Myosin-like proteins 1 and 2 are not required for silencing or telomere anchoring, but act in the Tel1 pathway of telomere length control

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Abstract

The positioning of chromosomal domains in interphase nuclei is thought to facilitate transcriptional repression in yeast. It has been reported that two large coiled-coil proteins of the nuclear envelope, myosin-like proteins 1 and 2, play direct roles in anchoring yeast telomeres to the nuclear periphery, thereby creating a subcompartment enriched for Sir proteins. We have created strains containing complete deletions of mlp1 and mlp2 genes, as well as the double null strain, and find no evidence for the disruption of telomere anchoring at the nuclear periphery in these cells. We also detect no disruption of telomere-associated gene silencing. We confirm, on the other hand, that mlp mutants are particularly sensitive to DNA-damaging agents, such as bleomycin. Moreover, we show that rather than having short telomeres as in yKu-deficient strains, the mlp1 mlp2 strains have extended telomeres, resembling phenotypes of mutations in rif1. Whereas the mlp1 mlp2 mutations act on a pathway of telomere length regulation different from that of yKu70, the effects of the tel1 deletion are epistatic to the mlp mutations, suggesting that the Mlp proteins restrict telomere length in wild-type cells by influencing the Rif–Tel1 pathway of telomerase regulation.

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1. Introduction

Yeast telomeres assume a nonrandom distribution in the interphase nucleus (reviewed in Heun et al., 2001c). Although the function of this spatial arrangement is largely unknown, recent evidence suggests that subnuclear compartments contribute to the establishment and maintenance of epigenetic controls over euchromatic gene expression (reviewed in Cockell and Gasser, 1999; Fisher and Merkenschlager, 2002). An example of this is the silencing of genes that occurs in subtelomeric regions in yeast, called TPE,1 or telomeric position effect. The clustering of telomeres at the nuclear periphery appears to facilitate the transcriptional repression by creating zones that contain critically high concentrations of Sir proteins, which are limiting for maximal repression efficiency in yeast (Maillet et al., 1996; Marcand et al., 1996; Renauld et al., 1993). The creation of subnuclear pools or reservoirs of general chromatin repressors may be widely conserved. In flies and higher eukaryotes similar pools of proteins such as heterochromatin protein 1 are observed at the chromocenter, where the repetitive DNA of centromeres form a large cluster in certain cell types (reviewed in Dernburg and Sedat, 1998). As in yeast, it has been proposed that juxtaposition in trans to this repetitive DNA facilitates transcriptional repression (reviewed in Fisher and Merkenschlager, 2002).

The initial observation that budding yeast telomeres form foci adjacent to the nuclear envelope was based on immunostaining of the telomeric repeat-binding protein Rap1p (Klein et al., 1992) and of the silent information regulators Sir3p and Sir4p (Palladino et al., 1993). These
punctate staining patterns were lost under a wide range of conditions that perturb TPE (Cockell et al., 1995; Enomoto and Berman, 1998; Gotta et al., 1996, 1998; Hecht et al., 1995). Recently we have demonstrated that two partially redundant pathways are implicated in the anchoring of yeast telomeres, one requiring the yKu heterodimer and the other requiring the Sir proteins themselves. This second pathway is cell-cycle dependent and correlates with improved repression of subtelomeric genes (Hediger et al., 2002b).

Both Sir proteins and yKu are constitutively bound to the natural yeast telomere in an interdependent manner. Loss of Sir function releases a pool of yKu, and loss of yKu derepresses silent chromatin at telomeres, releasing a pool of Sir proteins (Laroche et al., 1998; Martin et al., 1999). Nonetheless, residual amounts of yKu and of Sir4p remain associated at the telosome in such mutants (Bourns et al., 1998; Luo et al., 2002), perhaps leading to the redundancy of these anchoring pathways. Although it is thought that rigid perinuclear lamina meshwork stabilizes chromatin at the nuclear periphery in higher eukaryotes, yeast has no close homologue to the lamins, which are nuclear members of the intermediate filament family. Since neither yKu nor any of the Sir proteins has an inherent ability to associate with the lipid bilayer, it has been postulated that there must be another structural protein(s) at the nuclear envelope that anchors yeast telomeres.

