A DNA-binding protein from *Candida albicans* that binds to the RPG box of *Saccharomyces cerevisiae* and the telomeric repeat sequence of *C. albicans*

Nobuya Ishii,¹ Mutumi Yamamoto,¹ Hans-Werner Lahm,² Shinnji Iizumi,¹ Fumie Yoshihara,¹ Hironobu Nakayama,¹ Mikio Arisawa¹ and Yuhko Aoki¹

Author for correspondence: Yuhko Aoki. Tel: +81 467 47 6773. Fax: +81 467 46 5320.
e-mail: yuhko.aoki@roche.com

¹ Department of Mycology, Nippon Roche Research Center, Kajiwarra 200, Kamakura, Kanagawa-Prefecture, Japan

² Department of PRPB, F. Hoffman-La Roche AG, Postfach 4070, Basel, Switzerland

Electromobility shift assays with a DNA probe containing the *Saccharomyces cerevisiae* ENO1 RPG box identified a specific DNA-binding protein in total protein extracts of *Candida albicans*. The protein, named Rbf1p (RPG-box-binding protein 1), bound to other *S. cerevisiae* RPG boxes, although the nucleotide recognition profile was not completely the same as that of *S. cerevisiae* Rap1p (repressor-activator protein 1), an RPG-box-binding protein. The repetitive sequence of the *C. albicans* chromosomal telomere also competed with RPG-box binding to Rbf1p. For further analysis, we purified Rbf1p 57600-fold from *C. albicans* total protein extracts, raised mAbs against the purified protein and immunologically cloned the gene, whose ORF specified a protein of 527 aa. The bacterially expressed protein showed RPG-box-binding activity with the same profile as that of the purified one. The Rbf1p, containing two glutamine-rich regions that are found in many transcription factors, showed transcriptional activation capability in *S. cerevisiae* and was predominantly observed in nuclei. These results suggest that Rbf1p is a transcription factor with telomere-binding activity in *C. albicans*.

Keywords: *Candida albicans*, transcription factor, RPG box, nuclear protein

INTRODUCTION

Candida albicans, the major opportunistic fungal pathogen of humans, causes serious systemic disease in immunocompromised patients and topical infections in healthy individuals (Odds, 1988). The fungal cells can undergo morphogenesis under various conditions and it has been reported that transcription factors regulate these phenomena, including morphological change, in viable cells. Only a limited number of studies have been reported, however, about gene regulation in *C. albicans*. *SUC1*, a putative zinc finger protein (Kelly & Kwon-Chung, 1992), and *CPH1*, a homologue of *Saccharo-*

myces cerevisiae STE12 (Liu *et al.*, 1994; Malathi *et al.*, 1994), have been reported to be transcription factors in *C. albicans*. Northern blot analysis has revealed that the transcription level of several *C. albicans* genes is regulated (i) during the transition between yeast and hyphal forms (reviewed by Gow, 1994; Calderone, 1994; Cannon *et al.*, 1994a), (ii) during white–opaque switching (reviewed by Morrow *et al.*, 1994) and (iii) by the addition and/or deprivation of sugars or amino acids (MAL2, Geber *et al.*, 1992; HEX1, Cannon *et al.*, 1994b; ADH1, Bertram *et al.*, 1996; ARO3, Pereira & Livi, 1993). Promoter analysis of WH11 (Srikantha *et al.*, 1995), ACT1 (Delbruck & Ernst, 1993) and DUT1 (McIntosh *et al.*, 1994) was performed in *C. albicans*, and McIntosh *et al.* (1994) proposed a putative MCB (*Mlu*I cell-cycle box) element in the DUT1 promoter.

The role of Rap1p (repressor-activator protein 1) in regulating transcription has been intensively studied

Abbreviations: EMSA, electromobility shift assay; GST, glutathione S-transferase; UAS, upstream activation sequence.

The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is D85862.

Table 1. Strains used in this study

Strain	Genotype	Source/reference
<i>S. cerevisiae</i>		
YTF4	<i>MATaura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrap1::LEU2</i> <i>pRS416/RAP1Xb</i>	This study
YTF5	<i>MATaura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrap1::LEU2</i> <i>pRS413/RAP1Xb</i>	This study
YTF6	<i>MATaura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δrap1::LEU2</i> <i>pRS413/rap1-2Mn</i>	This study
SFY526	<i>MATaura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 can^r gal4-542 gal80-538</i> <i>URA3::GAL1-lacZ</i>	Bartel <i>et al.</i> (1993)
<i>C. albicans</i>		
IFO 1060	Wild-type	
ATCC 10231	Wild-type	

(reviewed by Shore, 1994). Rap1p works as an activator, usually together with other factors, at a large number of promoters in yeast, including many of the highly transcribed ribosomal protein and glycolytic enzyme genes (Drazinic *et al.*, 1996). The RPG (ribosomal protein genes) box was proposed as a high-affinity DNA sequence for Rap1p (Buchman *et al.*, 1988). In addition, Rap1p engages in regulating telomere length and initiating telomeric silencing through the binding to telomeric repeat sequences characterized by short stretches of G residues (reviewed by Shore, 1996). In this study, we have attempted to identify a DNA-binding protein of *C. albicans* that could have an important role in the regulation of telomere length as well as gene expression so as to obtain an in-depth insight into the mechanism of transcription in this clinically important

organism. Here we report the purification of a *C. albicans* protein (Rbf1p) that binds to the *S. cerevisiae* RPG box and *C. albicans* telomere sequence, and the expression cloning of its encoding gene. We found that the protein activates transcription in *S. cerevisiae* and is predominantly present in nuclei. This suggests that the factor is a DNA-binding protein that functions as a transcription factor and/or a telomere-length regulator in *C. albicans*.

