PAP1 [poly(A) polymerase 1] homozygosity and hyperadenylation are major determinants of increased mRNA stability of CDR1 in azole-resistant clinical isolates of Candida albicans

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Using genetically matched azole-susceptible (AS) and azole-resistant (AR) clinical isolates of Candida albicans, we recently demonstrated that CDR1 overexpression in AR isolates is due to its enhanced transcriptional activation and mRNA stability. This study examines the molecular mechanisms underlying enhanced CDR1 mRNA stability in AR isolates. Mapping of the 3′ untranslated region (3′ UTR) of CDR1 revealed that it was rich in adenylate/uridylate (AU) elements, possessed heterogeneous polyadenylation sites, and had putative consensus sequences for RNA-binding proteins. Swapping of heterologous and chimeric lacZ–CDR1 3′ UTR transcriptional reporter fusion constructs did not alter the reporter activity in AS and AR isolates, indicating that cis-acting sequences within the CDR1 3′ UTR itself are not sufficient to confer the observed differential mRNA decay. Interestingly, the poly(A) tail of the CDR1 mRNA of AR isolates was ~35–50 % hyperadenylated as compared with AS isolates. C. albicans poly(A) polymerase (PAP1), responsible for mRNA adenylation, resides on chromosome 5 in close proximity to the mating type-like (MTL) locus. Two different PAP1 alleles, PAP1-a/PAP1-α, were recovered from AS (MTL-a/MTL-α), while a single type of PAP1 allele (PAP1-α) was recovered from AR isolates (MTL-α/MTL-α). Among the heterozygous deletions of PAP1-a (Δpap1-a/PAP1-α) and PAP1-α (PAP1-a/Δpap1-α), only the former led to relatively enhanced drug resistance, to polyadenylation and to transcript stability of CDR1 in the AS isolate. This suggests a dominant negative role of PAP1-a in CDR1 transcript polyadenylation and stability. Taken together, our study provides the first evidence, to our knowledge, that loss of homozygosity at the PAP1 locus is linked to hyperadenylation and subsequent increased stability of CDR1 transcripts, thus contributing to enhanced drug resistance.

INTRODUCTION

One of the major mechanisms of the multidrug resistance (MDR) phenotype in azole-resistant (AR) Candida albicans

Abbreviations: AR, azole-resistant; AS, azole-susceptible; LOH, loss of heterozygosity; MDR, multidrug resistance; Nod1, nourseothricin-resistant; PAT, polyadenylation test; RACE, rapid amplification of cDNA ends; 3′ UTR, 3′ untranslated region.

A set of supplementary results, two supplementary figures and three supplementary tables, with references, are available with the online version of this paper. The supplementary results describe how 3′ UTR of CDR1 mRNA forms altered secondary structures. The supplementary figures show the predicted secondary structure of CDR1 3′ UTR and the results of a lacZ mRNA decay assay. The supplementary tables list primers, plasmids and strains used in this study.
in AR isolates are very critical in designing strategies for therapeutic interventions.

Transcriptional regulation of CDR1 has been extensively studied by several groups. It has been shown that CDR1 harbours various consensus (Sp1, AP-1, Y-box) sequences as well as specific basal (BRE), negative (NRE) and drug/ steroid response elements (DRE and SRE) in the 5′ flanking region (De Micheli et al., 2002; Gaur et al., 2004; Karnani et al., 2004; Purii et al., 1999). Trans-acting factors regulating CDR1 have also been identified. For example, non dityrosine 80 (CaNDT80), a homologue of a meiosis specific transcription factor (TF) of Saccharomyces cerevisiae, has been identified as a potential activator of CDR1 (Chen et al., 2004). Coste et al. (2004) identified a TF, transcriptional activator of CDR genes (TAC1), that binds to the DRE in both the CDR1 and the CDR2 promoters. Interestingly, a TF belonging to the zinc cluster family, fluconazole resistance 1 (FCR1) (Talibi & Raymond, 1999), as well as the global repressor thymidine promoters. Interestingly, a TF belonging to the zinc cluster family, fluconazole resistance 1 (FCR1) (Talibi & Raymond, 1999), as well as the global repressor thymidine uptake 1 (Upc1), acts as a negative regulator of CDR1 expression (Yang et al., 2006). Recent genome-wide location profiling (ChIP-chip) results show that another TF of the zinc cluster family, uptake control 2 (Ucp2), which regulates ERG genes, also targets CDR1 (Znaidi et al., 2008). The efforts of several groups have also led to the identification of various cis-acting elements such as the H₂O₂ responsive element (HRE) and the benomyl responsive element (BRE) in the MDR1 promoter region, mediating its upregulation in AS isolates and its constitutive activation in AR isolates (Rognon et al., 2006). Recently a zinc cluster TF, designated multidrug resistance regulator 1 (MRR1), has been identified, which is linked to the activation of MDR1 expression. Inactivation of MRR1 abolishes the resistance of Mdr1p-overexpressing strains (Morschhäuser et al., 2007).