It was recently reported that a conserved coiled-coil protein, myosin-like protein 2 (Mlp2), was able to bind yKu and that its mutation resulted in a dispersion of yKu70p from perinuclear foci (Galy et al., 2000). Fluorescence in situ hybridization (FISH) experiments using a probe specific for yeast subtelomeric repeats suggested that telomere position was disordered in a strain lacking both Mlp2 and a closely related protein, Mlp1 (Galy et al., 2000). Previous work had shown that Mlp proteins associate with nuclear pores, being restricted to a zone adjacent to the nuclear envelope in yeast (Galy et al., 2000; Kosova et al., 2000; Strambio-de-Castillia et al., 1999). Consistent with this potential link to nuclear pores, it was shown that the general disruption of nuclear pore architecture through mutation of Nup60 or Nup145 also seemed to disrupt telomere anchoring (Feuerbach et al., 2002). Contradictory data have been presented on the status of telomeric repression in mlp mutants, and the disruption of these genes was reported first to fully disrupt TPE (Galy et al., 2000) and later to partially impair silencing in a “tethering” assay (Feuerbach et al., 2002), in which repression is promoted by recruiting a reporter gene to the nuclear envelope through interaction with a nonspecific membrane protein (Andrulis et al., 1998). It was unclear whether the effects reflect a general disruption of nuclear stability or interference with telomere position and function.

To examine the effect of mlp1 and mlp2 deletions on telomeric positioning and repression, we have created complete deletions of each gene and of the two together in several strain backgrounds that are commonly used for the analysis of TPE, analysis of telomere length, and immunolocalization assays. Since FISH is prone to artifacts that arise from nuclear disruption during the labeling procedure (Gotta et al., 1999), we monitor the nuclear diameter through nuclear pore immunofluorescence as a control for nuclear integrity. We see no significant effect of the mlp1 mlp2 double mutant on TPE nor on telomere positioning. Our results are completely consistent with a study based on in vivo time-lapse microscopy of telomeres tagged through the binding of the GFP-lac repressor, a technique that enables the precise measurement of telomere movement in intact cells (Hediger et al., 2002b). We find that mlp mutants are slightly more sensitive than wild-type cells to bleomycin, a radiomimetic reagent that induces DNA strand breaks (Koling et al., 1993), and that telomeres have an extended steady-state length in Mlp-deficient strains. The observed extension of telomeric TG1–3 repeats in mlp mutants correlates with the persistence of both telomere anchoring and TPE and is the opposite of the phenotypes associated with yKu disruption (Boulton and Jackson, 1998; Gravel et al., 1998; Laroche et al., 1998; Polotnianka et al., 1998). Although the natural anchor for yKu remains unidentified, our data argue that it is unlikely to involve the myosin-like proteins.

2. Materials and methods

Standard culture conditions at 30 °C were used unless otherwise indicated. Repression assays on 0.1% 5-fluoro-orotic acid (5-FOA) were performed as described in Gotta et al. (1998).

Complete hdf1, mlp1, mlp2, and tel1 deletions were obtained using the PCR-based gene deletion technique with primers that were within 100 bp of the beginning and end of each gene (Longtine et al., 1999). Gene deletions were performed with the following oligo nucleotides: SG-741 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-743 HDF1, TTTCATGATTTAGGGATT GCTTTAAGGTAGCTACAAATAGCGGATCCCCC CGGTTAAATTA; SG-721 MLP1, TTACCGCAGCGG AGAGATCCACGTTAATTCAATTTTATTGATCG GTAACTTGAGCTTGTTAAATTAA; SG-722 MLP1, TTACCGCAGCGG AGAGATCCACGTTAATTCAATTTTATTGATCG GTAACTTGAGCTTGTTAAATTAA; SG-722 MLP1, TTACCGCAGCGG AGAGATCCACGTTAATTCAATTTTATTGATCG GTAACTTGAGCTTGTTAAATTAA; SG-718 MLP2, TTTCATGATTTAGGGATT GCTTTAAGGTAGCTACAAATAGCGGATCCCCC CGGTTAAATTA; SG-743 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-743 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-741 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-743 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-741 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG; SG-743 HDF1, CGCACTTAAACTTGCATCTGTGAA AACACGACCTTTGCAATGATTTGATGATA ATCG. 
Deletions were confirmed by PCR and independent phenotypes were checked when applicable (loss of TPE, temperature-sensitivity, DNA damage sensitivity). MLP1 and MLP2 deletions were also confirmed by Southern blot (see Fig. 2). All strains used are indicated in Table 1. LM11 was originally created in a W303 background and is the same as that used by Feuerbach et al. (2002).