METHODS

Strains and media. *S. cerevisiae* and *C. albicans* strains used in this study are listed in Table 1. YTF4 was generated by the method described previously by Shore & Nasmyth (1987)

Table 2. Sequences of the competitor oligonucleotides

Oligo	Sequence
scTPI	5'-CTATTGATGTTACACCTGGACACCCCTTTTCTGGCT-3'
scPGK	5'-CCAAACTGAAAAACCCAGACACGCTCGACTTCCTG-3'
scADH1	5'-TTTTCTGGCAACCAACCCATACATCGGGATTCCCTATA-3'
scRPC40	5'-GGATGGATAATTAACCCATACACCTCATACAAGATT-3'
scRP39A	5'-CCGTACAACGAGAACCCATACATTACTTTTTTTAAT-3'
scTEF1	5'-ACCACTTCAAAACACCCAAGCACAGCATACTAAATT-3'
scTEF2	5'-ACATTCATGTTGCACCCACACATTTATACACCCAGA-3'
scHIS4	5'-AATTAATTGCTAAACCCATGCACAGTGACTCACGTT-3'
scPMA1	5'-ACCGCAGAAATAAACCCATACACGAGACGTAGAACC-3'
scTelomere	5'-CCCACACCCACACACCCACACACCCACACACCCACC-3'
klTelomere	5'-(ACGGATTTGATTAGGTATGTGGTGT) ₂ -3'
caTelomere	5'-(ACGGATGTCTAACTTCTTGGTGT) ₂ -3'
NFκB	5'-GATCGAACTGACCGCCCGCGGCCCGT-3'
AP2	5'-AGTTGAGGGGACTTTCCAGGC-3'
C4G scENO1	5'-GATCTAGGATAGCAgCCAAACACCTGCATATTTGGA-3'
A11T scENO1	5'-GATCTAGGATAGCACCCAAACcCCTGCATATTTGGA-3'
C12G scENO1	5'-GATCTAGGATAGCACCCAAACAgCTGCATATTTGGA-3'

Fig. 1. (a) EMSA in which *C. albicans* total protein extracts (2 µg per assay) and the radiolabelled 36 bp oligonucleotide probe containing the *S. cerevisiae* ENO1 RPG box (the sequence in Methods) were used. Lanes 1–10 contained probe plus total protein extract; lanes 2, 4, 6, 8 and 10 contained a 10-fold molar excess of unlabelled oligonucleotides; lanes 3, 5, 7 and 9 contained a 100-fold molar excess of unlabelled oligonucleotides. Lanes: 2 and 3, *S. cerevisiae* ENO1 RPG box; 4 and 5, *S. cerevisiae* TPI RPG box; 6 and 7, *S. cerevisiae* PGK RPG box; 8 and 9, *S. cerevisiae* ADH1 RPG box; 10, *S. cerevisiae* RPC40 RPG box. (b) Quantitative EMSA in which *C. albicans* total protein extracts, the ENO1 RPG box probe and a 10-fold molar excess of unlabelled competitors were used. (c) Quantitative EMSA in which *S. cerevisiae* recombinant Rap1p (0.2 µg per assay) expressed in *E. coli* was used. After the electrophoresis, the radioactivity of the complex forms was quantified by an image analyser. The competition activities were calculated by setting the activity of the ENO1 RPG box as 100%.

except haploidal YPH499 (Sikorski & Hieter, 1989) transfected with pRS416/RAP1Xb was used as a host. Plasmid pRS416/RAP1Xb was generated by the insertion of a RAP1 EcoRI–XbaI fragment from D56, a gift from D. Shore (Columbia University, USA; Shore & Nasmyth, 1987), into pRS416 (Sikorski & Hieter, 1989). Plasmid pRS413/rap1-2Mn was generated by PCR with the mutagenized oligonucleotide 5' GAAGATTACACTTTGACaATTGCGGTC-AAGAAGC 3' [bold letter and underline indicates rap1-2 mutation (Sussel & Shore, 1991) and MnnI site, respectively]. YTF6 was generated from YTF4 by plasmid shuffling with pRS413/rap1-2Mn. Both *S. cerevisiae* and *C. albicans* were routinely grown non-selectively in YPD medium [1%, w/v, yeast extract (Difco); 2%, w/v, Bacto peptone (Difco); 2%, w/v, glucose]. Selection for amino acid prototrophy was performed on SD medium containing 0.67% Yeast Nitrogen Base (Difco), 2% glucose, and supplemented with appropriate amino acids. For plasmid shuffling, 0.1% 5-fluoroorotic acid was used. All media were solidified by 2% (w/v) agar if necessary. Yeast transformation was performed according to Geits *et al.* (1992). Bacterial transformation was according to Inoue *et al.* (1990).

DNA-binding assay. For the electromobility shift assay (EMSA), *S. cerevisiae* ENO1 upstream activation sequence (UAS) (36-mer: 5' GATCTAGGATAGCACCCAAACACCTGCATATTTGGA 3') was used as a probe. ³²P-Labelled oligonucleotide (10 fmol) was incubated with protein for 10 min at 30 °C in 10 µl 10 mM Tris/HCl (pH 7.5) buffer containing 0.5 mM EDTA, 1 mM MgCl₂, 0.5 mM DTT, 50 mM NaCl, 0.1 mg poly[dI-dC] ml⁻¹ and 4% (v/v) glycerol. The reaction mixture was subjected to electrophoresis on an 8% polyacrylamide gel in 25 mM Tris and 192 mM glycine at 4 °C. Following the electrophoresis, gels were dried and radioactivity was quantified by a Mac BAS 1000 image analyser (Fuji film). The sequences of the competitor oligonucleotides are shown in Table 2.

