yKu70/yKu80 and Rif1 Regulate Silencing Differentially at Telomeres in Candida glabrata

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Candida glabrata, a common opportunistic fungal pathogen, adheres efficiently to mammalian epithelial cells in culture. This interaction in vitro depends mainly on the adhesin Epa1, one of a large family of cell wall proteins. Most of the EPA genes are located in subtelomeric regions, where they are transcriptionally repressed by silencing. In order to better characterize the transcriptional regulation of the EPA family, we have assessed the importance of C. glabrata orthologues of known regulators of subtelomeric silencing in Saccharomyces cerevisiae. To this end, we used a series of strains containing insertions of the reporter URA3 gene within different intergenic regions throughout four telomeres of C. glabrata. Using these reporter strains, we have assessed the roles of SIR2, SIR3, SIR4, HDF1 (yKu70), HDF2 (yKu80), and RIF1 in mediating silencing at four C. glabrata telomeres. We found that, whereas the SIR proteins are absolutely required for silencing of the reporter genes and the native subtelomeric EPA genes, the Rif1 and the Ku proteins regulate silencing at only a subset of the analyzed telomeres. We also mapped a cis element adjacent to the EPA3 locus that can silence a reporter gene when placed at a distance of 31 kb from the telomere. Our data show that silencing of the C. glabrata telomeres varies from telomere to telomere. In addition, recruitment of silencing proteins to the subtelomeres is likely, for certain telomeres, to depend both on the telomeric repeats and on particular discrete silencing elements.

Candida glabrata is a common opportunistic fungal pathogen of humans, accounting for ca. 12% of Candida bloodstream infections worldwide (30, 33, 38). Phylogenetically, C. glabrata is closely related to S. cerevisiae in infections worldwide (30, 33, 38). Phylogenetically, C. glabrata is closely related to S. cerevisiae and C. glabrata genome.

C. glabrata is able to adhere tightly to mammalian epithelial cells both in vivo and in vitro. This attribute is thought to be important for Candida species to establish infection. Adherence of C. glabrata to mammalian epithelial cells in culture is mediated primarily by the adhesin Epa1, a glycosylphosphatidylinositol-anchored cell wall protein. EPA1 is a member of a large gene family: in strain BG2 of C. glabrata we have characterized at least 23 paralogues of EPA1, all encoding proteins highly related to Epa1 in the N-terminal ligand-binding domain. Interestingly, most of these EPA genes are encoded at regions immediately adjacent to the telomeres, where they are transcriptionally silenced (9, 11). Transcription of at least some of subtelomeric EPA genes can be derepressed by certain environmental signals, including limitation for vitamin precursors of NAD+ (12).

In eukaryotes, the telomeric region is condensed into a heterochromatin structure through the interaction between the telomere sequences and several protein factors that form non-nucleosomal protein-DNA complexes called telosomes (41). In S. cerevisiae the telomeres are around 350 bp in length and consist of short heterogeneous tandem repeats with the consensus sequence C1-3A/TG1-3 (10, 42). Rap1 and the Ku heterodimer, yKu70/yKu80 (encoded by HDF1 and HDF2, respectively) bind to telomeric DNA. Rap1 binds in a sequence-specific fashion to the telomeric repeats, whereas the yKu70/yKu80 complex binds to the telomere ends in a sequence-nonspecific fashion (17, 25). Additional proteins localize to the telomere through their interaction with Rap1; these include the silencing proteins Sir2, Sir3, and Sir4 and the telomere-length regulatory proteins Rif1 and Rif2 (6, 35).

Adjacent to the telomere repeats, the DNA is organized into nucleosomes in a heterochromatin-like conformation. The formation of subtelomeric heterochromatin in S. cerevisiae is modeled to initiate when Rap1 binds to the telomeric tracts (about 10 to 20 Rap1 molecules per telomere) (10), which recruits Sir3, Sir4, and Sir2 (a NAD+-dependent histone deacetylase). Sir2 catalyzes the deacetylation reaction of the amino-terminal tails of histones H3 and H4. Sir3 and Sir4 then spread to the adjacent chromatin through interactions with the deacetylated histones (20, 37). In S. cerevisiae, it has also been demonstrated that binding of yKu70/yKu80 to the telomere facilitates the recruitment of Sir3 and Sir4 (17, 23, 25, 34, 39), and in fact the yKu70/yKu80 complex is required for silencing at all telomeres tested to date (4, 23, 25, 26, 31). The concerted assembly of heterochromatin can propagate from 1 to 4 kb from the end of the telomere, and genes located in this region are consequently transcriptionally silenced. This silencing, also known as telomere position effect (TPE), decreases precipitously with distance from the telomere repeats (1, 16, 32). All
of the proteins mentioned above play important roles in TPE. In particular, S. cerevisiae mutants in RAP1, SIR2, SIR3, SIR4, HDF1, or HDF2 exhibit decreased levels of subtelomeric silencing (1, 4, 16, 26).