FISH and immunofluorescence (IF) assays were performed on exponentially growing cells that were subjected to fixation in 3.7% formaldehyde prior to spheroplasting, followed by staining using affinity-purified rabbit antisera whose specificities have been previously characterized (Gotta et al., 1996) or the anti-pore MAb414 monoclonal (BABCO, Berkeley, CA). The detailed protocols are described in Gotta et al. (1999) and Heun et al. (2001a).

3. Results

3.1. Telomere localization is not affected in mlp mutants

FISH studies have suggested that the perinuclear clustering of yeast telomeres depends on two groups of proteins, the yKu70/80 heterodimer (Laroche et al., 1998) and the two coiled-coil proteins Mlp1p and Mlp2 (Feuerbach et al., 2002; Galy et al., 2000). The facts that Mlp proteins localize to the inner face of the nuclear envelope (Kosova et al., 2000; Strambio-de-Castilla et al., 1999) and that Mlp2 can interact with yKu70p led to an attractive model in which telomeres are tethered to the periphery of the nucleus through an interaction of yKu with Mlp1p and Mlp2p, which in turn bind the nuclear pore protein Nup145p (Feuerbach et al., 2002; Galy et al., 2000). However, the effects observed in situ hybridization assays were partial, and controls for nuclear integrity were lacking (Galy et al., 2000). It is not clear whether the partial effects are simply an indication of redundant pathways for telomere anchoring or whether the apparent loss of anchoring reflects limitations of the techniques used.

If Mlp proteins and yKu were to act on the same pathway, as proposed by Galy et al. (2000), mlp mutations should have defects in telomere localization similar to those documented for yKu-deficient strains (Hediger et al., 2002b; Laroche et al., 1998; Maillet et al., 2001). To examine this, we have characterized telomere position by FISH with a subtelomeric and TG1–3-containing probe, in mlp-deficient strains, taking special care to preserve nuclear architecture throughout the assay (Heun et al., 2001a). During the preparation of cells for FISH, we noted that mlp mutant nuclei have a tendency to enlarge and eventually burst under in situ hybridization conditions. To ensure that we score only intact nuclei, cells were stained for nuclear pore components, enabling us to follow nuclear shape and size through the procedure. Scoring intact cells, we observe very little change in the number of telomeric foci and their distribution in relation to nuclear pore staining (Fig. 1A).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1B</td>
<td>MATα ura3-1 trpl-1 ade2-1 leu2-3,112 his3-11,15 can1-100</td>
<td>R. Rothstein</td>
</tr>
<tr>
<td>LM11</td>
<td>W303-1B adh4::ura3-TelVII-L lys2::hml::E-ADE2-I</td>
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<tr>
<td>GA1469</td>
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<td>This study</td>
</tr>
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</tr>
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<td>ura3-52 lys2-801 ade2-101 trp1-63 his3-200 leu2-1 ppn1::HIS3 adh4::ura3-TelVII-L Vp::ADE2-Tel (also called GA503)</td>
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<td>RS543 mnp133::URA3</td>
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Fig. 1. Deletion of *mlp1* and *mlp2* does not alter telomere distribution by Y' FISH in intact nuclei. (A) In situ hybridization with a probe recognizing TG₁₋₃ and Y' sequences and immunofluorescence with affinity-purified polyclonal antibodies specific for Sir4p were performed on wild-type LM11 (wt; Maillet et al., 2001) and an isogenic strain deleted for *mlp1* and *mlp2* (GA1471, *mlp1* *mlp2*). Telomeres and Sir4p signals are in green, while nuclear pore staining (Mab414) is in red. Shown are confocal images taken of the equatorial plane of yeast nuclei that are intact after the staining procedure. Spread or flattened nuclei were not analyzed. The insets show enlargements of three cells typical for each staining condition. Cells were prepared for IF/FISH as described under Materials and methods. Bars, 2 µm. (B) Position of telomeric foci was analyzed by assigning each focus within a wild-type or *mlp1* *mlp2* strain to a peripheral (p) or internal (i) zone, each containing half of the nuclear surface of the given focal plane. Percentage of foci in each zone is represented in a bar graph for wild-type (white) or *mlp1* *mlp2* (gray). A random distribution would be 50–50. For each strain, 100 cells were counted. (C) Nuclear diameters based on the nuclear pore staining (Mab414, see Materials and methods) were measured in wild-type or *mlp1* *mlp2* strains after FISH treatment and selection for intact and round nuclei (see A; gray bars). As a control for nuclear diameter in living cells we monitored the nuclear diameter based on the GFP-Nup49 fluorescence in appropriated modified wild-type or *mlp1* *mlp2* strains (GA1459 and GA1731, respectively, white bars). The average diameter is given in µm.
The number of foci per cell is increased by a factor of only 1.2 in the \textit{mlp1 mlp2} mutant, and the percentage of telomeric foci in an outer zone that contains half of the nuclear surface is not significantly different from the wild-type value (67\% for the wt and 73\% for the \textit{mlp1 mlp2} mutant; Fig. 1B). Consistent with the lack of telomere displacement in the \textit{mlp1 mlp2} mutant strain, we observe no significant change in the focal pattern of Sir4p localization (Fig. 1A).