Purification of Rbf1p. *C. albicans* IFO 1060 was grown aerobically at 30 °C in 150 l YPD medium. After cells had reached an OD₆₀₀ of 2.0, they were harvested (yield = 3.0 kg wet wt) and frozen. The cells (300 g) were used for one batch purification, and repeated for 15 cycles. Cells were suspended in 300 ml buffer C (10 mM Tris/HCl, pH 8.0; 1.5 mM KCl; 0.1 mM EDTA; 0.5 mM DTT; 10% glycerol; 0.3 M ammonium sulfate; 1 mM PMSF; 2 µg chymostatin ml⁻¹; 1.5 µg leupeptin ml⁻¹; 5 µg antipain ml⁻¹; 1 µg pepstatin A ml⁻¹; 0.01%, v/v, NP40), and passed twice through a French pressure cell (Ohtake; French Press 5502) at an output pressure of 1300–1500 kg cm⁻². The resulting lysate was spun for 120 min at 13000 g to remove cell debris. All subsequent purification steps were carried out at 4 °C unless stated otherwise. The supernatant was applied to a heparin-agarose column (Sigma) equilibrated in 50 mM KCl/buffer A (20 mM Tris/HCl, pH 8.0; 0.5 mM EDTA; 0.5 mM DTT; 10% glycerol; 50 mM ammonium sulfate; 0.5 mM PMSF; 0.01% NP40). Rbf1p was eluted with a linear gradient formed from 50 mM KCl/buffer A to 1.0 M KCl/buffer A (300 ml for each solution developed at 2 ml min⁻¹). Active fractions in an EMSA with the ENO1 RPG-box-containing oligonucleotides were collected and dialysed against 1 l buffer A, followed by three steps of column chromatography; Mono S (FPLC; Pharmacia), an ssDNA-Cellulose column (Sigma) and a dsDNA-Sepharose column (described below). They were all equilibrated with 50 mM buffer A and Rbf1p was eluted with

Table 3. Purification of Rbf1p from *C. albicans* IFO 1060

Fraction	Total protein (mg)	Specific activity (units mg ⁻¹)*	Total activity (units)	Purification (-fold)	Yield (%)
Total protein extract	17 400	0.010	174	1.00	100
Heparin-agarose	109	0.65	77	113	40.7
Mono S (FPLC)	6.6	3.9	26	685	14.8
ssDNA-cellulose	0.22	28	6.3	4860	3.60
dsDNA (<i>ENO1</i> UAS)-agarose	0.031	110	3.4	19200	1.97
Reverse-phase (C ₄ type; HPLC)	NT	330†	NT	57600	NT

NT, Not tested.

* One unit of Rbf1p activity is defined as the formation of 1 fmol bound forms with *ENO1* RPG-box oligonucleotide.

† Activity of refolded proteins.

a linear gradient from 50 mM KCl/buffer A to 1.0 M KCl/buffer A (30 ml for each solution developed at 1 ml min⁻¹). These activities were monitored by an EMSA. Desalting at each step was performed by dialysis against 1 l 50 mM KCl/buffer A. The dsDNA column was prepared by coupling the 36 bp *ENO1* RPG-box-containing oligonucleotides with CNBr-activated Sepharose 4B (Pharmacia) according to the method of Kadonaga & Tjian (1986). The active fractions eluted from the dsDNA column were loaded onto a C₄-type reverse-phase column (HPLC; COSMOSIL-C₄AR; Nakarai) and a linear acetonitrile gradient was developed from 25% acetonitrile/0.05% trifluoroacetic acid to 45% acetonitrile/0.05% trifluoroacetic acid. Each fraction was lyophilized, denatured with a guanidine-containing buffer (50 mM Tris/HCl, pH 7.9; 0.1 mM EDTA; 1 mM DTT; 0.1 mg BSA ml⁻¹; 0.15 NaCl, 0.1% NP40, 6 M guanidine.HCl) and refolded by the method described by Watanabe *et al.* (1990), followed by the measurement of DNA-binding activity by an EMSA. Active fractions of the HPLC column, which included 60 and 55 kDa proteins, were developed by SDS-PAGE; each band corresponding to 60 or 55 kDa proteins was cut out, and each protein was extracted from the acrylamide gel by incubation with the extraction buffer (0.5 M sodium acetate, pH 8.68; 10 mM magnesium acetate; 1 mM EDTA; 0.1% SDS). These extracted proteins were refolded and assayed by the same method described above.

Amino acid sequence analysis. Tryptic digestion of the purified samples (33 µg 60 kDa protein and 20 µg 55 kDa protein) onto PVDF membrane was performed by the method described by Fernandes *et al.* (1994). Micro-bore reverse-phase HPLC peptide mapping was carried out on a C₈ column (Browlee; HP1090A). Peptides were eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid and peptide-containing fractions were further purified by cation-exchange chromatography (Mini S, Smartsystem; Pharmacia). The fractions were rechromatographed on a Browlee C₈ column and then subjected to N-terminal amino acid sequence analysis (Edman sequencer model 470A; ABI).

