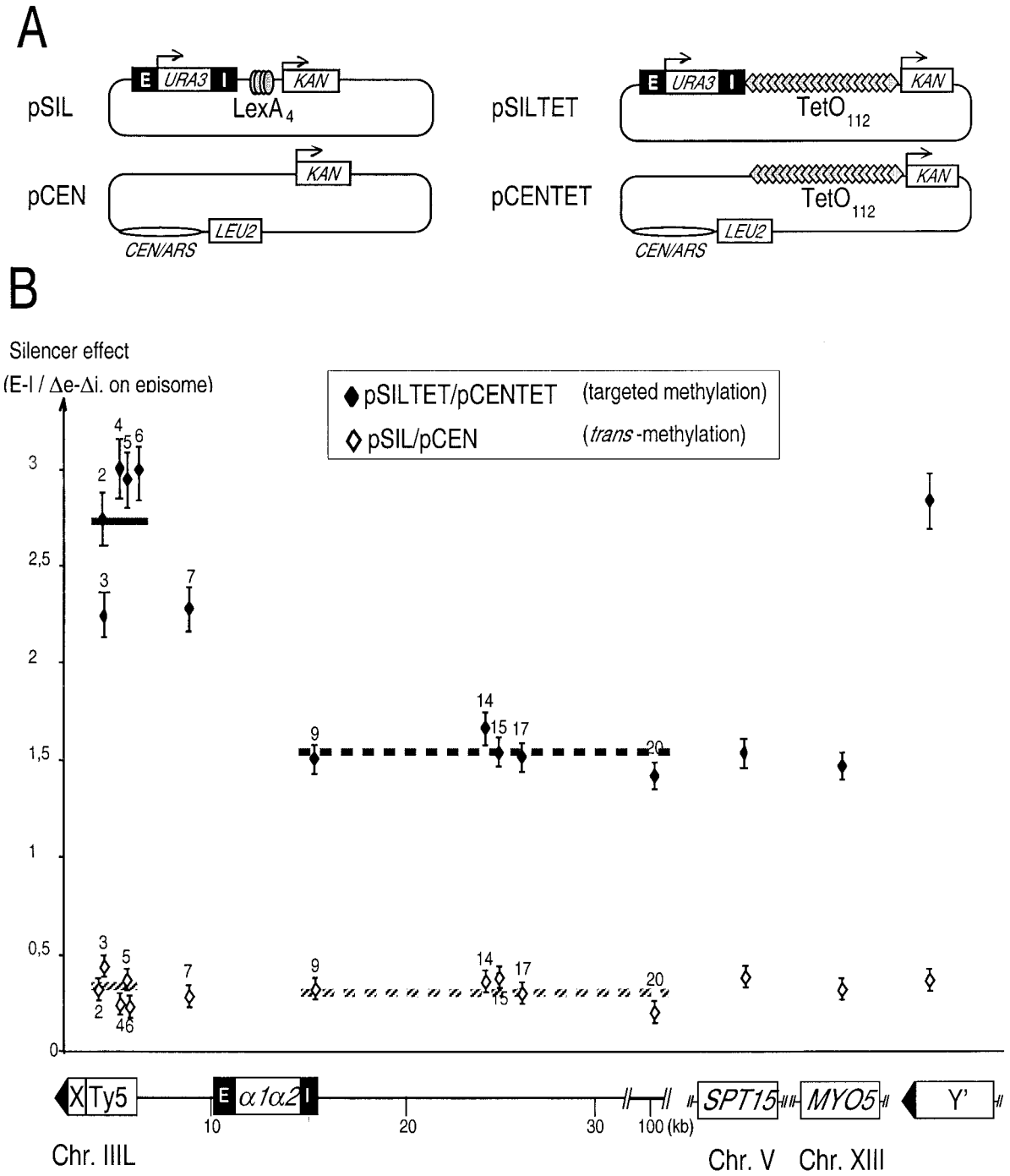


FIG. 5. Plasmid-borne *HML* associates with III-L subtelomere and Y′ chromosome ends. (A) Scheme of the plasmids used in this study. The thin lines indicate bacterial pUC vector sequences. pSIL and pSILTet derive from the same construct used to modify the *HML* locus, described in Fig. 1A. pCENTet includes the $TetO_{112}$ cluster and the *KAN* gene inserted in pRS315, a CEN-ARS vector. pCEN is identical to pCENTet except that it lacks the $TetO_{112}$ cluster. (B) Ratio of methylation frequency with the SIL to the CEN plasmids containing $TetO_{112}$ or not. A diagram of the left arm of chromosome III is shown below the graph, with the positions of the *HML* silencers and the two α1 and α2 mating type genes. Note that in that case, in which plasmids were transformed in the original S150-2B strain and selected for resistance to kanamycin, the chromosomal *HML* locus is not modified. Legend is otherwise the same as for Fig. 4 except that fewer sites were analyzed and reference and subtelomeric silencer effect levels are the averages of values obtained for sites 9, 14, 15, 17, 20, and 2 to 6.

that repression at *HML* is not affected by the expression of a *URA3* reporter gene located between *HML* and telomeres (30).

Many proteins, including Rap1, Sir2, Sir3, and Sir4, are known to associate both with silencers and with telomeres and could potentially bridge these two compartments. Interestingly, a Sir-dependent telomere folding in the immediate subtelomeric region has been described (11, 42). Although this looping occurs over much smaller distances than the interactions

