A Novel Downstream Regulatory Element Cooperates with the Silencing Machinery to Repress *EPA1* Expression in *Candida glabrata*

Verónica Gallegos-García,*[,1,2] Shih-Jung Pan,[†,1] Jacqueline Juárez-Cepeda,* Candy Y. Ramírez-Zavaleta,* Marcela Briones Martin-del-Campo,* Verónica Martínez-Jiménez,* Irene Castaño,* Brendan Cormack,[†] and Alejandro De Las Peñas*[,3]

*IPICYT, División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, San Luis Potosí, 78216, México and [†]Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT *Candida glabrata*, an opportunistic fungal pathogen, adheres to mammalian epithelial cells; adherence is mediated primarily by the Epa1 adhesin. *EPA1* is a member of a large gene family of ~23 paralogues, which encode putative adhesins. In this study, we address how *EPA1* transcription is regulated. Our data show that *EPA1* expression is subject to two distinct negative regulatory mechanisms. *EPA1* transcription is repressed by subtelomeric silencing: the Sir complex (Sir2–Sir4), Rap1, Rif1, yKu70, and yKu80 are required for full repression. Activation of *EPA1* occurs immediately after dilution of stationary phase (SP) cells into fresh media; however, transcription is rapidly repressed again, limiting expression to lag phase, just as the cells exit stationary phase. This repression following lag phase requires a *cis*-acting regulatory negative element (NE) located in the *EPA1* 3′-intergenic region and is independent of telomere proximity. Bioinformatic analysis shows that there are 10 copies of the NE-like sequence in the *C. glabrata* genome associated with other *EPA* genes as well as non-*EPA* genes.

CANDIDA glabrata, an opportunistic fungal pathogen normally present in the mucosal flora, can cause severe disseminated infections. *C. glabrata* is the second most common agent of candidiasis, accounting for 15–20% of *Candida* bloodstream infections worldwide (Pfaller and Diekema 2010). Some traits of *C. glabrata* that allow it to cause disease have been described (Kaur *et al.* 2005; Roetzer *et al.* 2011). These include resistance to oxidative stress (Cuellar-Cruz *et al.* 2008, 2009) and adherence to epithelial cells (Cormack *et al.* 1999; Castaño *et al.* 2005).

Adherence to host cells has been proposed to be an important initial step for virulence. Additionally, the ability to adhere to abiotic substrates and the adherence between microbial cells are essential attributes for biofilm formation in many pathogens. Adherence in pathogenic fungi has been shown to be mediated primarily by glycosylphosphatidylinositol-anchored cell wall proteins (GPI-CWPs) which are found broadly in different fungal species including *Saccharomyces cerevisiae*, *C. glabrata*, and *C. albicans* (Castaño *et al.* 2006). In *S. cerevisiae*, the *FLO* family of genes (*FLO1*, *FLO5*, and *FLO9–FLO11*) encode a group of (GPI-CWPs) that are required for flocculation, pseudohyphal growth, and biofilm formation on abiotic substrates (Kobayashi *et al.* 1998; Guo *et al.* 2000). *C. albicans* encodes ~104 putative GPI-CWPs including the Als family, the Hwp family, Hyr1, and Eap1 (De Groot *et al.* 2003a,b; Li and Palecek 2003), many of which are thought to mediate adhesion to host epithelial and endothelial cells as well as to extracellular matrix proteins (Hoyer 2001; Li and Palecek 2003; Sheppard *et al.* 2004; Klotz *et al.* 2004; Hoyer *et al.* 2008). *C. glabrata* can adhere to epithelial cells and also to inert surfaces. *EPA1* encodes the major epithelial adhesin in the BG2 strain, binding to *N*-acetyl lactosamine-containing glycoconjugates (Cormack *et al.* 1999; Zupancic *et al.* 2008). *EPA1* belongs to a large gene family (*EPA* family) of ~23 paralogues, of which *EPA6* and *EPA7* have also been shown to mediate

doi: 10.1534/genetics.111.138099
Manuscript received September 28, 2011; accepted for publication December 25, 2011
[1]These authors contributed equally to this work.
[2]Present address: Facultad de Enfermería, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, 78240, México.
[3]Corresponding author: Camino a la Presa San José 2055, División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, San Luis Potosí, 78216, México. E-mail: cano@ipicyt.edu.mx

adherence to epithelial cells *in vitro* (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; De Groot *et al.* 2008).

Proper regulation of the expression of the *EPA* adhesin genes is thought to be of importance for survival and proliferation in the host environment. One layer of *EPA* gene transcriptional regulation is related to the fact that most *EPA* genes are encoded in subtelomeric loci, where they are subject to chromatin-based silencing mediated by the Sir complex (Sir2, Sir3, and Sir4), yKu70, yKu80, Rif1, and Rap1 (De Las Peñas *et al.* 2003; Castaño *et al.* 2005). Deletion of genes encoding the silencing factors results in the cells becoming hyperadherent, due to overexpression of some *EPA* genes, including *EPA6* and *EPA7* (Castaño *et al.* 2005). Other subtelomeric *EPAs* are not expressed even in *sir* mutant backgrounds, indicating additional gene-specific regulation for individual *EPA* genes (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Domergue *et al.* 2005).

In this article, we focus on the detailed regulation of *EPA1* transcription. *EPA1* resides 20.7 kb upstream from the right telomere in chromosome E and forms a cluster with two other *EPA* genes (*EPA1–EPA2–EPA3* telomere, Figure 1A) (De Las Peñas *et al.* 2003). Our data show that *EPA1* expression is tightly controlled negatively and positively. *EPA1* transcription is repressed by the Sir complex (Sir2, Sir3, and Sir4) and by Rap1, Rif1, yKu70, and yKu80. Transcription of *EPA1* is induced immediately after dilution of stationary phase (SP) cells into fresh media and concomitantly, the cells become adherent. Interestingly, *EPA1* expression is limited to lag phase, and is tightly repressed in long-term log phase (LP) cultures as well as in SP. We show that a *cis*-acting regulatory negative element (NE) localized at the intergenic region between *EPA1* and *EPA2*, 300 bp downstream of *EPA1* stop codon (TAA), plays a major role in transcriptional repression of *EPA1*.

Materials and Methods

Strains

All strains used in the study are described in Table 1.

Plasmids

All plasmids used in this study are described in Table 2.

Primers

All primers used for cloning are summarized in Table 3.

Media and growth conditions

All cell cultures were grown for 48 hr at 30°. SP cells are cells grown for 48 hr. LP cells are dividing cells. Lag phase is considered when SP cells are diluted into fresh media and cells are preparing for cell division. Yeast media were prepared as described (Sherman *et al.* 1986), and 2% agar was added to plates. YPD media contains yeast extract 10 g/liter, peptone 20 g/liter, supplemented with 2% glucose. When needed, YPD plates were supplemented with 400 μg/ml of hygromycin (A. G. Scientific). Synthetic complete media (SC) contains YNB without amino acids and nitrogen source (1.7 g/liter), NH_2SO_4 (5 g/liter), supplemented with 0.6% of casamino acids and 2% glucose and, when needed, supplemented with 50 mg/liter uracil and 0.9 g/liter 5-fluoroorotic acid (5-FOA, Toronto Research Chemicals) for 5-FOA plates. Bacterial media were prepared as described (Ausubel *et al.* 2001), and 1.5% agar was used for plates. Luria-Bertani (LB) media contained yeast extract at 5 g/liter, bactopeptone at 10 g/liter, and NaCl at 10 g/liter. When needed, LB plates were supplemented with 100 μg/ml of carbenicillin (Cb100, Invitrogen). Phosphate buffer saline (PBS) was 8 g/liter NaCl, 0.2 g/liter KCl, 1.65 g/liter $Na_2HPO_4 \cdot 7H_2O$, and 0.2 g/liter KH_2PO_4.

Yeast transformation

Yeast transformation was performed using the lithium acetate protocol as described previously (Castaño *et al.* 2003).

Construction of deletion strains

To construct deletion strains in this study, we used the one-step gene replacement procedure. Briefly, we cloned two fragments (the promoter region and the 3′-UTR, flanking the gene to be deleted) at each side of the hygromycin resistance cassette in the integrative *URA3* plasmid, pAP599 (see Table 1). The plasmid was digested with restriction enzymes that cut within the two cloned fragments generating homologous ends. After inactivation of the enzymes, *C. glabrata* was transformed with the digestion mix and transformants were selected on YPD −hygromycin or SC −Ura plates. PCR analysis was done to confirm the structure of the deletion. The absence of each deleted gene was also verified by the inability to PCR amplify an internal fragment of the gene. Strains constructed in this way are described in Table 1.

S1 nuclease protection assay

BG14 cells were grown for 48 hr at 30°. RNA was extracted as previously described (De Las Peñas *et al.* 2003). The *ACT1* and the *EPA1* probes (Table 3) were end labeled using [γ-^{32}P]-ATP with T4 polynucleotide kinase. A total of 30 μg of RNA was hybridized with each end-labeled probe at 55° overnight. The mix was digested at room temperature with 150 units of S1 nuclease (Invitrogen) for 30 min. The samples were then extracted with phenol, precipitated, and resuspended in 17 μl of 1× loading buffer. A total of 5μl of each sample was separated by electrophoresis on a 10% acrylamide gel and the signal detected using a phosphorimager.