Anti-Rbf1p antibody generation. Mice were immunized with eluates of the HPLC C₈ column, the mixture of 60 and 55 kDa proteins (total 3 µg protein), in Freund's complete adjuvant

by interperitoneal injection, and boosted twice a month with Freund's incomplete adjuvant. mAbs were prepared from the spleens excised 4 d after the final boost followed by the method of Campbell (1984). A selection of appropriate hybridomas was performed by a solid-phase assay directly measuring RPG-box-binding activity (M. Yamamoto and others, unpublished). The conditioned media of the hybridomas were used for the immunoscreening and the immunofluorescence microscopy.

Immunoscreening. A *C. albicans* IFO 1060 cDNA library was generated in the λZAPII vector using the ZAP-cDNA synthesis kit (Stratagene) according to the manufacturer's instructions. mRNA was prepared from exponentially growing *C. albicans* IFO 1060 cells in YPD using Oligotex dT30-super (Nippon Roche). The library (10⁶ independent clones) was transfected to *Escherichia coli* XL1-Blue and spread onto NZY (Gibco) agar plates with 0.7% molten agarose-NZY. After a 4 h incubation at 37 °C, dried nitrocellulose filters, presoaked in 20 mM IPTG, were placed on the plates. After a 2 h incubation at 42 °C, the filters were removed and then soaked in 5% skimmed milk/1% casein for 1 h. Filters were subsequently incubated with anti-Rbf1p antibodies (OA4, OD5) overnight at 4 °C. Bound antibody was detected with alkaline-phosphatase-conjugated anti-IgG. Positive signals which appeared on duplicate filters were plaque-purified by two rounds of re-screening.

Expression of proteins in *E. coli*. *C. albicans* *RBF1* and *S. cerevisiae* *RAP1* genes were fused with glutathione S-transferase (GST) in a plasmid, pGEX-4T-1 (Pharmacia), and transformed into *E. coli* strain DH5. In both cases, after the overnight cultivation at 37 °C with 1 mM IPTG, purification of the soluble fractions was performed by GSH-agarose column chromatography (Sigma) according to Pharmacia's instructions.

In vivo transcriptional activation. The *RBF1* fusion with *GAL4_{BD}* was constructed on plasmid pGBT9 (Clontech), and transformed into *S. cerevisiae* SFY526. The plasmid-carrying yeast strains were grown in 2 ml selective medium at 30 °C to an OD₆₀₀ of 2.0 and then the specific β-galactosidase activities were determined as described by Guarente (1983). The A₄₁₅ was measured and assays were normalized to the OD₆₀₀ of the culture and to the assay time (min).

Fig. 2. RPG-box-binding activities and nucleotide recognition profile of 60 and 55 kDa proteins corresponding to Rbf1p activity. (a) SDS-PAGE of gel-purified 60 and 55 kDa proteins with silver staining. The 60 and 55 kDa proteins purified by five steps of column chromatography (Table 1 and Methods) were extracted from the acrylamide gel after SDS-PAGE. Lanes: M, molecular mass markers; 1, fraction 27 of the fifth column chromatography, the microbore C_4 -type reverse-phase HPLC; 2 and 3, extracted 60 and 55 kDa proteins. (b) EMSA of refolded proteins. Gel-purified 60 kDa (lanes 1–5) and 55 kDa (lanes 6–10) proteins were denatured in guanidine-containing buffer, refolded and assayed in an EMSA (0.2 μ g each protein per assay). Lanes 2–5 and 7–10 contained a 100-fold molar excess of unlabelled oligonucleotides. Lanes: 1 and 6, *ENO1* RPG box (ER) probe alone; 2 and 7, ER; 3 and 8, mutated ER (C4G); 4 and 9, mutated ER (C12G); 5 and 10, AP2.

Isolation of nuclei and immunofluorescence of *C. albicans* spheroplasts. For the isolation of nuclei, we used the protocol of Lue *et al.* (1991). Preparation of spheroplasts, fixation to poly-L-lysine-coated slides and staining by anti-Rbf1p mAbs and fluorescein-isothiocyanate-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratory) for Rbf1p and 4',6-diamidino-2-phenylindole (DAPI; Sigma) for nuclei were followed by the method described by Palladino *et al.* (1993).

The triple-stained cells were observed and photographed with an epi-illumination fluorescence microscope (SPM-RFL-II; Nikon), according to Yokoyama *et al.* (1990).

DNA manipulation. Standard recombinant DNA techniques were followed by the methods described by Sambrook *et al.* (1989). DNA fragments were radioactively labelled with 32 P using the Multiprime DNA labelling system (Amersham) and [α - 32 P]dCTP or T4 polynucleotide kinase and [γ - 32 P]ATP. Hybridization was carried out with rapid hybridization Buffer (Amersham) according to the manufacturer's instructions. The nucleotide sequence was determined with a 373A DNA sequencing system (ABI) by the Dye-terminator method according to the manufacturer's instructions.