In S. cerevisiae the Rif proteins compete with Sir3 and Sir4 for Rap1 binding and therefore have a negative effect on TPE (19, 40). It is thought that deletion of either Rif1 and/or Rif2 results in increased TPE because, first, more Rap1 is available to interact with Sir proteins in the absence of competition from Rap1-Rif1 or Rap1-Rif2 interactions and, second, because loss of Rif proteins leads to telomere elongation and the consequent ability to recruit additional Rap1-Sir complexes to the telomeres (14, 22, 40). Genetically, Rif1 and Rif2 are antagonized by yKu70/80 in TPE, since mutations in Rif1 and Rif2 restore wild-type levels of TPE to hsf1 and hsf2 mutants (26, 27).

Previously, we and others showed that in C. glabrata the subtelomeric silencing of several EPA genes depends on the functions of the homologues of Rap1, Sir3, Sir4, and Rif1, since null mutations in SIR3, SIR4, and Rif1 and deletion of the C-terminal 28 amino acids of Rap1 leads to expression of many EPA genes, resulting in a hyperadherent phenotype when cells are grown under standard laboratory conditions (9, 11, 21). Interestingly, Rif1 may have a different role in C. glabrata subtelomeric silencing than has been characterized in S. cerevisiae. C. glabrata rif1 Δ mutants display loss of silencing and increased expression of multiple EPA genes. This stands in contrast to S. cerevisiae rif1 Δ strains which show an increase in subtelomeric silencing as assayed by expression of the reporter gene URA3 placed at a truncated telomere (7, 9, 11, 19, 21, 22).

We describe here a systematic analysis of the role of Sir2, Sir3, Sir4, Rif1, yKu70, and yKu80 on subtelomeric silencing in four individual telomeres in C. glabrata. Our data show that the function of Sir2, Sir3, Sir4, and Rif1 are absolutely required for TPE and/or TPE at all of the telomeric regions tested. However, the requirement for Rif1, yKu70, and yKu80 varies between subtelomeric regions. In addition, we mapped a cis-acting DNA sequence located 1 329 kb upstream from the start of EPA3, which functions as a discrete silencing element and which could contribute to subtelomeric silencing at chromosome E. Together, these data suggest that the silencing landscape of C. glabrata telomeres is not homogenous and that unique sequence-specific characteristics of individual telomeres influence subtelomeric silencing and potentially the normal regulation of different EPA genes.

MATERIALS AND METHODS

Strains. All strains used in the present study are listed in Table S1 in the supplemental material.

Plasmids. All of the plasmids used in the present study are summarized in Table S2 in the supplemental material.

Oligonucleotides. All of the primers used in the present study are listed in Table 1.

Media. Yeast were grown on standard yeast media as described previously (36) with 2% agar added for plates. Synthetic complete (SC) contains yeast nutrient broth without NH4SO4 at 1.7 g/liter and NH4SO4 at 5 g/liter and supplemented with 0.6% of Casamino Acids and 2% glucose. When needed, SC was supplemented with 25 mg of uracil/liter. To score for resistance to 5-fluoroorotic acid (5-FOA; Toronto Research Chemicals, North York, Canada), 0.9 g of 5-FOA and 25 mg of uracil/liter were added to the SC. Yeast extract-peptone-dextrose (YPD) medium contains yeast extract at 10 g/liter and peptone at 20 g/liter and is supplemented with 2% glucose. When required, YPD plates were supplemented with hygromycin (Invitrogen) at 400 μg/ml.

Bacteria were grown in LB medium as described previously (2). All plasmid constructs were introduced into strain D100 by electroporation, and carbenicillin (Invitrogen) at a final concentration of 100 μg/ml was added for selection of plasmids. For LB plates, 1.5% agar was used.

Yeast transformation. Yeast transformation with digested or supercoiled plasmids was performed as previously described (8).

Construction of deletion strains. To generate all deletion derivatives in the present study, we first constructed disruption plasmids for each gene to be deleted (SIR2, SIR3, SIR4, HDF1, HDF2, and RIF1). Briefly, the 5’ and 3’ untranslated regions of each gene to be deleted were PCR amplified and cloned into pGEM-T (Promega). Each pair of fragments was subcloned into pAP599 (conserving the relative orientation of the chromosomal locus to be deleted) flanking the hygromycin expression cassette. The plasmids generated in this way were used to generate allele replacements of each gene to be deleted by homologous recombination in a one-step gene replacement procedure. Briefly, each plasmid was digested with enzymes that cut at both ends of the cloned 5’ and 3’ flanking fragments, generating ends homologous to each specific gene to be deleted in the C. glabrata genome. The released fragment was used to transform C. glabrata selecting on plates supplemented with 400 μg of hygromycin/ml. Homologous recombination and allele replacement of each locus was verified by PCR analysis using a primer that anneals in the sequences external to the cloned fragments and a primer annealing within the disrupted locus. We also verified the absence of each gene deleted by the inability to PCR amplify an internal fragment from each deleted gene.