EPA1 promoter::URA3 plate assay

Cells were grown for 48 hr at 30° in YPD and the cultures were adjusted to 0.5 OD_{600nm}. Ten-fold serial dilutions were spotted on YPD, SC −Ura, and SC +5-FOA plates and incubated at 30° for 48 hr. Ura^+ cells die on SC +5-FOA plates. Only cells with the *URA3* gene transcriptionally repressed can grow on SC +5-FOA.

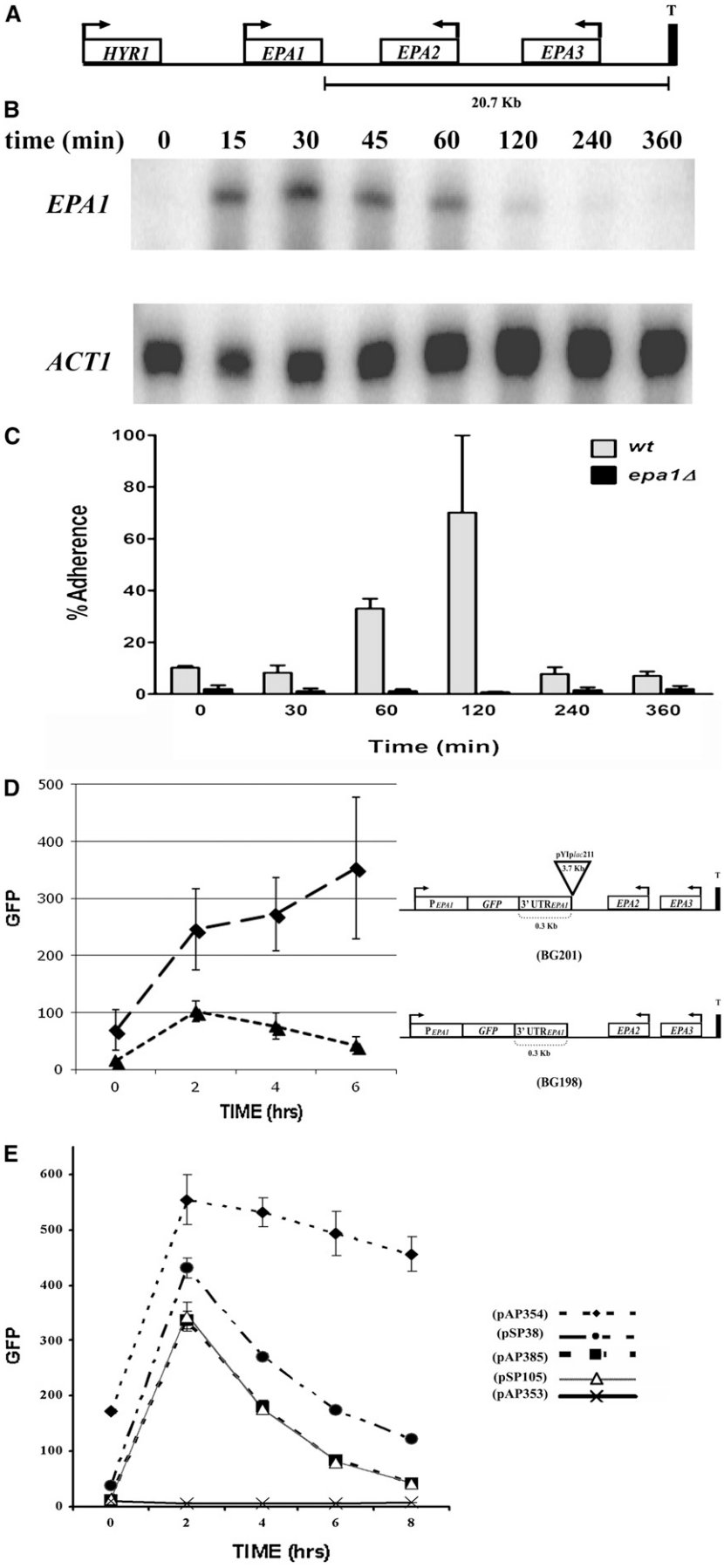

Figure 1 Regulation of the expression of *EPA1*. (A) Schematic representation of the *EPA1* genomic locus. (B) *EPA1* transcript levels measured by S1 nuclease protection. BG14 (WT) cells were grown for 48 hr and 30° in YPD media. Cells were diluted into fresh media and samples were taken at different time points (see *Materials and Methods*). Time 0 is undiluted stationary phase (SP) cells. (C) Adherence of *C. glabrata* cells to HeLa cells. *C. glabrata* wild-type strain BG14 (WT) and strain BG64 (*epa1*Δ) were grown for 48 hr at 30° in YPD media. Cells were diluted into fresh media and samples were taken at different time points. Cells were adjusted to OD_{600} of 1.0 in HBSS supplemented with 5 mM $CaCl_2$. Cell suspensions were diluted serially in sterile water and appropriate dilutions were made and plated on YPD plates to determine input colony forming units (CFU) (see *Materials and Methods*). Each experiment was made in triplicate. (D) *EPA1* promoter activity measured by FACS. Strains BG198 (P_{EPA1}::GFP) and BG201 (P_{EPA1}::GFP::pYIp*lac*211) were grown for 48 hr at 30° in YPD media. Cells were diluted into fresh media and samples were taken every 2 hr. Yeast cells were washed and resuspended in 1 ml PBS and fluorescence was assessed by FACS analysis using a BD FACSCalibur flow cytometer (see *Materials and Methods*). *EPA1* promoter GFP fusion is at the chromosomal *EPA1* locus. GFP was used as reporter of the activity of the *EPA1* promoter. (E) *EPA1* promoter activity measured by FACS as in D, but cells were grown in SC −Ura media and all constructs are borne in plasmids. Strain BG14 (WT) carrying plasmids pAP353 (promoterless control, GFP::3′UTR_{HIS3}), pAP354 (P_{EPA1}::GFP::3′UTR_{HIS3}), pAP385 (P_{EPA1}::GFP::3′$UTR_{EPA1}NE_{(3.1Kb)}$), pSP38 (P_{EPA1}::GFP::3′UTR_{HIS3}::$NE_{(200bp)}$), and pSP105 (NE_{200bp}::P_{EPA1}::GFP::3′UTR_{EPA1}).

Table 1 Strains used in this study

Strain	Parent	Genotype	Reference
Escherichia coli strain			
DH10B		F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*Δ*M15* Δ*lacX74* *deoR recA1 endA1 araD139* Δ(*ara,leu*)7697*galU* *galK* λ$^-$ *rpsL nupG*	Calvin and Hanawalt (1988)
Candida glabrata strains			
BG2		Clinical isolate (strain B)	Fidel *et al.* (1996)
BG14	BG2	*ura3*Δ::Tn*903* G418^R	Cormack and Falkow (1999)
BG64	BG14	*epa1*Δ *ura3*Δ::Tn*903* G418^R	Cormack *et al.* (1999)
BG198	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*GFP*. *GFP* under the control of the *EPA1* promoter (Figure 1C)	De Las Peñas *et al.* (2003)
BG201	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*GFP* pYIp*lac*211 integrated 300bp from TAA of *EPA1*. *GFP* under the control of the *EPA1* promoter (Figure 1C)	This work
BG509	BG14	*ura3*Δ::Tn*903* G418^R *rif1*Δ::*hph* Hyg^R	Castaño *et al.* (2005)
BG592	BG14	*ura3*Δ::Tn*903* G418^R *rap1-21*	De Las Peñas *et al.* (2003)
BG646	BG14	*ura3*Δ::Tn*903* G418^R Tn*7* at intergenic region between *EPA1* and *EPA2*	De Las Peñas *et al.* (2003)
BG676	BG14	*ura3*Δ::Tn*903* G418^R *sir3*Δ::*hph* Hyg^R	De Las Peñas *et al.* (2003)
BG1048	BG14	*ura3*Δ::Tn*903* G418^R *sir2*Δ::*hph* Hyg^R	Domergue *et al.* (2005)
BG1050	BG14	*ura3*Δ::Tn*903* G418^R *sir4*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1080	BG14	*ura3*Δ::Tn*903* G418^R *hdf1*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1081	BG14	*ura3*Δ::Tn*903* G418^R *hdf2*Δ::*hph* Hyg^R	Rosas-Hernandez *et al.* (2008)
BG1124	BG1212	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3*. *EPA1* replaced by *URA3*. *URA3* under the control of the *EPA1* promoter	This work
BG1132	BG14	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat*. *EPA1* replaced by *URA3* and NE (negative element) replaced by the bacterial *cat* gene, chloramphenicol acetyl transferase from pACYC184 *URA3* under the control of the *EPA1* promoter	This work
*sir2*Δ			
CGM172	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir2*Δ::*hph* Hyg^R	This work
CGM174	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir2*Δ::*hph* Hyg^R	This work
*sir3*Δ			
CGM283	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir3*Δ::*hph* Hyg^R	This work
CGM285	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir3*Δ::*hph* Hyg^R	This work
*sir4*Δ			
CGM188	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *sir4*Δ::*hph* Hyg^R	This work
CGM190	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *sir4*Δ::*hph* Hyg^R	This work
*hdf1*Δ			
CGM198	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hdf1*Δ::*hph* Hyg^R	This work
CGM184	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hdf1*Δ::*hph* Hyg^R	This work
*hdf2*Δ			
CGM187	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hdf2*Δ::*hph* Hyg^R	This work
CGM185	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hdf2*Δ::*hph* Hyg^R	This work
*rif1*Δ			
CGM210	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *rif1*Δ::*hph* Hyg^R	This work
CGM212	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *rif1*Δ::*hph* Hyg^R	This work
*hst1*Δ			
CGM213	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hst1*Δ::*hph* Hyg^R	This work
CGM214	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hst1*Δ::*hph* Hyg^R	This work
*hst2*Δ			