RESULTS

RPG-box- and telomeric-repeat-binding activity in the total protein extract of *C. albicans*

To test whether a specific protein in *C. albicans* binds to the *S. cerevisiae* RPG box, EMSAs were performed with an end-labelled 36 bp oligonucleotide containing the *S. cerevisiae* *ENO1* RPG box. One complex that disappeared by the addition of 10–100-fold unlabelled *ENO1* RPG box oligonucleotide was detected (Fig. 1a). Binding competition experiments indicated that this *ENO1* RPG-box-binding activity also competed with other RPG boxes in *S. cerevisiae*, *TPI*, *RPC40*, *RP39A*, *TEF2*, *HIS4* and *PMA1*. In addition, telomeric repeat sequences of *C. albicans* and *Kluyveromyces lactis* bound to the protein (Fig. 1b). Whereas other *S. cerevisiae* RPG boxes found in the telomere and promoter regions of the *PGK*, *ADH1* and *TEF1* genes had high affinity for the *S. cerevisiae* Rap1 protein (Fig. 1c), these sequences did not compete with this activity (Fig. 1b), suggesting that the nucleotide recognition manner of this RPG-box-binding factor (Rbf1p) in *C. albicans* was not completely the same as that of *S. cerevisiae* Rap1p. Oligonucleotides including the NF- κ B- or AP2-binding sequences unrelated to Rap1p-binding sites did not compete with the *ENO1* RPG box for either *C. albicans* Rbf1p or *S. cerevisiae* Rap1p. The affinity of Rbf1p for the 36 bp *ENO1* RPG box was comparable to that of *S. cerevisiae* Rap1p (K_d approximately 2–3 nM; data not shown).

Purification of Rbf1p from *C. albicans*

To further characterize and determine the amino acid sequence of Rbf1p, we purified it from *C. albicans* IFO 1060 as described in Methods and in Table 3. Four column chromatography steps resulted in a 19200-fold purification. In the fourth step, we used affinity chromatography employing the *ENO1* RPG-box-conjugated Sepharose column. The 19200-fold enriched preparation was then subjected to reverse-phase chromatography on a microbore C_4 HPLC column. Each fraction was assayed for Rbf1p activity in the EMSA after refolding of the protein and after denaturation in the guanidine buffer. Rbf1p activity was detected in association with 60 and 55 kDa proteins in SDS-PAGE. The two proteins had *ENO1* RPG-box-

Table 4. N-terminal amino acid sequences of tryptic fragments of Rbf1p

Fragment	Sequence
1	N-K-P-D-E-P-G-H-N-E-E-N-S-R
2	Q-A-Q-A-Q-A-Q-A-Q-A-Q-R
3	T-L-V-E-K-P-I-N-K-P-H-H-H-X-L-L
4	G-L-D-Q-P-D-A-A-V-I-A-A-I
5	I-S-E-M-W-V-L-R
6	E-H-Q-Q-L-Q-H-A-Y-Q-Q-Q-Q-L-H-Q-L-G-Q-L
7	D-A-L-T-T-D-E-F-D-T-N-E-D-L-R
8	I-V-I-N-S-S-K-P-K
9	L-V-A-N-L-Y-P-N-E-K

Fig. 3. Restriction map of the *C. albicans* *RBF1* locus. p12B (1.0 kbp) was cloned from a cDNA library derived from *C. albicans* IFO 1060 and p27a1 (2.8 kbp) and p8a1 (8.3 kbp) were from a genomic library of *C. albicans* ATCC 10231. Black rectangles indicate the locus of amino acid sequences determined from the purified Rbf1p. SI, *Sal*I; Hc, *Hinc*II; Ps, *Pst*I; Bg, *Bgl*II; Ec, *Eco*RI.

binding activity with the same sequence specificity as shown in Fig. 2; tryptic mapping by HPLC of the 60 and 55 kDa proteins also showed they were similar. In addition, the mass analysis of selected HPLC peak fractions revealed the presence of peptides with the same molecular mass (data not shown). These observations are consistent with the notion that the 60 kDa protein is Rbf1p and the 55 kDa protein may be a shortened fragment of it.

The tryptic fragments separated by the C_8 reverse-phase HPLC and cation-exchange chromatography were subjected to N-terminal Edman degradation. The sequences are listed in Table 4.

Immunoscreening of the *RBF1* gene

To clone the *RBF1* gene, we raised mAbs against the purified protein (a mixture of 60 and 55 kDa proteins), and a λ ZAPII *C. albicans* cDNA library was screened with a mixture of two anti-Rbf1p mAbs, both of which reacted with 60 and 55 kDa proteins in a Western blotting experiment using purified proteins (data not shown). Out of 10^6 plaques, we obtained one positive clone, p12B, which lacked the 3'-terminus of the coding region. By the screening of a genomic library of *C. albicans* with the p12B plasmid as a probe, we isolated overlapping DNAs (p27a1 and p8a1) representing approximately 2.8 and 8.3 kb inserts, respectively (Fig. 3). Nucleotide sequence analysis of a 3.5 kb *Sal*I-*Bgl*II fragment that reacted with p12B revealed a single ORF spanning 1494 nucleotides with no apparent introns. The ORF specifies a protein of 527 aa with a calculated

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TACAAAGAGAAAAAATCCCTCATTAAGACCTCTTATCTTTTCTCTCTTTCTACTTTTATTAATTCCTTCATATTAACA 90
ATTTCGTGTTCCAAAGAGATTTCCTAACCAATCCCAATGAGATTCTTATTCAGGAACAATTAATTTATTTATTCAGGATTAATGTT 180
TACAAAAAATTTACACAGATAGTTTGTCAACTTGAACCAATTTGAAGACAAATTTAGGTGGAAAGAGATAACATACGTATACACA 270
TATAAGAAATATTGGTGTGTTTATTTATTTAAGAAAGAGAGACCTCCGCCAAAAAACAAGCAAGATACGAGACTTTGAGATT 360
AATAGAACACAAATCTATCATCTCATATAGAACCAATGATTAAATATCCCACTAATTCGCACTCATCTTACAGCAAGAAC 450
MSSNKNQSDLNIEPTNSASLKKQR 24