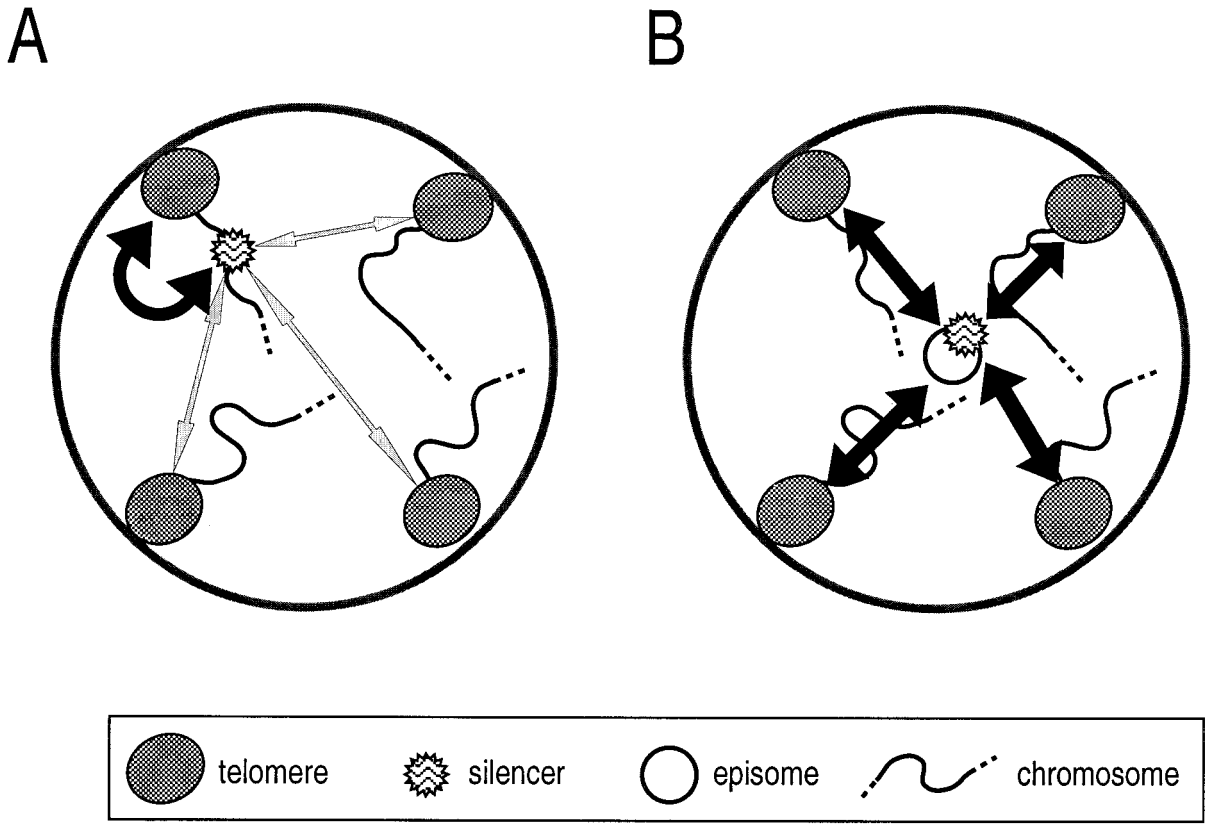


FIG. 6. Model for physical associations between silencers and telomeres. (A) A chromosomal silencer can interact with telomeres (bidirectional arrow) but displays strong preference for a proximal partner. (B) In contrast, a plasmid-borne silencer associates equally with all chromosome ends, and this difference likely reflects its free movement in the nucleoplasm.

described here, both phenomena could possibly rely on the same mechanisms.

In light of the physical associations between telomere and silencer described here, the role of telomeres in silencer-mediated repression certainly needs to be reconsidered. In its simplest form, the reservoir model assumes that silencers can function autonomously as long as the local concentration of silencing factors is sufficient. However, a situation in which a silent locus is located away from the nuclear periphery has never been reported (43), and the establishment of silencing appears to require an intact telomeric compartment (15). The existence of specific telomere-silencer associations points to a more direct role of the telomeric compartment than just as a storage space for silencing factors. The fact that overexpression or delocalization of the Sir proteins can rescue *HML* silencing at nontelomeric sites has been put forward as a strong argument in favor of the reservoir model (30, 32). However, this may also be explained by an improved association of the silencers with telomeres, which would emancipate them from the chromosomal context and mimic the situation observed with silencer-containing plasmids (Fig. 5).

Physical associations might also explain the functional cooperation observed between various types of silencing elements (5, 17, 27). These interactions could involve many of the factors present both at telomeres and at silencer elements. Although the association of silencers with telomeres might be essential for their silencing function, one may imagine that certain silencers may be less telomere dependent than others, requiring less frequent or less prolonged association. This might be the case with *HMR*-E, which