(continued)

Table 1, *continued*

Strain	Parent	Genotype	Reference
CGM208	BG1124	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* *hst2*Δ::*hph* Hyg^R	This work
CGM217	BG1132	*ura3*Δ::Tn*903* G418^R *epa1*Δ::*URA3* neΔ::*cat* *hst2*Δ::*hph* Hyg^R	This work
rap1-21			
CGM349	BG592	*ura3*Δ::Tn*903* G418^R *rap1-21 epa1*Δ::*URA3*	This work
CGM374	BG592	*ura3*Δ::Tn*903* G418^R *rap1-21 epa1*Δ::*URA3* neΔ::*cat*	This work

In vitro adherence assays

Cervical carcinoma cells (HeLa) were cultured in 24-well plates in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 80 units ml^{-1} of penicillin G, 50 $\mu g\ ml^{-1}$ of streptomycin sulfate, and 7% calf serum (HyClone Laboratories). The cells were maintained at 37° in 5% CO_2 until confluent growth. Each well was washed once with sterile water and PBS. Cells were fixed with 4% of paraformaldehyde. Plates were stored at 4° containing 1 ml of PBS supplemented with Pen/Strep (penicillin 100 units ml^{-1} and streptomycin 100 $\mu g\ ml^{-1}$). For adherence assay, *C. glabrata* cells were grown for 48 hr at 30° and, where indicated, diluted into fresh media. SP and LP cultures were adjusted to an OD_{600} of 1.0 with Hanks balanced salt solution (HBSS) supplemented with 5 mM $CaCl_2$. A total of 100 μl of cells was added to a 24-well plate containing fixed HeLa cells in 900 μl of PBS without antibiotics. The plates were incubated at room temperature for 1 hr. Nonadherent yeast cells were washed three times with HBSS with $CaCl_2$ and once with PBS. Adherent cells were recovered from the epithelial cells by scraping off the plate in 500 μl of PBS containing 0.1% Triton and 0.5% SDS. The cells were plated and viable accounts recorded.

FACS analysis of GFP expression

Strains were grown for 48 hr at 30° in SC −Ura media. Cells were diluted into fresh media to induce *EPA1* expression. *GFP* was used as reporter gene to measure the activity of the *EPA1* promoter. Yeast cells were washed with PBS and resuspended in 1 ml PBS and fluorescence was assessed by FACS analysis using a BD FACSCalibur flow cytometer with Cell Quest Pro software.

Results

EPA1 is induced in lag phase

C. glabrata strain BG14 SP cells do not adhere to cultured epithelial cells. However, upon dilution and growth in fresh media, BG14 cells become adherent. This *in vitro* adherence is mediated by the Epa1 adhesin. *epa1*Δ mutant cells are virtually nonadherent in any growth condition (Cormack *et al.* 1999). To begin to dissect the transcriptional regulation of *EPA1*, we measured transcript levels of *EPA1* in SP cells and in cells following dilution into fresh media. Little or no *EPA1* transcript is observed in *C. glabrata* SP cells, but dilution into fresh media results in significant *EPA1* transcription, which reaches maximal levels at 30 min following dilution (Figure 1B). However, 120 min after dilution, the *EPA1* transcript level has fallen significantly, and at 240 min postdilution, it returns to background level (Figure 1B). The cells become adherent at 60 min with maximal adherence at 120 min postdilution, but as cells grow in prolonged LP, they become nonadherent, since new *EPA1* transcription is repressed and preexisting Epa1 protein is diluted upon cell division (Figure 1C). These data indicate that *EPA1* expression is tightly regulated and is expressed neither in SP nor in long-term LP cultures, but rather is limited to lag phase, as cells exit SP.

EPA1 expression is controlled by positive and negative regulation

To analyze the regulation of *EPA1*, we replaced the *EPA1* ORF in its chromosomal location with the reporter gene GFP (BG198, P_{EPA1}::GFP). GFP fluorescence is measured in cells in SP and upon dilution into fresh media by fluorescence activated cell sorting (FACS). The expression profile of P_{EPA1}::GFP parallels that of *EPA1* transcript shown in Figure 1B. In SP cells, the P_{EPA1}::GFP reporter shows background levels of fluorescence; expression is highly induced immediately after dilution into fresh media (Figure 1D). We noticed that correct regulation of *EPA1* was lost in a mutant strain carrying an insertion of 3.797 kb (pYip*lac*211 plasmid, strain BG201) 300 bp downstream of the P_{EPA1}::GFP reporter stop codon: higher basal levels of expression and dilution of SP cells into fresh medium resulted in strong GFP induction; however, GFP reporter activity remained induced as cells divided, remaining elevated through LP and even in SP (Figure 1D). These data strongly suggest that normally, *EPA1* expression is repressed in LP cells and that in the insertion mutant strain, such repression is relieved (Figure 1D). The lack of repression of the reporter gene in the insertional mutant (BG201) could in principle be caused by disruption of a *trans*-acting repressor gene encoded downstream of *EPA1*. However, sequence analysis of the 3.1-kb intergenic region shows no evidence of any ORF sequences within the 3′-intergenic region; moreover, complementation experiments in which the entire 3.1-kb intergenic region was cloned on a plasmid and introduced into BG201 did not suppress the high GFP-expression phenotype (data not shown).

Next, we tested whether the transcriptional regulation of the *EPA1* locus can be reconstituted on a plasmid. We cloned in a *URA3* CEN-ARS plasmid (pGRB2.0) the P_{EPA1}::GFP

Table 2 Plasmids used in this study

Plasmid	Relevant genotype	Reference
pCYC184	Cloning vector Cm^R Tc^R	Chang and Cohen (1978)
pYlp*lac*211	Cloning vector, integrative vector *URA3* Ap^R	Gietz and Sugino (1988)
pMB11	Cloning vector, *sacB* counterselection Cm^R *ori* p15A	Lab collection
pGRB2.0	Cloning vector, pRS406 *URA3 C.g.CEN ARS* Ap^R	De Las Peñas *et al.* (2003)
pRS306	Cloning vector, integrative vector *URA3* Ap^R	Sikorski and Hieter (1989)
pAP599	Cloning vector for construction of knockout mutants with two *FRT* direct repeats flanking the hygromycin cassette to remove the selection marker for construction of multiple mutants in *C. glabrata*. [*FRT*-P_{PGK}::*hph*::3′UTR_{HIS3}-*FRT*] *URA3* Hyg^R Ap^R	Domergue *et al.* (2005)
pAP640	Cloning vector, pRS306 cut with *Nde*I/*Nae*I blunt ended with Klenow and religated. *ura3*Δ Ap^R	This work
pAP353	A 0.738-kb *Eco*RI/*Sal*I PCR fragment carrying *GFP* (no *Bst*XI site) and a 0.397-kb *Xho*I/*Kpn*I PCR fragment carrying *HIS3* 3′*UTR* were cloned into pGRB2.0. For *GFP* promoter fusions. *URA3* Ap^R. (Figure 1D)	This work
pAP354	A 2.5-kb *Bst*XI PCR fragment (primers 666/667) carrying the promoter region of *EPA1* was cloned into pAP353. *URA3* Ap^R P_{EPA1}::*GFP*::3′UTR_{HIS3} (Figure 1D)	This work
pAP385	A 3.1-kb *Xho*I PCR fragment (primers 723/724) carrying the 3′UTR_{EPA1} fragment cloned at *Xho*I site of pAP354. *URA3* Ap^R*EPA1*$_{Promoter}$::*GFP*::3′$UTR_{EPA1\text{-}3.1Kb}$ (Figure 1D)	This work
pAP407	Similar to pAP354 but 3′-UTR from *EPA1*.*URA3* Ap^R P_{EPA1}::*GFP*::3′$UTR_{EPA1\text{-}0.300Kb}$	Domergue *et al.* (2005)
pSP7	A 0.100-kb *Xho*I PCR fragment (primers 780/795) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+400 bp; Figure 2, line 4)	This work
pSP19	A 0.50-kb *Xho*I PCR fragment (primers 780/841) carrying the 3′ *EPA1* (NE mapped) was cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+350 bp; Figure 2, line 5)	This work
pSP20	A 0.150-kb *Xho*I PCR fragment (primers 780/842) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+450 bp; Figure 2, line 3)	This work
pSP21	A 0.200-kb *Xho*I PCR fragment (primers 780/843) carrying the 3′ intergenic region of *EPA1*cloned into pAP407 digested with *Xho*I. *URA3* Ap^R (+500 bp; Figure 2, line 1)	This work
pSP26	A 0.200-kb *Xho*I/*Sal*I PCR fragment (primers 776/839) carrying the 3′ UTR of *EPA1*cloned into pAP407 digested with *Xho*I/*Sal*I. *URA3* Ap^R (+200 bp; Figure 2, line 2)	This work
pSP31	A 0.250-kb *Xho*I PCR fragment (primers 843/913) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−50 bp; Figure 2, line 7)	This work
pSP32	A 0.200-kb *Xho*I PCR fragment (primers 780/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−100 bp; Figure 2, line 8)	This work
pSP33	A 0.150-kb *Xho*I PCR fragment (primers 914/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−150 bp; Figure 2, line 9)	This work
pSP34	A 0.100-kb *Xho*I PCR fragment (primers 915/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−200 bp; Figure 2, line 10)	This work
pSP35	A 0.50-kb *Xho*I PCR fragment (primers 916/843) carrying the 3′ intergenic region of *EPA1*cloned into pSP26 digested with *Xho*I. *URA3* Ap^R (−250 bp; Figure 2, line 11)	This work
pSP38	Similar to pAP354 with the NE cloned immediately after the *HIS3* 3′-UTR. *URA3* Ap^R P_{EPA1}::*GFP*::3′UTR_{HIS3}::NE^+.	This work
pSP105	A 0.300-kb fragment (primers 1135/1136) carrying the NE cloned 5′ the *EPA1* promoter in pSP26. *URA3* Ap^R (Figure 1D)	This work