GTCAACGATAGGAATTAATCCGAGATTGGTCTTCAACATCAGACGATATATGATCCCAAGTTCGTAATTTAAGTGCCTGGTATT 540
QQLGKISEIGASTSDVDPPQVASYLSAGDS 54
CACCATGACCAATTTGCCAACACTGCCCTTCATCATGTAATATGTTGTTTATTTCTGCTATGTCAGCTGTCAGCTGTCGGAATACAC 630
PSQFANTALHHSNSNVGYSASAAAAAELQH 64
ACCGTGCAGATTAACAAGAGGCAACAACATTCACCAACAGAAATTAACAATCAACAGGAACAGTTACAACAATATCAGAGGCTC 720
RAELQRRQQQLLQQQLLQHQQGRELQQLQYRQAQ 124
AAGCAGAGCTCAAGCCGAGCGAAGCTCAAGAGAACCAACAGATTAACAGATTCCTTCAACAGCAACAGCTACACCAATG 810
AQAQAQAQAQAQREHQQLQHAYVQQQQQLLHQLG 144
Q-rich I
GTCAACTTCTCAACGTTGGCAACACCATTTGTCAACATGAGCATGTGAGAGATGCGCTCACTACGATGAATTTGATCTAATG 900
QLSGQLLAQPHLSQHEHVRDALTTDEFDTNE 174
AAGATCTTGTTCACGATACATTTGAGATGAGATTGTAAGACATTTAAGCAATTAAGCCAAATTTGATCATTTGTTTAAATGAATAG 990
DLRSRYIENEIVKTPNSKAEELVHFVKNELG 204
GTCTCGAAGAGATTAATTAATTTATCATTAACAAAGAGCTGTTTATTCGAATGTGAAGATCGGATCATTTAGTAACA 1080
PERRCIKIVINSSKPRXAVYFQCERSGSFRT 234
CAGTAAGGATGCAATAGGACAAAGAAATGCTTACACCAAGAGAAACAAATGCTTATCCTTTGTTGTCGCAATTTGATCTCAATG 1170
VKDATKRQLATYTKERNKCA YRLVAHL YPN 264
basic region
AAGCAAGCTCAGAAAGAAAAAATTAACCGATGAACCTGGTCAATGAAGAGATTTCTGATTTCCGAATGTGGGTTTGAAGATGA 1260
KDKKRNKKNKPD EFGHNENENSRISEM WVLRLMI 294
loop
TCACCTCTCAACATTAATCATGCTGATCCATTAACAAAGAGAGACAAAAAATCTCAGAACATTTGTTGTAAGAGCAATTAACA 1350
NPQHNNHAPDPINKKERQKTSRTLVLVEKPIN 324
AACCTCATCACCATCTTCTTCAACAGAACAGCAGCAGCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAAC 1440
PHHHLLLQQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQL 354
ACATATGCCAGCAGCAACAGCAGCTGCTGATGTCAGCAACAAATGCAACAGCAATGCAAGCAATGCTGCTGCTGCTGCTGCT 1530
NANSQAQQAALQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQLQL 384
CAACTATCTGATTTAGTTCATTTAGGCAATTTAGGCAATTTAGGCAATTTAGGCAATTTAGGCAATTTAGGCAATTTAGGCAAT 1620
NYSBELLGQLGLQLSQQQSQQQLLHHLIPQQQR 424
AAGCAACCCAGATCAACCACTGCAACCACTCAACCACTGCAACCACTGCAACCACTGCAACCACTGCAACCACTGCAACCACT 1710
RTQSQQSQQQFPQT A HGLDQPDAAVIAAIE 444
AAGCTATGTCAGCAGCTGCTGCTCAAGAGTACCTATGTCAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1800
ASAAAAVASQGSFPNVTA AVAALQHNTQGN 474
AGCAGATGCTCAACCAACAGATCTGTTGTAATACGTTGCTATTTGATTTCAATATGTCAGCAATGCTGCTGCTGCTGCTGCT 1890
HDAQQQDRGGNNGGATIDSNVDPSLDPNVD 504
ACCTATGTCAGCTATGATCTTCTATGATTAAGAAATTCGATGGAAGAAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG 1980
PNVOAHDHSHGLRLNSYQKRSQFL 527
AGTTGTAATTTTTCACCAAGCTATGAACTCTCTACAGCTCTACAGCTCTGATTTGATTTTCTTATTTGTTTATTTATTTAT 2070
GTATTTGATTTTATTTTCTTAAAGATTTATTTATTTAGATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTT 2160
CAGGTATTAATAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG 2250
TATATTCATTTATTAATGCTTTCTTAAAGGTTAACCATTTTAAAGCTTTTATTAAGCTCTGATTTATTTATTTATTTATTT 2337

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Fig. 4. DNA sequence of the *C. albicans* *RBF1* gene and the predicted amino acid sequence of the encoded protein. Putative TATA boxes are thick underlined. The 5'-end of the cDNA clone p12B is indicated with a bent arrow.

Fig. 5. RPG-box-binding activity and nucleotide recognition profile of the recombinant GST-Rap1p fusion protein. (a) SDS-PAGE of the recombinant protein which was expressed in *E. coli* and purified by a GSH-agarose column. Lanes: M, molecular mass markers; 1, GST protein alone; 2, GST-Rbf1p fusion protein. (b) EMSA using a *C. albicans* total protein extract (lanes 1–7) and the recombinant protein (lanes 8–14). Lanes 2–7 and 9–14 contained a 100-fold molar excess of unlabelled oligonucleotides. Lanes: 2 and 9, *ENO1* RPG box (ER); 3 and 10, mutated ER (C4G); 4 and 11, mutated ER (A11T); 5 and 12, mutated ER (C12G); 6 and 13, NFκB; 7 and 14, AP2.