The difference between our data and those previously published (Feuerbach et al., 2002; Galy et al., 2000) could be explained by a general instability of \textit{mlp1 mlp2} nuclei in cells that are exposed to spheroplasting and FISH protocols. To test this we scored the diameter (based on nuclear pore staining with Mab414; BABCO) of intact nuclei of both wild-type and \textit{mlp1 mlp2} strains after FISH. The nuclear diameter in these fixed and spheroplasted \textit{mlp} mutant cells is larger than that in identically treated wild-type cells (average wt nucleus diameter is 2.06 ± 0.17\,μm, whereas it is 2.45 ± 0.34\,μm in \textit{mlp1 mlp2} mutant, Fig. 1C). Moreover, the frequency of flattened or spread nuclei, which are not counted in our analyses, is significantly elevated in the mutant. On the other hand, if we score for the average diameter of the nucleus in living cells using the direct fluorescence of a GFP-Nup49 fusion (Heun et al., 2001a) in \textit{mlp1 mlp2} mutant strains, we detect no difference in nuclear diameter (open bars, Fig. 1C). These results suggest that loss of the two Mlp proteins renders the nuclear envelope more fragile, such that it distorts or disrupts during the IF and/or FISH protocol. This underscores the fact that pore staining and measurement of the nuclear diameter is an essential control in studies of nuclear organization. We have previously shown that permeabilization of the nuclear envelope during spheroplasting readily induces the spreading of chromatin and perturbs its spatial organization. Furthermore, mechanical stress on spheroplasted wild-type cells results in the spreading of nuclear contents, leaving telomeric foci more numerous and more dispersed (Heun et al., 2001a).

Because we saw no significant differences between wild-type cells and the \textit{mlp} deletion strains, we have checked that we have actually eliminated the full \textit{MLP1} and \textit{MLP2} reading frames at the respective chromosomal loci by Southern blot. The bands detected by Southern confirm that this is the case (Fig. 2).

3.2. Telomeres do not cluster with pores in \textit{nup133} mutants

\textit{Nup133} is a conserved and integral nuclear pore component that is nonessential for cell growth at 24 or 30\,°C (Doye et al., 1994). In \textit{nup133}-disrupted yeast strains the nuclear pores cluster constitutively at one side of the nucleus, although this can be reversed by inducing intact Nup133p (Belgareh and Doye, 1997). If telomeres are localized to the nuclear periphery through association with nuclear pores, as suggested by Galy et al. (2000), then one would predict that telomeres