Table 3 Oligonucleotides used in this study

Primer	Sequence	Sites
666	TCGTTAAGCCATTGTGTTGGATCACTTTCAACACCAAATG	*Bst*XI
667	AAAATCATCCATTGTGTTGGGTTAATTGCAAAGACTAAAT	*Bst*XI
723	TGGTACTTCTCGAGTCCCACCAGTTGG	*Xho*I
724	CATAATAGTGATGAACATAGGGACCTAAAACCAGAAAAT	*Xho*I
776	CATAGGGGTCGACAACCAGAAAATATAATAAC	*Sal*I
777	AAAGTTCTCGAGTCTGGGAGAATAGAAAAGGCA	*Xho*I
795	ATTGACCTCTCGAGGAAGTTTAATTCGAGATT	*Xho*I
796	ACCTGAAACTCGAGAATAGTCCGTTACCTACC	*Xho*I
780	CTCCCAGACTCGAGAACTTTTGAGCAGGGACCA	*Xho*I
839	TAATATCTCTCGAGTCAAGTGTGACCAGGAAT	*Xho*I
841	GTGATTTGCTCGAGCTTTCTCTTGCTTTTGAA	*Xho*I
842	ATGATCATCTCGAGTTAGAATAATAAGTTGTT	*Xho*I
843	CCTTGCACCTCGAGCGTATAAACTCTCATATT	*Xho*I
913	TTCAGGGTCTCGAGTTTACATACGAAGCCTAA	*Xho*I
914	AAAGTTAGCTCGAGTCACGAAAATCCAGAAGA	*Xho*I
915	TAAACTTCCTCGAGAGGTCAATTGTCAAAAAA	*Xho*I
916	TATTCTAACTCGAGATGATCATATGAACATAC	*Xho*I
1135	CTCCCAGAGGATCCAACTTTTGAGCAGGGACCA	*Bam*HI
1136	CCTTGCACGGATCCCGTATAAACTCTCATATT	*Bam*HI
ACT1	CTCAAAATAGCGTGTGGCAAAGAGAAACCGGCGTAAATTGGA ACAACGTGGGTAACACCGTCACCAGAGTCCTTTTG	Probe for S1
EPA1	GCCAGTTCTAGGGTAATTGGGATCTAAATATGCTGCATCCCAA CATGGGTACGAACCCTTCTTCCGAAAATCTATCC	Probe for S1

fusion followed by the 3.1-kb *EPA1–EPA2* intergenic region (P_{EPA1}::GFP::3.1-kb intergenic region, pAP385). When this plasmid was transformed into *C. glabrata*, the GFP expression profile mirrored that observed from the chromosomal *EPA1* locus (BG198): activation of the *EPA1* promoter upon dilution into fresh media, followed by immediate repression (compare Figure 1, D and E, BG198 *vs.* pAP385). This suggests that the *EPA1* regulatory regions in pAP385 contain the *cis*-acting elements required for the positive as well as negative regulation in response to the growth state of cells, and that both regulatory mechanisms can function independently of the chromosomal location. When the 3.1-kb intergenic region was replaced with the *HIS3* 3′-UTR (P_{EPA1}::GFP::3′UTR_{HIS3}, pAP354), the resulting GFP expression profile mimics that derived from the insertional mutation in the chromosome (BG201): higher basal levels of expression, normal induction of *EPA1* transcription upon dilution, and loss of LP transcriptional repression (compare Figure 1, D and E, BG201 *vs.* pAP354). These results further support the important role for the 3.1-kb intergenic region in the transcriptional repression of *EPA1* locus, and suggest the presence in this 3′ region of a *cis*-acting element (NE) responsible for the repression of the expression of *EPA1*.

Mapping of the NE in the intergenic region between EPA1 and EPA2

To map the NE present in the intergenic region between *EPA1* and *EPA2*, we constructed a series of deletions in the 3.1-kb intergenic region carried on pAP385 (P_{EPA1}::GFP::3.1-kb intergenic region), transformed the resulting plasmids into strain BG14 and screened for loss of repression by monitoring GFP expression by FACS. As described above, when GFP is controlled by *EPA1* regulatory regions on the chromosome or in a plasmid, the GFP activity reaches the maximum at 2 hr after diluting SP *C. glabrata* cells into fresh media, then dropping to background levels by 6 hr postdilution. In Figure 2, the extent of *EPA1* transcriptional repression is indicated by the ratio (repression index, RI) of GFP reporter activity at 2 hr *vs.* 8 hr postdilution. First, we mapped the 3′-UTR of *EPA1* from +1 to +200 bp. This construct (pSP26, P_{EPA1}::GFP::+200 bp) is induced but has a RI of only 1.66 (compare with parental plasmid carrying the 3.1-kb intergenic region, RI of 8.01) (Figure 2, line 12). A construct with only 500 bp of the 3′-UTR, however, is fully induced and repressed, with a RI of 8.06 (Figure 2, line 1) at the same level as the parental plasmid carrying the 3.1-kb intergenic region (pAP385; Figure 2, line 12) or the P_{EPA1}::GFP in the chromosome (BG198) (Figure 1D). These data suggest that the NE may reside in the region between 200 to 500 bp downstream of *EPA1* ORF. Within this defined region, we further generated constructs containing deletions in 50-bp increments from either the 5′ or 3′ (Figure 2, line 3–11). These deletion series showed that the NE is contained in this +500-bp region. The first 50-bp deletion (from +450 to +500, relative to the stop codon) completely eliminate repression (RI of 2.79; Figure 2, line 3). Further 50-bp deletions from +300 to +450 had no additional impact (Figure 2, lines 3–6). The 5′ to 3′ deletion series revealed that the NE begins at +300 bp since deletion of +200 to +300 had no impact on the RI (Figure 2, lines 7 and 8), but deletion of +300 to +350 completely eliminated repression (RI of 1.96; Figure 2, line 9). These experiments indicate that the minimal region (NE), which confers the LP-specific repression of *EPA1*, is 200 bp long and is localized between +300 and +500 bp downstream of *EPA1* ORF, indicating a complex *cis*-acting element.

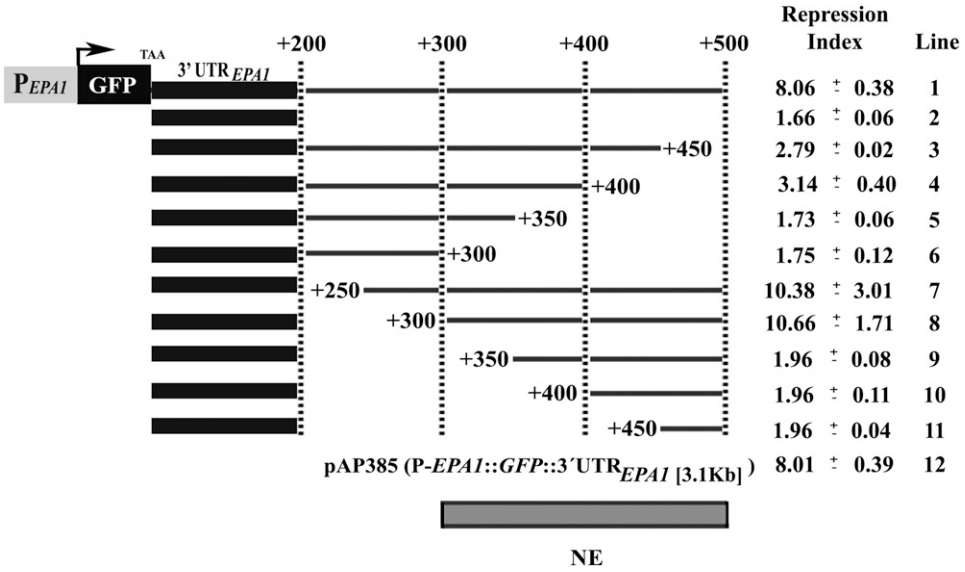

Figure 2 Mapping of the negative element (NE). The activity of the NE was assayed by measuring *EPA1* promoter activity by FACS analysis of the GFP reporter fused to the *EPA1* promoter. BG14 (WT) strain carrying a collection of plasmids containing serially deleted fragments (5′ to 3′ and 3′ to 5′) of the intergenic region between *EPA1* and *EPA2*, were grown in SC −Ura media and assayed as described in Figure 1D legend. Fold of repression is the maximal expression of *EPA1* at 2 hr divided by the expression at 8 hr. Line 1 is pSP21, line 2 is pSP26, line 3 is pSP20, line 4 is pSP7, line 5 is pSP19, line 6 is pAP407, line 7 is pSP31, line 8 is pSP32, line 9 is pSP33, line 10 is pSP34, line 11 is pSP35, and line 12 is pAP385 (Table 2). Experiments were done in triplicate and SDs are shown.