Fig. 6. *RBF1* genes complementing the growth-defect phenotype of *S. cerevisiae* *rap1* mutants. (a) The *S. cerevisiae* *RAP1*, *KIRAP1* and *C. albicans* *RBF1* genes in a low-copy-number (YCp) or a high-copy-number (YEp) plasmid were transformed into the *S. cerevisiae* *rap1*^{ts} strain YTF6 (*rap1-2*). Cells were grown at the permissive temperature (25 °C) or at the restrictive temperature (36 °C). (b) The *S. cerevisiae* *RAP1* (wild-type, *rap1-2* or *rap1-4*) or *C. albicans* *RBF1* genes were transformed into the *S. cerevisiae* *rap1* disruptant strain YTF5 (*rap1::LEU2*) harbouring a plasmid, pRS413/*RAP1*, and the plasmid shuffling was performed by the addition of 5-fluoroorotic acid (5-FOA) at 25 °C.

molecular mass of 59440 Da (Fig. 4). This figure agrees with the previous size estimate of purified Rbf1p. All nine tryptic fragments from which unambiguous sequence information was obtained are precisely represented in different regions of the deduced translation product (Fig. 3). Among these is a peptide that occurs between the first two in-frame N-terminal methionine residues, allowing us to identify the initiator AUG codon. Potential TATA transcriptional regulatory elements are located 228 and 297 nucleotides upstream from the initiator codon. The cDNA clone p12B includes the 145 bp 5'-flanking region.

The FASTA and BLAST programs did not reveal any

significant similarities between the primary amino acid sequence of the translation product of *RBF1* and any entry in the SWISS-PROT database, including *S. cerevisiae* Rap1p (17% identity). One characteristic of the sequence is that Rbf1p includes many glutamine residues (21.5%). As shown in Fig. 4, Rbf1p has two glutamine-rich regions which are seen in many transcription factors. Another sequence characteristic is its high content of alanine residues (10.6%).

To directly establish that the product of the cloned gene was Rbf1p, we expressed it in *E. coli* as a GST fusion protein and looked for the *ENO1* RPG-box-binding activity in an EMSA. Fig. 5 indicates that the expressed

Table 5. Effects of the overexpression of the *RBF1* gene on the growth rates of *S. cerevisiae* strains

The *C. albicans* *RBF1* gene under the control of the galactose-inducible *GAL1* promoter was transformed into *S. cerevisiae* YTF5 (wild-type) and YTF6 (*rap1-2*) and cells were grown in a galactose- or glucose-containing medium at 30 °C. Growth rates were monitored by measuring the OD₆₀₀.

Conditions	Doubling time (h)			
	<i>S. cerevisiae</i> YTF5		<i>S. cerevisiae</i> YTF6	
	pGAL1	pGAL1- <i>RBF1</i>	pGAL1	pGAL1- <i>RBF1</i>
With galactose	5.9	6.5	5.7	7.4
With glucose	6.9	7.0	6.9	6.6

protein did have binding activity with the same nucleotide recognition manner as that of the native Rbf1p protein.

RBF1 did not complement the *rap1^{ts}* mutation in *S. cerevisiae*

The *RBF1* gene did not complement the growth of *S. cerevisiae* *rap1^{ts}* strain YTF6 whether it was put in low-copy-number (YCp) or high-copy-number (YEpl) plasmids, as was the case with the *K. lactis* *RAP1* gene (Larson *et al.*, 1994) (Fig. 6a). The plasmid-shuffling experiment also indicated that the *RBF1* gene did not rescue the *S. cerevisiae* *rap1* disruptant YTF4 (Fig. 6b). The overexpression of Rbf1p under the control of the galactose-inducible *GAL1* promoter, however, affected the growth of *S. cerevisiae* (Table 5). While the doubling time of Rbf1p-expressing cells was slightly prolonged in a wild-type strain, the growth inhibition was more severe in a mutant strain, *rap1-2* (Sussel & Shore, 1991).

Rbf1p activated transcription in *S. cerevisiae*

The function of *RBF1* in the transcriptional activation in *S. cerevisiae* was tested by fusing parts of the gene to sequences encoding the Gal4pDNA-binding domain (Gal4_{BD}) (Ma & Ptashne, 1987). We expected that the hybrid protein would be targeted to UAS_{GAL} elements in the chromosome and therefore tested them for the ability to activate a UAS_{GAL}-*lacZ* reporter gene in an *S. cerevisiae* strain lacking wild-type Gal4p. The hybrid protein was able to activate the reporter gene to significant levels ($A_{415}/OD_{600} \text{ min}^{-1} 40.3 \pm 4.85$), about ten times higher than that of Gal4_{BD} alone ($A_{415}/OD_{600} \text{ min}^{-1} 4.06 \pm 0.49$). Since it has been reported that the glutamine-rich regions of transcription factors act as transcription activation domains (Mitchell & Tjian, 1989), it is considered that the N- and C-terminal glutamine-rich regions fulfil this function.

Fig. 7. Rbf1p activity in the nuclear fraction of *C. albicans*. (a) SDS-PAGE of the total protein (lane 1) and the nuclear (lane 2) fractions. M, Molecular mass markers. Histones, prominent components in nuclei, are indicated by two dots. (b) EMSA of the total protein (lanes 1–4) and the nuclear (lanes 5 and 6) fractions. Lanes: 1, 80 µg protein; 2, 3 and 5, 8 µg protein; 4 and 6, 0.8 µg protein.