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig2.pdf}
\caption{Confirmation of \textit{MLP} gene deletion by Southern. The complete deletion of \textit{MLP1} and \textit{MLP2} genes is demonstrated by Southern hybridization of total genomic DNA digested with \textit{BglII} or \textit{ScaI}, respectively. Samples were run on a 0.8 or 1\% agarose gel, transferred to a nitrocellulose membrane, and blotted with a probe containing \textit{MLP1} (probe 1, 4114–5620) or \textit{MLP2} (probe 2, 3724–5036). Digestion of a wild-type \textit{MLP1} locus with \textit{BglII} produces a 5.6-kb fragment containing the 3.2-kb C-terminal fragment of \textit{MLP1}, while the deletion strain (\textit{mlp1}) should yield a 3.8-kb fragment containing only 30 bp of the very C-terminal part of the \textit{MLP1} gene. This small fragment is not revealed by blotting with probe 1, but correct insertion of the selectable marker into the \textit{MLP1} locus was tested by PCR. Digestion of a wild-type \textit{MLP2} locus with \textit{ScaI} produces a 5.4-kb fragment containing the 4.3-kb C-terminal part of \textit{MLP2}, while the deletion strain (\textit{mlp2}) yields a 1.4-kb fragment retaining only the extreme C-terminal 60 bp of \textit{mlp2}. Here and throughout, the nomenclature \textit{mlp1/2} indicates the complete deletion of both \textit{mlp1} and \textit{mlp2}.}
\end{figure}
would cluster with pores in the nup133 mutant. Alternatively, if Nup133 itself were part of the anchor, one might expect telomeres to become dispersed in the mutant. We tested this by immunostaining telomeres with affinity-purified anti-Rap1 and anti-Sir4 antibodies, while counterstaining nuclear pores (Nup116 and Nup96, Mab414) in both wild-type and nup133 strains. We confirm that nuclear pores do cluster in one or two zones of the nuclear envelope (Fig. 3; Doye et al., 1994). Telomeres, however, remain distributed around the nuclear periphery in bright foci that only rarely colocalize with the clustered pores. They maintain a distribution largely indistinguishable from that seen in wild-type cells (compare Figs. 3a and d with insets). Since neither the pore-associated Mlp proteins nor the clustering of nup133-deficient pores affects yeast telomere distribution, we find it unlikely that nuclear pores play a direct role in telomere positioning.

3.3. Disruption of MLP genes does not perturb repression at telomere-proximal reporters

Several lines of evidence suggest that transcriptional repression by Sir proteins is influenced by the spatial position of the silencer-containing locus (Andrulis et al., 1998; Maillet et al., 1996). Consistent with the proposed positioning defect of mlp1 mlp2 mutants, it was reported that subtelomeric reporter genes were derepressed in the absence of these two proteins (Galy et al., 2000). Because we detected no delocalization of Sir proteins (Fig. 1), it seemed unlikely to us that telomeric silencing would be compromised in these mutants. The LM11 strain used above allows us to monitor subtelomeric transcription levels through the expression of a reporter gene, URA3. URA3 expression can be tested by plating cells on 5-FOA; in the presence of the URA3 gene product cells convert 5-FOA to a toxic compound (5-fluorouracil), impeding growth. Thus, transcriptional silencing of URA3 enables cells to grow on 5-FOA (see wt strain, Fig. 4A).

Compared to the wild-type strain, the disruption of HDF1 (which encodes yKu70p) dramatically reduces URA3 repression, leading to very little cell growth on 5-FOA. In contrast, the single and the double mlp mutants have wild-type levels of telomeric repression (Fig. 4A). To test whether this lack of effect is strain- or telomere-specific we scored TPE levels in a second background (UCC3505; Singer and Gottschling, 1994) that contains both a URA3 and an ADE2 reporter integrated at telomeres VII-L and V-R, respectively. The repression of ADE2 allows cells to accumulate a red pigment that is not generated in ADE2⁺ cells, which form uniformly white colonies. The variegated repression of a subtelomeric ADE2 gene gives rise to red and white sectored colonies. In this UCC3505 background, we again see that growth on 5-FOA is as efficient for mlp1⁻, mlp2⁻, and mlp1 mlp2⁻ deficient strains as for wild-type cells (Fig. 4B), and all combinations of mlp mutants show a red/white sectorsing typical of subtelomeric ADE2 repression (Fig. 4B). In conclusion, and in agreement with the normal focal staining shown in Fig. 1 for telomeric FISH and Sir4p IF, we see no defect in telomeric re-

Fig. 3. Mutation of nup133 does not affect telomere position nor Sir4p localization. Immunofluorescence was performed on fixed cells using affinity-purified polyclonal antibodies specific for Rap1p (a), Sir4p (d), and the monoclonal Mab414 (b, e) that recognizes the pore proteins Nup116 and Nup96. Strains used were either wild type (RS453, shown in insets) or an isogenic strain deleted for nup133 (GA-380; Doye et al., 1994). In the merged images (c, f) Rap1p and Sir4p signals are in green, while nuclear pore staining (Mab414) is in red. Cells were grown at 24°C and were prepared for IF as described under Materials and methods. Bars, 2µm.
pression in either single or double mlp mutants, at two different telomeres and in different genetic backgrounds.