5-FOA sensitivity assays. To assess the level of silencing of the URA3 gene inserted at different positions throughout the four telomeres, we carried out a

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**Table 1. Oligonucleotides used in this study**

| Primer | Sequence (5’–3’) | Site(s) added |
|--------|-----------------|---------------|
| 962    | AATCCGTGACGCTATACAGG | PstI          |
| 963    | AGGCATGTCGACATCGATCAA | SalI          |
| 1952   | CCAATACCGGATCGAGTCACGG | Xho          |
| 2050   | TAAAGTCTCCGGGACAGCCAGAAGCTT | Xmal       |
| 2051   | CCCAAAGCTTAATGCCTATTTTCGAT | HindIII     |
| 2059   | ATCTTCGTCGATGTTGCTTG | Xhol        |
| 2238   | CGGGAATTCCTCGACTGGTTCAT | KpnI, BclI   |
| 2239   | CCAAGCCTATATGCTATTTTCGAT | HindIII     |
| 2240   | GGAGATCTCCCATCCTTTTTT | BglII        |
| 2241   | CAAGGAGCTTCGATGTTGCTG | SacI, BclI   |
| 2244   | CCGGTACATTCGACTAGTGCCA | KpnI, BclI   |
| 2245   | CCAAACGCTTAGGCTTTTTCGAT | HindIII     |
| 2246   | CTAGAGATTCCAGGTCTATATA | BglII        |
| 2247   | CAAGGAGCTTCGATGTTGCTG | SacI, BclI   |
| 2248   | GATTCTAATTTGGGACATTAGAAGCCTTTT | KpnI, BglI |
| 2249   | TATGACCATTTGCTGCC | HindIII     |
| 2250   | CCCAAAGCTTTTTTATTTATGATAC | BamHI       |
| 2253   | CCGGATCCATTCATTACATTTAATATGCTATTAAT | SacI, BglI |
| 2254   | CAAGGAGCTTCGATGTTGAGTACAGCA | AATATGCCATATATTCCAA |
plate growth assay as described previously (9, 11). Briefly strains containing the different URA3 insertions were grown in YPD for 36 h to stationary phase. The cultures were adjusted to an optical density at 600 nm of 1 with sterile water, and 10-fold serial dilutions were made in 96-well plates. Then, 5 µl of each dilution was spotted onto YPD, SC lacking uracil (SC−Ura), and SC−5-FOA plates, followed by incubation 48 h at 30°C, and photographed.

Telomere length determination by Southern blotting. Genomic DNA from wild-type (BG2), rif1Δ (BG509), hfl1Δ (BG1800), hfd2Δ (BG1801), sir2Δ (BG1048) and sirΔ (BG1050) strains (see Table S1 in the supplemental material) was digested with either Aadapter1 or Sau3AI, run on a 0.8% agarose gel, and transferred to a Amersham Hybond-N membrane (General Electric). The blot was hybridized to a 32-mer probe (CACCCAGACCCCACGAGGCCGACG CCAAG) end labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP.

In vivo nonhomologous end-joining assay. Plasmid pGRB2.0 (C. glabrata CEN ARS URA3; see Table S2 in the supplemental material) was linearized with either BamHI or SmaI to generate cohesive or blunt-ended plasmid molecules, respectively. Supercoiled and linearized plasmids were gel purified by using a Quiaquick gel extraction kit (Qiagen). Then, 500 ng of each gel-purified DNA was used to transform the wild-type (BG14), hfd2Δ (BG1800), and hfd2Δ (BG1801) strains. Serial dilutions of transformed cells were plated on SC−Ura plates, followed by incubation at 30°C for 2 days. Colonies were counted on each plate, and the relative efficiency of end joining was calculated as follows. First, the efficiency of end joining (transformant recovery) was calculated for each strain as the number of transformants obtained with linearized plasmid divided by the number obtained with supercoiled plasmid. The wild-type efficiency for BamHI-digested plasmid was then normalized to 1, and the relative efficiencies were calculated dividing the transformant recovery in each case by the wild-type efficiency obtained with each type of digested DNA.