Fractionation of *C. albicans* and confocal immunofluorescence microscopy suggested that Rbf1p was primarily a nuclear protein

If Rbf1p is a transcription factor or telomere-binding protein, it should be found in nuclei. We used two methods for the analysis of the subcellular location of Rbf1p: (i) fractionation of *C. albicans* proteins and (ii) confocal immunofluorescence microscopy. In the first method, total cell lysates were fractionated by ultracentrifugation to isolate the nuclei. The resulting fractions in which histones, prominent components in the low-molecular-mass regions of nuclei (Zinser &

Fig. 8. Confocal immunofluorescence microscopy of *C. albicans* spheroplasts using anti-Rbf1p mAb. Spheroplasts of *C. albicans* fixed to poly-L-lysine-coated slides were stained by the anti-Rbf1p mAb followed by the fluorescein-isothiocyanate (FITC)-conjugated anti-mouse IgG and 4',6-diamidino-2-phenylindole (DAPI) treatment. (a, d) Staining with DAPI; (b) staining with anti-Rbf1p mAb and FITC-conjugated anti-mouse IgG; (e) staining with FITC-conjugated anti-mouse IgG alone; (c, f) phase-contrast.

Daum, 1995), were enriched (Fig. 7) were assayed for RPG-box-binding activity in the EMSA. The specific activity of Rbf1p was tenfold higher in the nuclear fraction (Fig. 7). The *cis*-element recognition profile was determined to be the same as that of purified Rbf1p (data not shown). More direct evidence was obtained by the second method. Incubation of permeabilized *C. albicans* spheroplasts with the mAb (OA4) preparation revealed diffuse nuclear staining with a weak discernible concentration of antigen at the cytoplasm (Fig. 8). The data presented in Fig. 8 were consistent with the result of the cellular fractionation studies in Fig. 7.

DISCUSSION

In this paper, we report the purification and cloning of a *C. albicans* gene encoding a protein that binds to *S. cerevisiae* RPG boxes and *C. albicans* telomeric repeats. This protein, Rbf1p, was considered to be a new *C. albicans* transcription factor on the basis of several lines of experimental results: (i) it bound to a specific nucleotide sequence; (ii) it could activate transcription in *S. cerevisiae*; (iii) it contained glutamine-rich regions characteristic of transcription factors; and (iv) it was found predominantly in nuclei.

Rap1p proteins from *S. cerevisiae* and *K. lactis* have been identified as RPG-box-binding proteins. Despite a high degree of conservation between the *S. cerevisiae* and *K. lactis* Rap1p proteins (48.2% identity in amino acid sequences), especially within the DNA-binding domain (68.9% identity), *K. lactis* Rap1p failed to rescue several *S. cerevisiae* *rap1^{ts}* mutants at the non-permissive temperature (Fig. 6a; Larson *et al.*, 1994). The functions that Rap1p plays within a host may be highly specialized within a given species. *C. albicans* Rbf1p did not have homology with *S. cerevisiae* or *K. lactis* Rap1p proteins (~17% identity), showed a different nucleotide recognition profile from them, and did not functionally complement *S. cerevisiae* *rap1* mutants. These results suggest that its function is distinct from that of the Rap1p family. It was demonstrated, however, that the overexpression of the *RBF1* gene caused a growth defect in *S. cerevisiae*. Freeman *et al.* (1995) reported that the Rap1p DNA-binding domain causes dominant negative growth inhibition when overexpressed, and have speculated that this toxic effect would come from the disturbance of interactions with other proteins after binding to the RPG box or telomeres. This may be considered as one of the reasons for the growth defect in *S. cerevisiae* caused by Rbf1p.

Rbf1p showed binding activity to the *C. albicans* telomeric repeat sequence. While the repeat in *S. cerevisiae* consists of the TG₁₋₃, the telomeres of other fungi including *C. albicans* often have either irregular or much longer repeats [(ACGGATGTCTAACTTCTTGGTGT)_n for *C. albicans*] (McEachern & Hicks, 1993; McEachern & Blackburn, 1994). In *S. cerevisiae*, Rap1p also binds along the telomeric TG₁₋₃ tracts, where it regulates telomere functions (reviewed by Shore, 1994). Recently, the human telomeric repeat [(TTAGGG)₁₂] binding factor (hTRF) has been cloned (Chong *et al.*, 1995). Although the amino acid sequences gave little or no hint about the structural relationship between *S. cerevisiae* Rap1p and hTRF because there was no homology between them (17%), the three-dimensional structure of the Rap1p DNA-binding domain revealed that myb-type helix-turn-helix (HTH) motifs are concerned with telomere binding (Konig *et al.*, 1996). Rbf1p does not contain this HTH motif, but a variant of the basic helix-loop-helix sequence was suggested between amino acid residues 236 and 295 of Rbf1p (Fig. 4) by secondary structure prediction based on the algorithm of Chou & Fasman (1978). Further structural com-

parisons among these telomere-binding factors including those from *Schizosaccharomyces pombe* (Duffy & Chambers, 1996), *Oxytricha nova* (Gray *et al.*, 1991) or *Euplotes* (Price *et al.*, 1992), whose encoding genes have not yet been cloned, should be of interest, as should the analysis of the effect of Rbf1p on *C. albicans* telomere length regulation.

In summary, several lines of evidence suggest that Rbf1p is a transcription factor of *C. albicans* which may be involved in the regulation of telomere function. The phenotypic analysis of the *RBFI* disruptants and the identification of the target genes regulated by the factor should provide clues towards understanding the mechanism of regulation of gene expression and telomere control in *C. albicans*.

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