Characterization of the NE

We asked whether function of the NE depends on native polyadenylation sequences. To do this, we replaced the 200 bp *EPA1* 3′-UTR sequence immediately after the stop codon of GFP with the 200 bp from the 3′-UTR of *C. glabrata HIS3*. Following the *HIS3* 3′-UTR, we placed the 300-bp NE region (+200 to +500, pSP38). In this construct, pSP38, *EPA1* promoter is induced upon dilution into fresh media, followed by immediate repression; however, repression of this construct does not follow that of the *EPA1* promoter in pSP385 (P_{EPA1}::GFP::3.1-kb intergenic region) or pSP105 (see below). This result indicates that the *EPA1* polyadenylation sequences contribute partially to the overall negative regulation of *EPA1* (Figure 1E). The next question we asked was whether the LP-specific repression mediated via the NE depends on its location relative to the ORF. To test this, we placed the 300-bp negative element region (from +200 to +500 bp downstream of *EPA1* ORF) immediately in front of the *EPA1* promoter fused to GFP (NE::P_{EPA1}::GFP::3′UTR_{EPA1}, pSP105). The GFP expression profile is nearly identical to the parental construct (Figure 1E, compare pSP105 *vs.* pAP385) in which the *EPA1* regulatory elements are in the original order. This shows that the regulation exerted by the NE can be location independent. These experiments confirm that the NE itself is a *cis*-acting regulatory element mediating the LP-specific repression. While the proximity of the NE to the 3′-UTR raised the possibility that the NE functions to regulate transcript stability, the fact that the NE can function upstream of the ORF suggests rather that it acts at the level of transcription initiation.

The effect of the NE on transcription of the *EPA1* locus has a substantial impact on adherence, as would be expected. We compared adherence of a wild-type strain BG14 with strain BG646. Both strains contain the functional *EPA1* ORF at the normal chromosomal locus, except that the latter carries a Tn*7* insertion localized at 300 bp downstream of *EPA1* ORF which separates the *EPA1* ORF from the NE by 3.4 kb. As expected, the parental strain BG14 in SP does not express *EPA1* and showed little adherence to HeLa cells (Figure 3) but SP cells of the mutant strain BG646 were hyperadherent (Figure 3). Consistent with this experiment, RT–PCR analysis of strain BG646 showed an increased transcript level of *EPA1* (data not shown). This experiment indicates that the regulation mediated through the NE influences the adhesion phenotype of *C. glabrata* cells.

EPA1 is controlled by the Sir complex and by yKu70, yKu80, Rap1, and Rif1

We have previously shown that the *EPA1-3* loci (Figure 1A) is under the control of subtelomeric silencing. In those

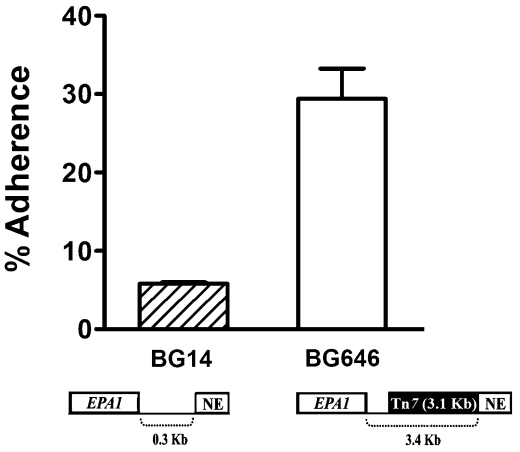

Figure 3 NE effect on adherence. *C. glabrata* wild-type strain BG14 (WT) (hatched bar) and strain BG646 containing a Tn*7* insertion between *EPA1* and the NE (open bar) were grown for 48 hr at 30° in YPD media. Cells were diluted to OD_{600} of 1.0 in HBSS supplemented with 5 mM $CaCl_2$. The adherence assays were done as described in the Figure 1C legend. Adherent cells were recovered from the epithelial cells and were plated for viable accounts. See *Materials and Methods*.

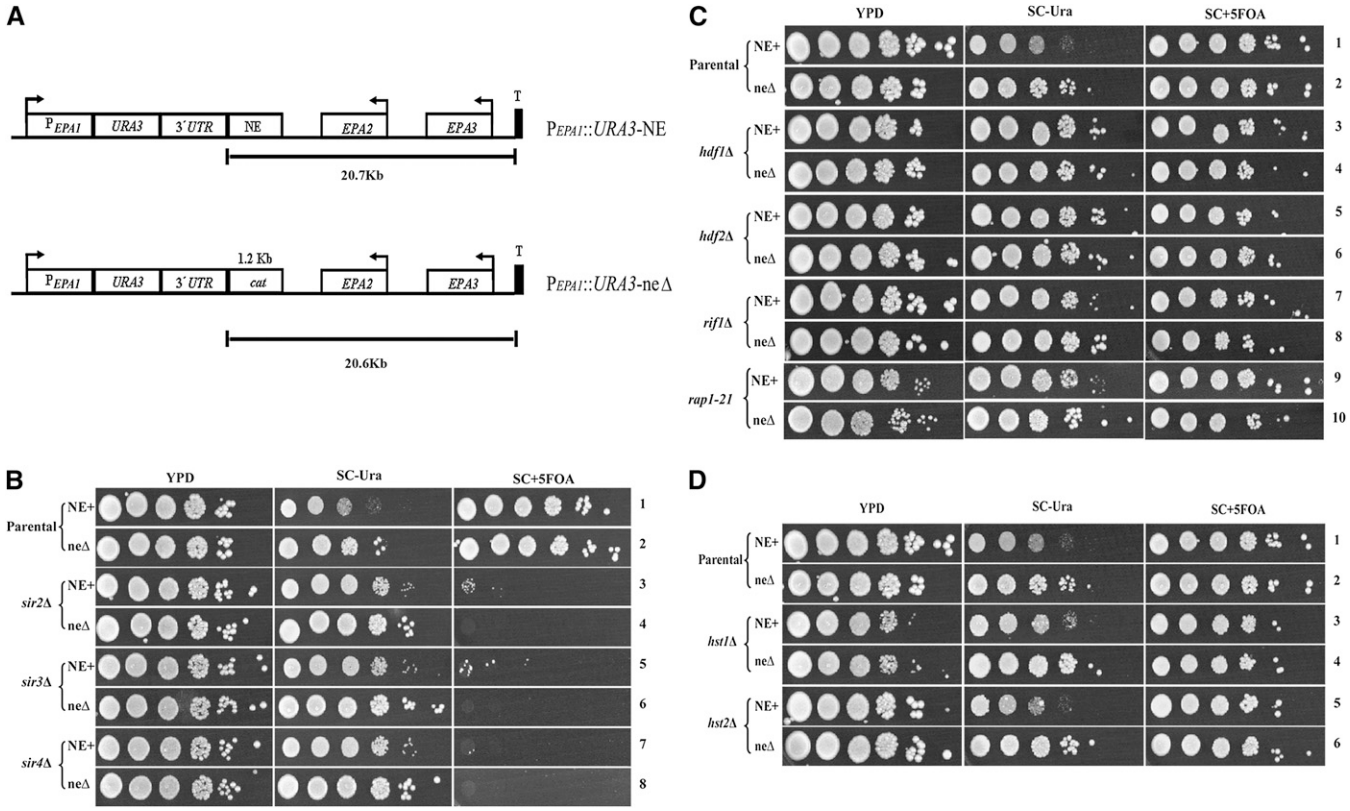

Figure 4 Silencing effect on the expression of *EPA1*. Schematic representation of the reporter strains. (A) *EPA1* was replaced by the *URA3* gene and the NE was replaced by the bacterial *cat* gene and recombined in the chromosome. *URA3* reports the activity of the *EPA1* promoter. (B) The parental strains NE$^+$ and neΔ and the strains carrying null mutations in *SIR2–SIR4* (*sir2*Δ–*sir4*Δ), (C) *HDF1* (yKu70), *HDF2* (yKu80), *RIF1*, and *rap1-21* (*hdf1*Δ, *hdf2*Δ, and *rap1-21*), and (D) *HST1* and *HST2* (*hst1*Δ and *hst2*Δ) were grown for 48 hr in YPD. Strains were diluted to OD_{600nm} 0.5 with distilled water and 10-fold serial dilutions were spotted onto YPD, SC −Ura and SC +5-FOA plates. Plates were incubated at 30°. Ura$^+$ cells die on SC +5-FOA plates. Only cells with the *URA3* gene transcriptionally repressed can grow on SC +5-FOA. See *Materials and Methods*.

experiments, a *URA3* reporter gene inserted at 20.7 kb from the telomere (300 bp from the TAA of *EPA1*), was largely not silenced, suggesting that the telomere position effect (TPE) ends before the *EPA1* locus (De Las Peñas *et al.* 2003; Rosas-Hernandez *et al.* 2008). However, in contrast to those data, mutations in *SIR2–SIR4* in fact increase the expression of *EPA1* (Castaño *et al.* 2005; Domergue *et al.* 2005). We therefore revisited whether the *EPA1* locus itself is controlled by silencing, by assessing the role of the Sir complex, yKu70, yKu80, Rap1, and Rif1 as well as the sirtuins Hst1 and Hst2. To monitor silencing of the *EPA1* locus we constructed a sensitive silencing reporter strain in which the *EPA1* ORF in its chromosomal location was replaced with the *URA3* ORF. Separately, we replaced a 1.0-kb fragment (carrying the NE) from the *EPA1/EPA2* intergenic region with a 1.2-kb fragment containing the *cat* gene (chloramphenicol acetyl transferase gene) from the bacterial plasmid pACYC184 to maintain the same distance from the telomeric repeats (Figure 4A). This pair of constructs permits assessment of silencing of the *EPA1* locus as well as the impact of the NE on that silencing.