appears to serve as a dominant repressor of expression (7, 14, 44, 48). Overall, we propose that telomeres form a platform that facilitates the coalescence of silencers and consequently organize the yeast silencing compartment. Such long-range interactions are likely to play a key role in the establishment and/or maintenance of silenced domains in other organisms like *Drosophila melanogaster* (37).

When *HML* silencers were placed on a plasmid, we observed that these now interacted not only with the Ty5 III-L region but also with other telomeric regions. It is unlikely that the silencers in an episomal state lack some chromatin structure necessary to direct interaction with the III-L telomere, since these can still interact with various telomeres and appear functional in imposing silencing on a reporter gene. Rather, the episome might lack some intrachromosomal constraint that causes *HML* to interact preferentially with its proximal telomere (Fig. 6A). For instance, when carried on a plasmid, the movements of the *HML* region are likely to be less constrained in the nucleoplasm. The sequence would then be free to interact with several, if not all, chromosome ends (Fig. 6B). This is in full agreement with the fact that plasmid-borne silencers are more active than those inserted far away from a telomere (30). Therefore, the dynamic properties of a chromosomal segment determine its capacity to be silenced. In agreement with this idea, it was recently demonstrated that telomeres provide

strong constraints on chromosome movements that contrast with the ability of other parts of the genome to diffuse rapidly in G_1 phase (23).

By using a methyltransferase targeting assay, we showed the existence of direct interactions between *HML* silencers and telomeres. This finding closes the circle of evidence suggesting that functional cooperation between silencing elements in *S. cerevisiae* relies at least in part on direct physical interactions. We have brought direct evidence for the existence of silencing compartments that contain telomeres and *HM* silencers.

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