Reverse transcription-PCR (RT-PCR). RNA was extracted from stationary grown cells (36 h in YPD) using TRizol reagent (Invitrogen) according to the manufacturer’s instructions and treated with DNase I (Invitrogen). Synthesis of cDNA and PCR were carried out as previously described using the ThermoScript RT-PCR system (Invitrogen) (11). The RT primers used for each gene were as follows: EPA1, TAAACATGTTTTCGTTTGAT; EPA2, GAATGATTCCTTC ATAAATA; EPA3, TAATTTGATCAGTACCAGCG; EPA4/5, GCTAAAATT GCTGATGAAAG; EPA6, GACCTAGTACCCATTCTG; EPA7, GCTGTCGGGTAAAGTGT; and ACT1, CTGGATGAGTGTCCGT. The cDNA synthesis reaction was carried out at 55°C for EPA1, EPA2, EPA3, EPA4/5, EPA5, and EPA6; at 59°C for EPA7; and at 50°C for ACT1. We used the same reverse primer for each gene of the PCR and the following forward primers: EPA1, GGCGTCAAAACGCTAAAG; EPA2, GGATGATGATGTTCAAAAG; EPA3, GCTGTTGATGATTCCAAA; EPA4/5, GCTTAACATTGCTGATT TCT; EPA6, GACCTAAAAACGACCATCTG; EPA7, TACGGAAAGTGGTTCGTAC; and ACT1, CGCCGCGTACGGTACTCCC. The PCR was carried out at 52°C for EPA4, EPA1, and EPA4/5; at 57°C for EPA3 and EPA4; and at 55°C for EPA5, and at 50°C for ACT1.

In all of the RNA samples and with every pair of primers, a no-reverse transcribease reaction was included as a negative control. No bands were obtained, indicating that the RNA preparations had no DNA contamination.

RESULTS

Subtelomeric regions at different telomeres are silenced in C. glabrata. In order to analyze silencing at different subtelomeric regions in C. glabrata, we chose to use a URA3 reporter assay. Briefly, URA3 expression can be scored both positively by growth on medium lacking uracil (SC−Ura plates) and negatively by growth on medium containing uracil and 5-FOA (5-FOA plates). 5-FOA is converted into a toxic product by orotidine-5′-phosphate decarboxylase, encoded by the URA3 gene; consequently, cells expressing URA3 are unable to grow on plates containing 5-FOA, and growth on 5-FOA plates is a measure of the extent of transcriptional silencing. The URA3 gene was integrated at nine positions across four telomeres. The telomeres chosen are the right end of chromosome E (Chr E-R), where the EPA1, EPA2, and EPA3 are localized; the right end of chromosome I (Chr I-R), where the EPA4 and EPA5 are located; and both ends of chromosome C (Chr C-L and Chr C-R), where EPA6 and EPA7, respectively, are found (Fig. 1A).

As shown in Fig. 1B, on Chr E-R, URA3 inserted at a position 31.5 kb from the telomeric repeats (insertion 1) is transcriptionally active and not silenced, as measured by the failure to grow on 5-FOA plates. As the distance between the URA3 reporter and the telomere repeats decreases, the level of gene silencing increases. Low levels of silencing are seen for insertion 2 (20.8 kb from the telomere repeats), whereas insertions 3 and 4 (13.39 and 1.32 kb, respectively, from the telomere) are strongly silenced. The three insertions throughout the EPA4 and EPA5 loci (Chr I-R) are all transcriptionally silent. Insertion 5, located 23.69 kb from the telomere repeats, is completely silenced, as are insertions 6 and 7 (11.5 and 0.9 kb, respectively, from the telomeric repeats). It is interesting that insertion 5 is farther away from its telomere than insertion 2 (23.69 kb versus 20.86 kb), and yet the region downstream from EPA5 is silenced, whereas the URA3 insertion in the region downstream from EPA1 is largely expressed (insertion 2). This suggests already that silencing in C. glabrata is not purely a product of distance from the telomeric repeats. EPA6 and EPA7 are located close to their respective telomeres on chromosome C. These two loci in strain BG2 are nearly identical to each other, including 2.3 kb of the 3’ untranslated region (representing the entire region between the open reading frames and the telomeric repeats) (9). Insertions 8 and 9 are positioned 2.53 and 2.29 kb from their respective telomeres on chromosome C, and both of these are silenced.

Expression of the native EPA1, EPA2, EPA3, EPA4, EPA5, EPA6, and EPA7 genes as measured by semiquantitative RT-PCR of the wild-type strain is in good agreement with what is seen with the reporter (see Fig. 5). EPA1 expression is detectable in stationary-phase cultures, whereas EPA2, EPA3, EPA4/5, and EPA7 are not expressed. In wild-type cells, there was detectable expression of EPA6, even though a URA3 gene integrated at the EPA6 locus is strongly silenced (see Fig. 4B).