3.4. Reestablishment of silencing is not affected by mlp mutations

It was reported that mlp1, mlp2, and mlp1 mlp2 deletions are more deficient in an assay that monitors the reestablishment of subtelomeric repression, rather than its maintenance (Feuerbach et al., 2002). In this study, the authors grew cells on synthetic medium lacking uracil to force derepression of the subtelomeric URA3 gene and subsequently plated onto synthetic complete medium (SC) and SC containing 5-FOA to monitor the efficiency of URA3 repression. We performed this assay as described, but still saw no effect of any combination of mlp deletion on either the establishment or the maintenance of telomeric repression (Fig. 5). The absence of an effect was also observed when YPAD containing 5-FOA was used for the plating assay.

In the mlp mutants we do detect an enhanced repression of an ADE2 reporter that is flanked by silencers and inserted at the LYS2 locus, located >200 kb from a telomere, as previously reported (Maillot et al., 2001). In the latter two mutants, internal silencing was attributed to a well-documented redistribution of Sir proteins from telomeres that occurs in yKu mutants, allowing a more efficient recruitment of Sir proteins to internal sites. We performed the internal ADE2 silencing assay in the same strain background used to monitor silencing (Fig. 4) and Sir protein localization (Fig. 1). Both these assays reveal no significant delocalization of Sir proteins from telomeres in strains lacking the Mlp's. Thus, we conclude that the
phenotype of enhanced repression of the internal ADE2 reporter observed in the mlp mutants reflects an indirect effect, such as a decrease in RNA Pol II activity or an increase in Sir protein levels.

3.5. The loss of Mlp proteins affects cellular resistance to bleomycin

The double mlp1 mlp2 disruption has been shown to have growth defects and irregular-shaped colonies and to be sensitive to DNA-damaging agents such as UV irradiation and bleomycin (Galy et al., 2000; Kolling et al., 1993; Kosova et al., 2000). As our data do not agree with the published data on the telomeric effects of mlp mutations, we examined whether we could reproduce the reported DNA-damage sensitivity. We tested resistance to bleomycin by plating wild-type and hdf1 or mlp mutants on YPAD alone or YPAD containing 20 mU/ml bleomycin. We found that cells carrying the single mlp1 mutation are more sensitive to DNA damage than wild-type cells, while the mlp2 mutant is not (Fig. 6). Furthermore, the double mutant is significantly less viable than either single mutant in the presence of bleomycin. Whether this enhanced sensitivity reflects interference in a checkpoint pathway, enhanced chromosome loss, or altered permeability to the drug is unknown.

3.6. Mlp1 and Mlp2 negatively regulate telomere length

yKu is thought to regulate telomere length both by protecting chromosome ends from degradation and by recruiting telomerase (reviewed in Dubrana et al., 2001). yKu mutants have very short telomeres with long extensions of the G-rich strand that persist throughout the cell cycle (Gravel et al., 1998). We examined telomere length in MLP mutants and found that deletion of either MLP1 or MLP2 resulted in telomeres that are consistently ~50 bp longer than wild type (Fig. 7A). The effect of the two genes is additive, since the disruption of both leads to even longer telomeres (100 bp longer than wild type, Fig. 7A). It is noteworthy that the loss of Mlp proteins affects telomere length maintenance in a way opposite to that of loss of yKu, again suggesting that the two factors act on different pathways. Mlp proteins seem either to impair telomere elongation or to promote telomere degradation, whereas yKu does the opposite.

To determine if the mlp effect is epistatic to the yKu pathway, we combined the hdf1 mutants with deletions...
of mlp1, mlp2, or both genes. In all three combinations, the telomeres are longer than those in an isogenic strain containing the hdf1 mutation alone, indicating that the pathway that leads to telomere lengthening in the absence of Mlp function is independent of yKu (Fig. 7A).