The two strains (P_{EPA1}::*URA3*::NE$^+$, BG1124, and P_{EPA1}::*URA3*::neΔ, BG1132) were grown in YPD media and spotted onto YPD (for viable counts), SC −Ura (to assess activity of the *EPA1* promoter) and 5-FOA plates (to assess silencing of the *EPA1*::*URA3* locus, since the synthesis of Ura3 is toxic to the cell in the presence of 5-FOA). The NE$^+$ strain (P_{EPA1}::*URA3*::NE$^+$) grew poorly on SC −Ura medium (Figure 4B, line 1), whereas the neΔ strain (P_{EPA1}::*URA3*::neΔ) grew better but not to the same extent as the viable count on YPD (Figure 4B, line 2). This is consistent with the repressor role of the NE on the expression of *EPA1*. Both parental strains (P_{EPA1}::*URA3*::NE$^+$ and P_{EPA1}::*URA3*::neΔ) grew on 5-FOA plates at almost the same extent as the viable count on YPD, suggesting that *EPA1* is subject to silencing independent of the NE (Figure 4B, lines 1 and 2). Consistent with this, even the neΔ strain (P_{EPA1}::*URA3*::neΔ) did not grow as well on the SC −Ura plates as on the YPD or 5-FOA plates (Figure 4B, line 2), suggesting NE-independent repression of *EPA1*. These results suggest two levels of negative regulation of *EPA1*. One depends on the NE; in addition, a NE-independent repression is indicated by the growth of both strains (NE$^+$ and neΔ) on 5-FOA plates and by the reduced growth of the neΔ strain (P_{EPA1}::*URA3*::neΔ) on SC −Ura (Figure 4B, line 2).

To assess the impact of known silencing genes on repression of the *EPA1* locus, we deleted the *SIR2–SIR4*, *HDF1*

Table 4 Role of silencing proteins on NE function independently of telomere

Strain	Genotype	Plasmid	Repression index
BG14	WT	pSP21	16.26 ± 2.00
BG509	*rif1*Δ	pSP21	15.06 ± 1.83
BG592	*rap1-21*	pSP21	13.04 ± 1.87
BG676	*sir3*Δ	pSP21	16.71 ± 2.10
BG1048	*sir2*Δ	pSP21	14.62 ± 1.78
BG1050	*sir4*Δ	pSP21	15.78 ± 0.78
BG1080	*hdf1*Δ	pSP21	8.74 ± 1.54
BG1081	*hdf2*Δ	pSP21	8.66 ± 2.10
BG14	WT	pSP26	2.54 ± 0.40

(yKu70), *HDF2* (yKu80), *RIF1*, *HST1*, and *HST2* genes in the strains carrying the P_{EPA1}::*URA3*::NE and P_{EPA1}::*URA3*::neΔ constructs on the chromosome. In addition, in both strains, we replaced the essential *RAP1* gene with the silencing defective *rap1-21* allele. Strains were grown and spotted onto YPD, SC −Ura and 5-FOA plates. *sir2*Δ–*sir4*Δ mutant strains did not grow on 5-FOA plates, indicating that silencing is completely relieved independently of the presence of the NE (Figure 4B, lines 3–8). Consistent with this, growth of the *sir2*Δ–*sir4*Δ mutant strains on SC −Ura plates was better than the corresponding parental strains. Notably, growth of *sir2*Δ, *sir3*Δ, or *sir4*Δ NE^+ strains on SC −Ura plates was consistently worse than the corresponding *sir2*Δ, *sir3*Δ, or *sir4*Δ neΔ strains (Figure 4B, lines 3, 5, and 7 and lines 4, 6, and 8 on SC −Ura plates). This suggests that the function of the NE is independent of the Sir proteins.

We analyzed the effect of yKu70, yKu80, Rif1, and Rap1 on the expression of *EPA1*. Loss of *HDF1* (yKu70), *HDF2* (yKu80), *RIF1*, and *RAP1* in the presence (NE^+) or absence (neΔ) of the NE did not substantially affect silencing of *EPA1* since the vast majority of *hdf1*Δ, *hdf2*Δ, *rif1*Δ, or *rap1-21* mutant strains grow on 5-FOA plates (Figure 4C, compare lines 1 and 2 with lines 3–10). The *hdf1*Δ, *hdf2*Δ, *rif1*Δ, and *rap1-21* NE^+ strains grew better than the parental NE^+ strain on SC −Ura plates (Figure 4C, compare line 1 with lines 3, 5, 7, and 9), suggesting a derepression of *EPA1* transcription in these backgrounds, even though the effect on silencing was minimal. Notably, for the *hdf1*Δ and *hdf2*Δ mutants, growth of the NE^+ and neΔ strains on SC −Ura plates were the same, suggesting that *HDF1* (yKu70) and *HDF2* (yKu80) are required for function of the NE (Figure 4C, lines 3–6). The sirtuins Hst1 and Hst2 do not participate in the regulation of *EPA1* since *hst1*Δ and *hst2*Δ strains in the presence (NE^+) or absence (neΔ) of the NE behave the same as their corresponding parental strains (Figure 4D).

To assess the role of Sir2–Sir4, yKu70, yKu80, Rap1, and Rif1, on NE function independently of the telomere, we assayed the repression index of P_{EPA1}::GFP::+500 bp (pSP21; Figure 2, line 1) in each mutant background. Consistent with no effect of Sir2–Sir4, Rif1, and Rap1 on NE function, the RI in these backgrounds was no different than wild-type strain BG14. By contrast, for yKu70 and yKu80, the RI was decreased to 8.74 and 8.66, respectively, consistent with a role for yKu70 and yKu80 in NE-mediated repression (Table 4).

The NE is present 10 times in the C. glabrata genome

Finally, we asked whether additional copies of the NE were present in the *C. glabrata* genome and associated to other *EPA*s. We carried out a Blast search with the 200-bp sequence of the NE and found that the last 60 bp are present 10 times in the *C. glabrata* genome. This sequence is associated both with *EPA* genes as well as other genes (Figure 5).

Discussion

Adherence to specific cell tissues is important for pathogens. Their capacity to adhere to cells, tissues, abiotic surfaces, as well as their ability to form biofilms is often tied to expression of families of cell surface proteins whose transcription is tightly controlled. In *C. glabrata*, the *EPA* genes encode GPI-anchored cell wall proteins of which *EPA1*, *EPA6*, and *EPA7* have been shown to mediate adherence to epithelial cells *in vitro* (Cormack *et al.* 1999; De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Domergue *et al.* 2005). Interestingly, many of these *EPA* genes are subject to chromatin-based subtelomeric silencing. This epigenetic regulation of adhesins is advantageous, since pathogens need not commit all cells in the population to express a particular adhesin, allowing a balance between adherence, colonization, and dissemination (De Las Peñas *et al.* 2003; Halme *et al.* 2004; Domergue *et al.* 2005).

A 3′ cis-acting element (NE) negatively regulates the expression of EPA1

In this study, we showed that *EPA1* expression is negatively regulated by two independent mechanisms: subtelomeric silencing and a telomere-independent, novel mode of negative regulation, dependent on a *cis*-acting NE contained in a 200-bp fragment required for full activity, located 300 bp downstream from the stop codon of *EPA1* in the intergenic region between *EPA1* and *EPA2* (Figure 2). The NE can still repress transcription independent of the 3′-UTR used and if placed upstream of the *EPA1* promoter (Figure 1E), underscoring that the NE is a transcriptional, rather than a post-transcriptional regulatory element. Furthermore, the *EPA1* 3′-UTR has a partial contribution on the expression of *EPA1*.

To our knowledge, *cis*-acting elements located outside promoters, or 3′ of the ORF affecting the expression of promoters have not previously been reported in yeast for Pol II promoters, though similar *cis*-acting elements have been described in yeast for Pol III transcribed genes and in other organisms, some localized inside introns (Errede *et al.* 1987; Martin *et al.* 2001; Stark *et al.* 2001; Calderwood *et al.* 2003; Delaloy *et al.* 2008). In yeast, activators or repressors generally function when their corresponding *cis*-acting elements are located at distances no greater than 700 bp (Guarente and Hoar 1984; Struhl 1984; Keegan *et al.* 1986) upstream of the start site of transcription. Notably, *cis*-acting elements