yKu70 and yKu80 are required for maintaining telomere length and for nonhomologous end joining (NHEJ). To investigate the function of C. glabrata Sir2, Sir3, Sir4, Rif1, Ku70, and Ku80 in subtelomeric silencing, we generated null alleles in each of the corresponding genes in each of the eight strains carrying the URA3 insertions in the intergenic regions of the genes EPA1 to EPA7, generating a total of 48 strains in addition to the parental strains containing the reporter insertions that are wild-type for silencing function (see Materials and Methods and Table S1 in the supplemental material). Many of the C. glabrata silencing proteins have been shown to be functional equivalents to the corresponding orthologues in S. cerevisiae. In C. glabrata, as in S. cerevisiae, mutations in RIF1 have been previously shown to be altered in subtelomeric silencing and to have an increased telomere length (9, 19). In an analogous way, sir2Δ, sir3Δ, and sir4Δ mutants in C. glabrata show the same defective silencing phenotypes as the corresponding S. cerevisiae sir mutants (9, 11, 21). Mutants in HDF1 and HDF2, however, have not been described in C. glabrata. We generated deletion/insertion alleles of the C. glabrata orthologues of HDF1 and HDF2 (CAGL002b62g and CAGL0k03443g), which show 46 and 40% amino acid identity (and 64 and 59% similarity), respectively, to S. cerevisiae yKu70 and yKu80. In addition, the genes are syntenic with their S. cerve- siiae orthologues. To further verify that CAGL002b62g and
CAGL0K03443g correspond to HDF1 and HDF2 orthologues, we also characterized phenotypically yKu mutants in C. glabrata. In S. cerevisiae, yKu70 and yKu80 are required for maintenance of telomere structure and length and for double-strand break repair by NHEJ (3, 5, 17, 26). To determine whether the hdf1 and hdf2 mutants in C. glabrata shared these phenotypes, we first performed Southern blot experiments to analyze the telomere length in the wild-type strain and the yKu mutants digested with either of two enzymes and hybridized to a probe containing two copies of the telomere repeats (see Materials and Methods). As can be seen, the smear that corresponds to the smaller telomere fragments form an inhomogeneous population of telomere bands that hybridize to the probe. The average molecular weight of the smaller fragments is 250 to 300 bp smaller for both yKu mutants than it is for the wild-type strain or sir2Δ and sir4Δ strains, which were included for comparison and are known not to be defective for maintenance of telomere length in

FIG. 1. The subtelomeric regions of four telomeres in C. glabrata containing the genes EPA1 to EPA7 are subject to subtelomeric silencing.

(A) Schematic representation of the positions of nine different insertions of Th7 (containing the URA3 reporter gene) throughout four separate telomeres of C. glabrata. The EPA1 cluster is located at the right telomere of chromosome E, the EPA4/5 cluster is located at the right telomere on chromosome I, and EPA6 and EPA7 are located at either telomere of chromosome C. (B) Transcription of the URA3 reporter gene is subject to TPE when placed in the intergenic regions between EPA genes and the EPA genes and their respective telomeres. Strains containing URA3 insertions 1 to 9 (Fig. 1A) were grown to stationary phase in YPD, and 10-fold serial dilutions were made. Equal numbers of cells of each dilution were spotted onto SC−Ura and SC plates containing 5-FOA, and the plates were incubated for 48 h at 30°C and photographed (see Materials and Methods).
S. cerevisiae. As a reference, we also included a rif1Δ mutant in which the average telomere length is increased by 300 to 400 bp (9). Thus, similar to S. cerevisiae hdf1 and hdf2 mutants, the C. glabrata hdf1 and hdf2 mutants have a substantially shorter average telomere length. Next, we measured the ability of hdf1Δ and hdf2Δ mutants to repair in vivo double-strand breaks, as determined by the repair of a linearized plasmid transformed into C. glabrata. In order for the plasmid to replicate in C. glabrata and to obtain stable transformants, the plasmid must be recircularized. Therefore, the number of transformants obtained in each strain with linearized plasmid normalized to the number of colonies obtained with uncut plasmid and the wild-type value was normalized to 1.

In backgrounds mutated for any of the SIR genes, the native genes EPA1 to EPA7 are derepressed in stationary phase, which is in good correlation with the data for the URA3 reporter strains (see Fig. 6).

Subtelomeric silencing at the right telomere of chromosome E (EPA1 telomere) does not require yKu70/yKu80 proteins, but three other telomeres do. In order to assess whether the C. glabrata yKu proteins are required for subtelomeric silencing, we assayed the expression of the URA3 reporter genes placed at all of the positions across the four telomeres shown in Fig. 1A. Although silencing of the URA3 insertions at telomeres Chr I-R, Chr C-L, and Chr C-R depends strongly on functional yKu70 and yKu80 (Fig. 4B and 5B), we found that, surprisingly, subtelomeric silencing over the entire EPA1 telomere region (Chr E-R) does not depend on the yKu70 and yKu80 proteins. The degree of silencing of each insertion is almost the same in the presence or absence of HDF1 or HDF2 (insertions 2, 3, and 4, Fig. 3B).