Only a few mutations have been shown to lead to telomere lengthening. Among these are the elimination of Pif1p, a 5′–3′ DNA helicase (Zhou et al., 2000), and mutations or deletions in the C-terminus of the yeast telomere repeat binding protein, Rap1 (Lustig et al., 1990). The deletion of two factors that interact with Rap1C, Rif1p, and Rif2p (Hardy et al., 1992; Wotton and Shore, 1997) has the same effect. The Rap1p–Rif1/2 pathway senses the length of the telomeric TG1–3 repeat through Rap1 binding sites and restricts telomere length through a feedback inhibition of telomerase (Marcand et al., 1997). It has been shown genetically that the Rif1p counting mechanism requires Tel1p, a homologue of the human ATM kinase that was shown to positively regulate telomerase (Ritchie and Petes, 2000; Ritchie et al., 1999). Strains carrying a deletion of TEL1 have short but stable telomeric repeats. Importantly, Tel1p and yKu effects on telomere length regulation have been shown to be independent (Gravel et al., 1998).
To see if the telomere lengthening due to the loss of Mlp’s might be acting through the inhibition of Tel1p, we tested combinations of the mlp1 and/or mlp2 mutations in a tel1 deletion strain (Fig. 7B). The elimination of either mlp gene alone did not alter the short telomere phenotype of the tel1 strain, and a very minor extension of the shortened telomere was detectable upon disruption of both mlp genes. The effect of mlp1 mlp2 disruption was significantly less in the tel1 background than in a wild-type context or in the hdf1 mutant (Fig. 7B); that is, telomeric repeats in the tel1 mlp1 mlp2 mutant extend ~40 bp, while we see an increase >100 bp when mlp1 and mlp2 are deleted in wt strains. In conclusion, the regulation of telomere length by Mlp proteins is independent of yKu, but is at least partially dependent on Tel1p. We do not know whether this reflects direct or indirect interactions. It is likely that Mlp’s do not act exclusively through Tel1p, however, for the effect of the triple deletion of tel1 mlp1 mlp2 on TPE is more pronounced than that of the tel1 mutation alone (data not shown).

4. Discussion

The mechanisms that position chromosomes in the interphase nucleus are still essentially unknown. While chromosomes tend to occupy discrete zones in mammalian nuclei (Cremer et al., 1993; Marshall, 2002), it is not clear to what extent chromosomal position is fixed in simpler organisms. Even in complex organisms it has been difficult to determine whether the spatial organization of chromatin is conserved from cell to cell. In budding yeast the best evidence for a nonrandom organization comes from the detection by FISH of repetitive domains of chromatin—notably the rDNA within the nucleolus and the telomeric repeats which are grouped in clusters near the nuclear envelope (reviewed in Heun et al., 2001c). In fission yeast and in Drosophila, satellite-containing centromeres also cluster in interphase nuclei (Cook and Karpen, 1994; Funabiki et al., 1993).

Recent studies have also shown that internal regions of yeast and Drosophila chromosomes are highly mobile in interphase nuclei, with frequent displacements of >0.5 μm within seconds (Heun et al., 2001b; Vazquez et al., 2001). On the other hand, yeast telomeres and centromeres were seen to impose constraints on this movement in both G1 and S phase (Heun et al., 2001b; Marshall et al., 1997). Using live GFP fluorescence and quantitative microscopy, we have recently shown that yKu is a key element in the anchorage of yeast Tel VI-R at the nuclear periphery (Hediger et al., 2002b). GFP-tagged telomeres do not remain attached to the nuclear envelope in strains lacking yKu and move randomly throughout the nucleus. The deletion of rif1 in the hdf1-null background has revealed a second anchoring pathway that correlates with the restoration of TPE (Hediger et al., 2002b). This second pathway requires Sir proteins and is partially redundant with that mediated by yKu. The yKu pathway is able to anchor telomeres in both G1 and S phase, while Sir proteins or silenced chromatin function most efficiently in S phase. Using live microscopy, no role could be demonstrated for Mlp proteins in either anchoring pathway for Tel VI-R (Hediger et al., 2002b).

4.1. Mlp proteins do not anchor telomeres

The current data show that the absence of an anchoring defect in a double mlp1 mlp2 mutant is true not only for a GFP-tagged telomere but also for the bulk of yeast telomeres, as detected by Y’ and TG1–3 FISH. In the strains used here, roughly 70% of the chromosomal ends contain Y’ repeats, but by combining this with a TG1–3 repeat, we are likely to detect all telomeres. Our FISH data are in direct contradiction with results reported (Feuerbach et al., 2002; Galy et al., 2000). We suggest that the partial delocalization observed by others is due to the disruption of nuclear integrity during the hybridization procedure. This is supported by data showing that mlp-deficient nuclei expand more under conditions of FISH than wild-type nuclei (Fig. 1C). The lack of telomere delocalization is consistent with data showing that the double mlp1 mlp2 cells are fully competent for TPE (Fig. 4) and have slightly longer than wild-type terminal TG1–3 repeats (Fig. 7). Sir proteins also retain their localization in perinuclear foci in the mlp1 mlp2 deletion strain, a distribution that correlates consistently with the efficient telomeric repression. We are unable to explain the published data showing a loss of TPE (Galy et al., 2000). Their reported delocalization of Sir3p may reflect the use of a GFP-Sir3 protein, since fusions to either the N- or the C-terminus of Sir3p are known to partially impair its function (S.M.G., unpublished results).