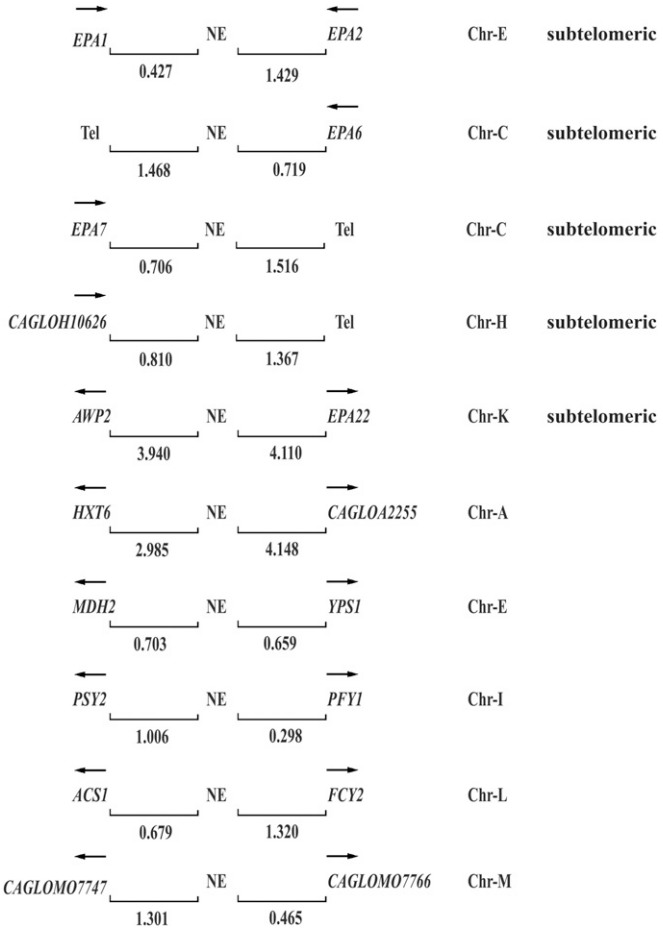

Figure 5 Blast analysis of the NE in the *C. glabrata* genome. The last 60 bp of the NE is associated with other *EPA*s and non-*EPA* genes. *EPA1*, *EPA2*, *EPA6*, *EPA7*, *AWP2*, and *CAGLOH10626* (15) encode cell wall proteins, all are subtelomeric and the NE associated is localized at their 3′ ends, except for *AWP2* and *EPA22*, which is localized between these two divergently transcribed genes. The NE associated to non-*EPA* genes is located at the 5′ regions (promoters) of these divergently expressed genes. *HXT6* (high-affinity glucose transporter of the major facilitator superfamily), *MDH2* (cytoplasmic malate dehydrogenase), *YPS1* (GPI-anchored aspartyl protease), *PSY2* (putative subunit of an evolutionarily conserved protein phosphatase complex containing the catalytic subunit Pph3p and the regulatory subunit Psy4p), *PFY1* (profilin, binds actin involved in cytoskeleton organization), *ACS1* (acetyl-coA synthetase isoform), *FCY2* (purine-cytosine permease), and CAGLOA2255, CAGL0M07747, and CAGL0M07766 are of unknown function (*Saccharomyces* Genome Database http://www.yeastgenome.org/ and *C. glabrata* Genome Database http://www.genolevures.org/cagl.html). Arrows indicate direction of transcription; numbers show the distance in kilobases between the negative element (NE) and the genes or the telomere (Tel); and Chr-(letter) denotes chromosome notation.

(UAS or operator) heterologously positioned at distances >700 bp on the 3′ end of a gene, can activate/repress if the reporter gene and the *cis*-element are localized near a telomere (de Bruin *et al.* 2001; Zaman *et al.* 2002). One explanation is that yeast telomeres can fold back and form a higher order structure or loop, allowing the *cis*-acting element with a tethered *trans*-acting factor to interact with the promoter and in this way activate or repress transcription of that gene. A similar mechanism could occur between the NE and some element in the *EPA1* promoter, in which looping of the DNA would establish an interaction leading to repression of *EPA1*. In support of this hypothesis, the NE does not function to repress transcription of five other promoters (*HHT2*, *MET3*, *PGK1*, *PDC1*, and *EGD1*) when carried on a CEN ARS plasmid (data not shown).

Interestingly, there have been no reports in other *Candida* species of transcriptional regulation dependent on a *cis*-element 3′ to the ORF. However, in an example with clear parallels to *EPA1* regulation, *C. albicans*, *ALS1* and *ALS7*, which, like *EPA1*, encode cell surface adhesions, are also induced in lag phase (Green *et al.* 2005). Whether this regulation depends on a 3′ element has not, to our knowledge, been tested.

Repression of EPA1 expression by the NE depends on HDF1 (yKu70) and HDF2 (yKu80) but is independent of the telomere

EPA1 repression after induction analyzed in the context of the chromosome, can be recapitulated in a plasmid (BG198 *vs.* pAP385, Figure 1, D and E), indicating that all regulatory elements for proper regulation of *EPA1* are contained in the plasmid (pAP385) and that NE-mediated repression of *EPA1* transcription is telomere independent. This telomere-independent regulation of *EPA1* expression must coordinate with telomere-dependent silencing.

Surprisingly, our genetic data show that the NE-mediated repression depends on *HDF1* (yKu70) and *HDF2* (yKu80) (Figure 4C, lines 3–6). yKu70 and yKu80 are essential to repair double strand breaks by nonhomologous end joining (Rosas-Hernandez *et al.* 2008), prevent native chromosome ends from degradation and fusion, and initiate silencing by recruiting Sir4 to the telomere (Tham and Zakian 2002). Interestingly, *C. glabrata* yKu70/yKu80 are not required for subtelomeric silencing at the *EPA1* telomere (chromosome E Right) (Rosas-Hernandez *et al.* 2008). How might yKu70 and yKu80 repress transcription of *EPA1* through the NE? Given that it has been shown that the Ku complex also associates with subtelomeric regions (Martin *et al.* 1999) and can nucleate silencing when tethered (Tham and Zakian 2002; Rusche *et al.* 2003), a possible model is that Ku associates directly with the NE ultimately leading to repression. We suggest that the repression mechanism might result from Ku-mediated interactions between the NE and elements in the *EPA1* promoter that form a transcriptionally repressed chromatin loop.

EPA1 expression is silenced

We have shown previously that *EPA1* expression is regulated by the silencing machinery (Castaño *et al.* 2005; Domergue *et al.* 2005). However, the analysis of *URA3* reporter genes inserted in the *EPA1–EPA3* region suggested that subtelomeric silencing ends in the intergenic region between *EPA1* and *EPA2* (De Las Peñas *et al.* 2003). We revisited this in the current study, and our genetic experiments confirm that Sir2–Sir4 silence the expression of *EPA1*. We suggest that differences in the reporter constructs used affect the

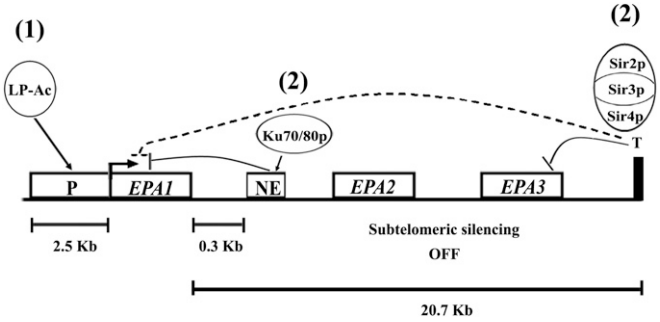

Figure 6 Model of *EPA1* regulation. In stationary phase (SP), *EPA1* is not expressed and cells are nonadherent. Upon dilution into fresh media, (1) a log-phase specific transcriptional activator (LP-Ac) induces expression of *EPA1* and cells become adherent. This transcriptional activation is counteracted in log phase (LP) by the concerted action of silencing and the NE (2). The Sir complex silences the expression of *EPA1* and yKu70/yKu80 repress *EPA1* expression through the NE. These two regulatory mechanisms assure that *EPA1* is not expressed even in the presence of the LP-Ac, which is active throughout LP.

silencing assay. The *URA3* reporter silencing experiments were done with two different promoters—the *EPA1* promoter fused to the *URA3* gene (shown in this article) and the *URA3* gene driven by its own promoter in the previous analysis (De Las Peñas *et al.* 2003; Castaño *et al.* 2005; Rosas-Hernandez *et al.* 2008). We suggest that the *EPA1*–*URA3* construct more faithfully reported silencing than the heterologous *URA3* gene either because the *EPA1* promoter is weaker and more sensitive to silencing or potentially because the *URA3* reporter inherently can be an imperfect measure of silencing (Stillman *et al.* 2011).

Our data suggest, therefore, that there are two distinct mechanisms that maintain the expression of *EPA1* tightly controlled: negative regulation by silencing, which depends on the Sir complex and telomere proximity, and a telomere-independent, Sir-independent repression by a NE at the 3′ end of the gene that depends on yKu70 and yKu80. These mechanisms operate independently of one another but in conjunction tightly control expression of *EPA1*. In our current model of *EPA1* transcriptional regulation, upon dilution into fresh media, a log-phase–specific transcriptional activator (LP-Ac, Figure 6) induces *EPA1* expression (Figure 1B). After one cell division, both silencing (Sir complex dependent) and the NE with yKu70/80 cooperate to repress transcription. These two regulatory mechanisms counteract the putative LP-Ac that is present and active throughout LP, keeping the expression of *EPA1* repressed. This mode of regulation keeps the expression of *EPA1* tightly repressed, but poised to be transiently induced in the appropriate environment. We do not know the identity of the activator; however, the transcription factors Flo8 and Mss1 are candidates since both transcription factors are normally required for the expression of *EPA6* under inducing environmental conditions (Mundy and Cormack 2009).

It is worth pointing out that traditional gene expression analyses in yeast do not usually use cognate 3′-UTR/3′-intergenic regions or assess their potential impact on regulation. Our data suggest that it might be worth doing so.

Acknowledgments

We thank B. Ma for careful reading of the manuscript. This work was funded by a Consejo Nacional de Ciencia y Tecnología (CONACYT) fellowship to V.G.-G. (143037), J.J.-C. (48549), M.B.-M.-C. (209276) and V.M.-J. (205619). This work was funded by CONACYT grant no. CB-2005-48279 to A.D.L.P and by National Institutes of Health grant 5R01AI046223 to B.C. We will always remember Meg.