Transcription levels of the native EPA genes in the absence of yKu70 and yKu80 is in good agreement with the expression of corresponding reporter; notably, EPA1, EPA2, and EPA3 are essentially not induced in the hdf1Δ or hdf2Δ mutants, whereas the expression of EPA4, EPA5, EPA6, and EPA7 is markedly induced in the absence of the Ku proteins (Fig. 6).

We conclude that different telomeres vary significantly in their dependence on the yKu70/yKu80 complex for silencing, as measured both by silencing of the native EPA genes and by silencing of the URA3 reporter. Specifically, silencing of the Chr E-R subtelomeric region is not dependent at all on yKu70 and yKu80.

Rif1 is required differentially at several telomeres in C. glabrata. We next determined the degree of silencing at all four telomeres in the absence of Rif1. The URA3 reporter gene,
located 1.32 kb from the Chr E-R telomere, is silent in the wild-type strain but in the absence of Rif1 is strongly derepressed (insertion 4, Fig. 3B). The URA3 reporter, placed 13.9 kb from the Chr E-R telomere (insertion 3, Fig. 3B), was strongly silenced in the parental strain, and this silencing was largely unaffected by the loss of RIF1. In the case of insertion 2, however (20.86 kb from the telomere), though the reporter is only slightly silenced in the parental strain, this small amount of silencing depends completely on RIF1. These results suggest that the dependence on RIF1 for silencing at this telomere may be discontinuous. The expression of EPA1, EPA2, and EPA3 is induced in the absence of RIF1 consistent with a role in silencing for Rif1 (Fig. 6).

In the case of Chr I-R telomere, Rif1 is required for silencing across the telomere as measured by derepression in a rif1Δ background of EPA4/5 transcription, as well as of the three URA3 reporters inserted in this region (insertions 5, 6, and 7; Fig. 4B). The dependence on Rif1 for silencing at each telomere of chromosome C is different. Figure 5B shows that absence of RIF1 results in almost complete expression of the URA3 reporter placed at the end of EPA6 (insertion 8), but it is still strongly silenced when the re-
porter is localized between EPA7 (insertion 9) and the Chr C-R telomere. These results show that different telomeres display different levels of TPE in response to some of the proteins involved in subtelomeric silencing. EPA6 and EPA7 are both equally derepressed in a rif1/H9004 background as measured by RT-PCR (Fig. 6). However, as measured by S1 mapping, EPA6 is strongly induced in a rif1Δ background, whereas EPA1 and EPA7 are more modestly induced (9, 21), a finding which correlates well with the expression of the reporter at these positions.

**FIG. 5.** Rif1 regulates differentially subtelomeric silencing at each telomere of chromosome C. Map of both telomeres of chromosome C showing the localization of EPA6 and EPA7, as well as the URA3 reporter insertions constructed in these loci. The distances of the insertions to the telomeres are indicated. (B) Deletion alleles of hdf1Δ (yKu70), hdf2Δ (yKu80), rif1Δ, sir2Δ, sir3Δ, and sir4Δ were introduced in each of the strains carrying the URA3 insertions 8 and 9 (panel A). The experiment was done as described in the legend to Fig. 1.

**FIG. 6.** Expression analysis by RT-PCR of EPA1, EPA2, EPA3, EPA4/5, EPA6, and EPA7 in the wild-type and in sir2Δ, sir3Δ, sir4Δ, hdf1Δ (yKu70), hdf2Δ (yKu80), and rif1Δ mutant strains. All strains were grown to stationary phase, and the total RNA was isolated and used for RT-PCR (see Materials and Methods). Lane 1, DNA, as a positive control for PCR and ACT1 RT-PCR, was used as internal control. Controls with no RT were also made and showed no bands (data not shown).
DISCUSSION

*C. glabrata* adheres efficiently to mammalian epithelial cells in vitro, an interaction that depends on the adhesin Epa1. Epa1 is a member of a large family of putative cell wall proteins in *C. glabrata*, most of which are localized to subtelomeric regions and are negatively controlled by subtelomeric silencing, which depends on the proteins Sir3, Sir4, Rap1, and Rif1 (9, 11, 21). In the present study, we determined in a systematic way the role of Sir2, Sir3, Sir4, Rif1, yKu70, and yKu80 in subtelomeric silencing of a reporter *URA3* gene placed at several positions in four different telomeres. We found that the silencing at different telomeres is subject to different genetic requirements, which points out a potential complexity in the regulation of the subtelomeric *EPA* genes. Some of the differences in silencing between telomeres may be explained by the action of telomere-specific cis-acting elements.

*C. glabrata* hdf1/H9004 and hdf2/H9004 mutants have shortened telomeres and are defective at NHEJ. yKu mutants in *S. cerevisiae* display shorter average telomere length and are compromised for subtelomeric silencing (4, 23, 25, 26, 31). Here we show that *C. glabrata* yKu orthologues are the functional equivalents of *S. cerevisiae* proteins, since hdf1Δ (yKu70), hdf2Δ (yKu80), rif1Δ, and sir3Δ mutant strains. In contrast, telomere length is normal in *sir2*Δ/*H9004*, *sir3*Δ/*H9004*, and *sir4*Δ/*H9004* mutant strains (9, 11, 21). Similar to the *S. cerevisiae* yKu genes, *HDF1* and *HDF2* are required for efficient NHEJ (Fig. 2B).