Given their perinuclear localization, Mlp proteins may be involved in some aspect of nuclear envelope organization, but we believe there is no convincing evidence that they mediate the positioning or anchoring of native telomeres. If the Mlp proteins play any role in anchoring, it must be fully redundant with another pathway. We also find little support from our studies for the model that telomeres are physically associated with nuclear pores, since nuclear pore complexes can be clearly distinguished from telomeric foci in both mutant and wild-type cells, and mutations that lead to a displacement of pores do not delocalize telomeres (Fig. 3; Gotta et al., 1996).

A few years ago, an imaginative study from the Sternaglantz laboratory showed that by artificially “tethering” a reporter gene to the yeast nuclear envelope by a
membrane-spanning polypeptide encoded by YIF1, transcriptional repression could be favored (Andrulis et al., 1998). This required the presence of a silencer, the specific regulatory element that nucleates Sir-dependent repression. The membrane-targeted repression assay was recently used to argue for a role of Mlp proteins in telomere clustering, since a strain lacking the Mlp proteins was partially compromised for Yif1p-mediated silencing (Feuerbach et al., 2002). Our data show that neither telomeres nor Sir proteins are delocalized in mlp mutants, leading one to wonder if perhaps the Yif1 fusion is mislocalized in the absence of Mlp proteins (discussed in Hediger and Gasser, 2002a). We predict that the yeast nuclear periphery is more complex than is generally imagined and that Mlp proteins may be involved in the organization of domains that are not directly involved in telomere positioning, but which influence Yif1p distribution.

The tethering of chromatin at the nuclear envelope is a universal phenomenon that has long been observed in higher eukaryotes (Goldman et al., 2002; Marshall et al., 1996). In mammalian nuclei the perinuclear lamina meshwork is bound to the nuclear membrane bilayer through lamin receptors, and the points of contact within chromosomes are thought to occur at irregularly spaced sites along the chromosomal arm. Unfortunately, there is to date no molecular characterization of these sites. Yeast has no homologue of the nuclear lamin protein, a fact that is also true for higher plant cell nuclei (Gindullis and Meier, 1999; Meier et al., 1996), and the absence of this perinuclear intermediate filament network may be responsible for the lability of nuclei in yeast peroxisome mutants.

### 4.2. Mlp proteins affect telomere length regulation

We show here, nonetheless, that mlp mutants do have an effect on telomere length maintenance (for reviews see Dubrana et al., 2001; Shore, 2001). In mlp-deficient strains, telomeres are extended more than 100 bp, precisely the opposite of the changes seen in yKu-deficient strains. While the lengthening observed in mlp mutants occurs independent of yKu, the extension is at least partially dependent on Tel1p, a PI3-like kinase implicated in both a checkpoint response and telomerase regulation (Mallory and Petes, 2000; Ritchie and Petes, 2000; Ritchie et al., 1999). Since Tel1p is required for the downregulation of telomerase signaled by Rap1p and Rif1p binding, the Mlp proteins may directly interact with either Tel1p or Rif1p, modulating its activity. A connection between Mlp proteins and Tel1p may also be at the root of the hypersensitivity to DNA-damaging reagents (Kolling et al., 1993), shown here in Fig. 6. Consistent with earlier data, we are able to fully separate the length of the telomeric TG1–3 tract from silencing efficiency by the analysis of the mutants shown here: tell and mlp1 mlp2 mutants both retain TPE, although the former mutation shortens TG1–3 tracts and the latter extends them.

Our vision of yeast nuclear envelope structure to date consists of pores, telomeric foci, and membrane anchorage sites, but it must certainly contain differentiated zones that facilitate more diverse nuclear activities (Hediger and Gasser, 2002a). For instance, telomere replication may require peripheral domains different from those required for transcriptional repression. While a global disruption of pore organization may influence multiple perinuclear events, one must look carefully at how differently the individual domains and regulatory events are independently organized, to be able to define what differentiates one peripheral zone from another on a molecular level. Such information may also reveal why the silencing at clustered telomeres is different from that of reporters that are anchored to the periphery by nonspecific membrane domains (Hediger and Gasser, 2002a).

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