Literature Cited

Ausubel, F., R. Brent, R. E. Kingston, D. Moore, J. G. Seidman *et al.*, 2001 *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Calderwood, M. S., L. Gannoun-Zaki, T. E. Wellems, and K. W. Deitsch, 2003 Plasmodium falciparum var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. J. Biol. Chem. 278: 34125–34132.

Calvin, N. M., and P. C. Hanawalt, 1988 High-efficiency transformation of bacterial cells by electroporation. J. Bacteriol. 170: 2796–2801.

Castaño, I., R. Kaur, S. Pan, R. Cregg, A. De Las Peñas *et al.*, 2003 Tn7-based genome-wide random insertional mutagenesis of Candida glabrata. Genome Res. 13: 905–915.

Castaño, I., S. J. Pan, M. Zupancic, C. Hennequin, B. Dujon *et al.*, 2005 Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata. Mol. Microbiol. 55: 1246–1258.

Castaño, I., A. De Las Peñas, and B. Cormack, 2006 Function and regulation of adhesin gene families in *Saccharomyces cerevisiae*, *Candida albicans*, and *Candida glabrata*, p. 684 in *Molecular Principles of Fungal Pathogenesis*, edited by J. Heitman, J. E. Edwards, S. G. Filler, and A. P. Mitchell. ASM Press, New York.

Chang, A. C., and S. N. Cohen, 1978 Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134: 1141–1156.

Cormack, B. P., and S. Falkow, 1999 Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen Candida glabrata. Genetics 151: 979–987.

Cormack, B. P., N. Ghori, and S. Falkow, 1999 An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells. Science 285: 578–582.

Cuellar-Cruz, M., M. Briones-Martin-del-Campo, I. Canas-Villamar, J. Montalvo-Arredondo, L. Riego-Ruiz *et al.*, 2008 High resistance to oxidative stress in the fungal pathogen Candida glabrata is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. Eukaryot. Cell 7: 814–825.

Cuellar-Cruz, M., I. Castaño, O. Arroyo-Helguera, and A. De Las Peñas, 2009 Oxidative stress response to menadione and cumene hydroperoxide in the opportunistic fungal pathogen Candida glabrata. Mem. Inst. Oswaldo Cruz 104: 649–654.

de Bruin, D., Z. Zaman, R. A. Liberatore, and M. Ptashne, 2001 Telomere looping permits gene activation by a downstream UAS in yeast. Nature 409: 109–113.

De Groot, P. W. J., K. J. Hellingwerf, and F. M. Klis, 2003a Genome-wide identification of fungal GPI proteins. Yeast 20: 781–796.

De Groot, P. W. J., H. L. Dekker, A. D. De Boer, K. J. Hellingwerf, C. G. De Koster *et al.*, 2003b Identification of cell wall proteins

of the fungal pathogen Candida albicans and other fungi using mass-spectrometric and genome-wide computational approaches. Yeast 20: S57–S57.

De Groot, P. W. J., E. A. Kraneveld, Q. Y. Yin, H. L. Dekker, U. Gross *et al.*, 2008 The cell wall of the human pathogen Candida glabrata: differential incorporation of novel adhesin-like wall proteins. Eukaryot. Cell 7: 1951–1964.

De Las Peñas, A., S. J. Pan, I. Castaño, J. Alder, R. Cregg *et al.*, 2003 Virulence-related surface glycoproteins in the yeast pathogen Candida glabrata are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. Genes Dev. 17: 2245–2258.

Delaloy, C., E. Elvira-Matelot, M. Clemessy, X. O. Zhou, M. Imbert-Teboul *et al.*, 2008 Deletion of WNK1 First Intron Results in Misregulation of Both Isoforms in Renal and Extrarenal Tissues. Hypertension 52: 1149–1154.

Domergue, R., I. Castaño, A. De Las Peñas, M. Zupancic, V. Lockatell *et al.*, 2005 Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. Science 308: 866–870.

Errede, B., M. Company, and C. A. Hutchison 3rd. 1987 Ty1 sequence with enhancer and mating-type-dependent regulatory activities. Mol. Cell. Biol. 7: 258–265.

Fidel, P. L. Jr. J. L. Cutright, L. Tait, and J. D. Sobel, 1996 A murine model of Candida glabrata vaginitis. J. Infect. Dis. 173: 425–431.

Gietz, R. D., and A. Sugino, 1988 New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74: 527–534.

Green, C. B., X. Zhao, K. M. Yeater, and L. L. Hoyer, 2005 Construction and real-time RT-PCR validation of Candida albicans PALS-GFP reporter strains and their use in flow cytometry analysis of ALS gene expression in budding and filamenting cells. Microbiology 151: 1051–1060.

Guarente, L., and E. Hoar, 1984 Upstream activation sites of the CYC1 gene of Saccharomyces cerevisiae are active when inverted but not when placed downstream of the "TATA box". Proc. Natl. Acad. Sci. USA 81: 7860–7864.

Guo, B., C. A. Styles, Q. H. Feng, and G. R. Fink, 2000 A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. Proc. Natl. Acad. Sci. USA 97: 12158–12163.

Halme, A., S. Bumgarner, C. Styles, and G. R. Fink, 2004 Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. Cell 116: 405–415.

Hoyer, L. L., 2001 The ALS gene family of Candida albicans. Trends Microbiol. 9: 176–180.

Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao, 2008 Discovering the secrets of the Candida albicans agglutinin-like sequence (ALS) gene family—a sticky pursuit. Med. Mycol. 46: 1–15.

Kaur, R., R. Domergue, M. L. Zupancic, and B. P. Cormack, 2005 A yeast by any other name: Candida glabrata and its interaction with the host. Curr. Opin. Microbiol. 8: 378–384.

Keegan, L., G. Gill, and M. Ptashne, 1986 Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. Science 231: 699–704.

Klotz, S. A., N. K. Gaur, D. F. Lake, V. Chan, J. Rauceo *et al.*, 2004 Degenerate peptide recognition by Candida albicans adhesins Als5p and Als1p. Infect. Immun. 72: 2029–2034.

Kobayashi, O., N. Hayashi, R. Kuroki, and H. Sone, 1998 Region of Flo1 proteins responsible for sugar recognition. J. Bacteriol. 180: 6503–6510.

Li, F., and S. P. Palecek, 2003 EAP1, a Candida albicans gene involved in binding human epithelial cells. Eukaryot. Cell 2: 1266–1273.

Martin, M. P., V. L. Gerlach, and D. A. Brow, 2001 A novel upstream RNA polymerase III promoter element becomes essential when the chromatin structure of the yeast U6 RNA gene is altered. Mol. Cell. Biol. 21: 6429–6439.

Martin, S. G., T. Laroche, N. Suka, M. Grunstein, and S. M. Gasser, 1999 Relocalization of telomeric Ku and SIR proteins in response to DNA strand breaks in yeast. Cell 97: 621–633.

Mundy, R. D., and B. Cormack, 2009 Expression of Candida glabrata adhesins after exposure to chemical preservatives. J. Infect. Dis. 199: 1891–1898.

Pfaller, M. A., and D. J. Diekema, 2010 Epidemiology of invasive mycoses in North America. Crit. Rev. Microbiol. 36: 1–53.

Roetzer, A., T. Gabaldon, and C. Schuller, 2011 From Saccharomyces cerevisiae to Candida glabrata in a few easy steps: important adaptations for an opportunistic pathogen. FEMS Microbiol. Lett. 314: 1–9.

Rosas-Hernandez, L. L., A. Juarez-Reyes, O. E. Arroyo-Helguera, A. De Las Peñas, S. J. Pan *et al.*, 2008 yKu70/yKu80 and Rif1 regulate silencing differentially at telomeres in Candida glabrata. Eukaryot. Cell 7: 2168–2178.

Rusche, L. N., A. L. Kirchmaier, and J. Rine, 2003 The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annu. Rev. Biochem. 72: 481–516.

Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu *et al.*, 2004 Functional and structural diversity in the Als protein family of Candida albicans. J. Biol. Chem. 279: 30480–30489.

Sherman, F., G. R. Fink, and J. B. Hicks, 1986 *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122: 19–27.

Stark, K., D. L. Kirk, and R. Schmitt, 2001 Two enhancers and one silencer located in the introns of regA control somatic cell differentiation in Volvox carteri. Genes Dev. 15: 1449–1460.

Stillman, B., M. P. Rossmann, W. J. Luo, O. Tsaponina, and A. Chabes, 2011 A common telomeric gene silencing assay is affected by nucleotide metabolism. Mol. Cell 42: 127–136.

Struhl, K., 1984 Genetic properties and chromatin structure of the yeast gal regulatory element: an enhancer-like sequence. Proc. Natl. Acad. Sci. USA 81: 7865–7869.

Tham, W. H., and V. A. Zakian, 2002 Transcriptional silencing at Saccharomyces telomeres: implications for other organisms. Oncogene 21: 512–521.

Zaman, Z., C. Heid, and M. Ptashne, 2002 Telomere looping permits repression "at a distance" in yeast. Curr. Biol. 12: 930–933.

Zupancic, M. L., M. Frieman, D. Smith, R. A. Alvarez, R. D. Cummings *et al.*, 2008 Glycan microarray analysis of Candida glabrata adhesin ligand specificity. Mol. Microbiol. 68: 547–559.

Communicating editor: F. Winston