Subtelomeric silencing and TPE at loci *EPA1* to *EPA7* depend on Sir2, Sir3, Sir4. Subtelomeric silencing affects larger regions of the telomere in *C. glabrata* than in *S. cerevisiae*. For example, at the *EPA1* cluster and the *EPA4-5* cluster, this regulation extends >20 kb from the telomere repeats (Fig. 3 and 4) (9, 11). The insertion between *EPA1* and *EPA2* (20.8 kb...
from the telomere; Fig. 3B, insertion 2) is subject to weak silencing, which for this telomere might delimit the propagation of silencing from this telomere. Silencing in C. glabrata over 20 to 25 kb of the subtelomeric region stands in contrast with the 4- to 8-kb subtelomeric region typically subject to TPE in S. cerevisiae (31).

The URA3 insertions we analyzed all display a high degree of silencing that depends on the Sir2, Sir3, and Sir4 proteins (Fig. 3B, 4B, and 5B); therefore, the Sir proteins appear to be absolutely required for silencing of the native subtelomeric genes, as well as for TPE at these telomeres. This is similar to S. cerevisiae, where subtelomeric silencing also depends absolutely on Sir2, Sir3, and Sir4 (reviewed in reference 35). In this regard, repression of the FLO10 gene in S. cerevisiae, which encodes a cell wall protein, is interesting. FLO10 is encoded at a locus 17.9 kb from the right telomere in chromosome XI. FLO10 is subject to epigenetic repression that depends on SIR3, HDF1, and HDF2 but, unlike classic subtelomeric silencing, requires the native FLO10 promoter. Repression does not require Sir2; instead, it requires Hst1 and Hst2, NAD+-dependent histone deacetylases related to Sir2 (18).

C. glabrata and S. cerevisiae have different genetic requirements for silencing at telomeres. In S. cerevisiae, subtelomeric silencing requires not only the Sir proteins but also Rap1, Rif1, Rif2, and the yKu70/yKu80 heterodimer. In C. glabrata the same proteins are important for silencing, but there are some apparent differences. Rap1 and the Sir proteins are also absolutely required for silencing in C. glabrata (9, 11, 21). It is noteworthy in this regard that C. glabrata does not have the SIR1 gene (13) but nevertheless can establish and maintain subtelomeric silencing. In the case of yKu proteins, it is surprising that, while they are essential for TPE at all S. cerevisiae telomeres tested (natural or truncated) (4, 23, 25, 26, 31), they are not required for silencing at the C. glabrata Chr E-R telomere (for the reporter URA3 or for EPA1, EPA2, and EPA3 expression) (Fig. 3B and 6). This is not a general effect since silencing of the other three telomeres we tested depends completely on the yKu proteins (Fig. 4B, 5B, and 6). In this regard, it is interesting that in Schizosaccharomyces pombe, yKu70 is not required for TPE at the one telomere that was studied (24). It remains to be tested whether other telomeres (in addition to the Chr E-R telomere) do not require the Ku proteins for TPE (we have only tested 4 of 26). The fact that yKu70 and yKu80 are required for TPE and the expression of native subtelomeric genes in some but not all of the telomeres in C. glabrata indicates that the telomeres in C. glabrata are not equivalent and that both TPE and the expression of native subtelomeric genes are subject to complex regulation of expression that differs from telomere to telomere and even from gene to gene. This is parallel to what has been characterized in S. cerevisiae. The sequence of the subtelomeric regions of S. cerevisiae are different from one another and consist of repetitive elements immediately adjacent to the telomere tract and do not have the same level of TPE (27, 28). In addition, at two different truncated telomeres, the levels of TPE vary ~10-fold in the same strain background (16). Even when the subtelomeric elements of two different telomeres are identical in sequence, the TPE levels can be different. Therefore, there must be other factors (other than the sequence of the subtelomeric elements) that contribute to the final expression level of marker or native subtelomeric genes at particular subtelomeres (27, 28).

Rif1, yKu70, and yKu80 differentially regulate some telomeres of C. glabrata. We found that different telomeres in C. glabrata respond differently to Rif1 and the yKu proteins. In the case of the Rif1, C. glabrata encodes only the Rif1 gene, whereas S. cerevisiae encodes both Rif1 and Rif2. Rif1 is quite divergent (24% identical in amino acid sequence) between S. cerevisiae and C. glabrata, but as for the S. cerevisiae Rif proteins (19, 40), C. glabrata Rif1 is required for correct telomere length regulation in C. glabrata (9). In S. cerevisiae, the Rif proteins play a negative role in subtelomeric silencing (19, 40). In contrast, C. glabrata Rif1 seems to play a positive role, since several subtelomeric EPA4 genes are derepressed in a rif1Δ background (9, 21). Rif1 has a strong positive role in silencing the reporter placed at the EPA6 locus on Chr C-L but has only a modest effect on the reporter placed at the EPA7 locus on Chr C-R (Fig. 5B). In addition, we found that at the Chr E-R telomere, Rif1, is required for silencing discontinuously across the telomere (Fig. 1B). This is reminiscent of discontinuous silencing in S. cerevisiae, which is attributed to telomere-specific elements (15). This may suggest the presence of cis-acting elements in the Chr E-R region that serve to recruit Rif1 and/or other silencing proteins.

Chr E-R telomere contains a discrete cis-acting silencer element. It is notable that the Rif1 and yKu proteins, which differentially affect different telomeres in C. glabrata, also affect telomere length. An attractive model to explain the differential effects of Rif1 and yKu proteins on silencing at different telomeres in C. glabrata is that certain telomeres encode cis-acting elements, analogous to silencers or proto-silencers previously described in S. cerevisiae. These sequences could provide an additional means of recruiting silencing complexes to certain telomeres, making these telomeres more or less sensitive to the effects of changes in telomere length. Indeed, we found a novel cis-acting element between EPA3 and the telomere that can silence a URA3 reporter inserted 31.9 kb from the telomere repeats, sufficiently distant from the telomeric repeats that it is not subject to subtelomeric silencing (Fig. 1B, insertion 1). In preliminary experiments, we have tested the function of this cis element at a second locus entirely removed from the normal subtelomeric context (400 kb from the telomere in chromosome F). Interestingly, in this position, the element does not confer silencing of a reporter URA3 (data not shown). This position dependence suggests that this element may be similar to the subtelomeric proto-silencers described in S. cerevisiae (4, 23, 25, 26, 31), although this needs to be investigated further.

The silencing exerted by this cis element depends on Sir3 and partially on Rif1 but not on yKu70 or yKu80; in fact, silencing mediated by the element apparently increases in the hdl1Δ and hdl2 mutant strains (Fig. 7B). The genetic requirements for this element are similar to those described in S. cerevisiae for the silencers that flank and silence the silent copies of the mating type information cassettes (HML and HMR) (4, 29). Our data in C. glabrata are consistent with a model in which the effect of hdl1Δ, hdl2Δ, and rif1Δ mutations on silencing mediated by the cis-acting element is the indirect result of changes in telomere length. The telomeric repeats bind Rap1 which in turn binds the Sir complex. In the current model from S. cerevisiae (35), telomere length affects silencing
because Sir proteins bound to the telomere are removed from the pool available to bind to a silencer or protosilencer element. Accordingly, in hdf1Δ and hdf2Δ mutants, decreased telomere length would increase the concentration of Sir proteins available to interact with a cis-acting element, thereby increasing silencing; by contrast in rif1 mutants, the longer telomere length would decrease the concentration of Sir proteins available to interact with the cis-acting element, thereby decreasing silencing.

The silencer/proto-silencer element found next to EPA may not be the only one that regulates the expression of EPA genes at this or other telomeres. We are currently in the process of mapping this element and testing whether there are other such elements at other positions near other EPA genes. Initial experiments have failed to find a functionally equivalent silencer downstream of EPA6 or EPA7 (data not shown).

The regulation of expression of the subtelomeric EPA family of adhesins is complex and includes several layers of regulation such as subtelomeric silencing, cis-acting subtelomeric silencer or proto-silencer elements, and complex promoters (longer than 2 kb in some cases) that may impose specific requirements or proto-silencer elements, and complex promoters (longer than 2 kb in some cases) that may impose specific requirements on the expression of these genes. Even in terms of silencing, the four telomeres in C. glabrata are not equivalent in that they are differentially regulated by the silencing proteins yKu70, yKu80, and Rif1p. This complexity may be important in the regulation of EPA genes during infection since it may mean that even though the EPAs are silenced by the general silencing machinery, expression of EPAs can be individually modulated in response to the host environment. For example, EPA6, which is transcriptionally repressed by subtelomeric silencing, has been shown to be induced during murine urinary tract infections in response to limitation for environmental nicotinic acid (NA). NA is a precursor of NAD+, which is a cofactor of the NAD+-dependent histone deacetylase Sir2, and under conditions of NA limitation Sir2 is not fully functional, leading to the derepression of some EPA genes (12). We suggest that different telomeres, which have different genetic requirements for silencing may, as a result, respond differently to environmental cues, including NAD+-limiting environments. This might allow C. glabrata to modulate the expression of different adhesins according to the signals that it receives from the environment during an infection.

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