It is thus apparent that transcriptional regulation plays an important role in the mechanism underlying the overexpression of MDR genes; however, the relevance of the post-transcriptional events associated with it is poorly understood. By employing two pairs of matched C. albicans clinical isolates in which azole resistance developed due to the overexpression of CDR1 during prolonged azole therapy, we have recently shown that the high mRNA levels in AR isolates are predominantly contributed by both enhanced transcriptional activation and mRNA stability (Manoharlal et al., 2008). In this study, we further dissected the molecular basis of CDR1 mRNA turnover in matched clinical isolates. For this, we have identified and characterized the complete 3′ end of the CDR1 mRNA by the 3′-rapid amplification of cDNA ends (3′-RACE)-PCR method in these matched isolates. Our observations with chimeric lacZ–CDR1 3′ UTR transcriptional reporter fusion transformants ruled out the participation of the 3′ UTR in transcriptional as well as post-transcriptional control of CDR1. However, there was an increase in poly(A) tail length, which coincided with the enhanced CDR1 mRNA stability in AR isolates. Poly(A) tail synthesis in C. albicans is catalysed by the nuclear poly(A) polymerase that resides within the mating type-like (MTL) locus on chromosome 5.

The MTL loci of C. albicans span approximately 9 kb and contain S. cerevisiae mating-type homologues MAT-a1, MAT-a2 and MAT-a2 (Hull & Johnson, 1999), as well as genes unique to fungal mating loci, including OBP (an oxysterol-binding protein), PAPI [a poly(A) polymerase 1] and PIKI (a phosphoinositol kinase). A copy of each of these three genes is present in both the MTL-a and the MTL-α locus. The MTL genes have no significant homology to each other. The a and α versions of PAPI, PIKI and OBP have approximately 60% homology to each other. Most wild-type C. albicans strains are diploid and have both the MTL-a and MTL-α loci (Magee & Magee, 2000). However, our analysis of PAPI alicic status revealed that MTL heterozygosity (MTL-a/MTL-α) and homozygosity (MTL-α/MTL-α) are linked to the acquisition of PAPI heterozygosity (PAPI-a/PAPI-α) and homozygosity (PAPI-a/PAPI-a) in AS and AR isolates, respectively. We also show that heterozygous disruption of PAPI-a conferred relatively enhanced drug resistance, hyperadenylation and increased stability of CDR1 transcripts in AS isolates. However, heterozygous disruption of PAPI-α in AS and AR affected neither the drug resistance nor the polyadenylation or stability of CDR1 transcripts. Therefore, our results provide evidence that loss of heterozygosity (LOH) at the PAPI locus is associated with the hyperadenylation and increased stability of CDR1 transcripts and that this helps the development of drug resistance in AR isolates.

**METHODS**

**Materials.** Media chemicals were obtained from HiMedia. Luria–Bertani broth and agar media were purchased from Difco, BD Biosciences. Restriction endonucleases, DNA-modifying enzymes, Taq DNA polymerase and ultrapure deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) for PCR were purchased from New England Biolabs. Moloney murine leukaemia virus (M-MuLV) reverse transcriptase, IPTG, X-Gal, T7 and Sp6 promoter primers were obtained from Promega. A copy of each of these three genes is present in both the MTL-a and the MTL-α locus. We have identified and characterized the complete 3′ end of the CDR1 mRNA by the 3′-rapid amplification of cDNA ends (3′-RACE)-PCR method in these matched isolates. Our observations with chimeric lacZ–CDR1 3′ UTR transcriptional reporter fusion transformants ruled out the participation of the 3′ UTR in transcriptional as well as post-transcriptional control of CDR1. However, there was an increase in poly(A) tail length, which coincided with the enhanced CDR1 mRNA stability in AR isolates. Poly(A) tail synthesis in C. albicans is catalysed by the nuclear poly(A) polymerase that resides within the mating type-like (MTL) locus on chromosome 5.

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**Bacterial and yeast strains and growth media.** Escherichia coli DH-5α was used as a host for plasmid construction and propagation. Plasmids and C. albicans strains used in this study are listed in Supplementary Tables S2 and S3. E. coli was cultured in Luria–Bertani broth media to which ampicillin was added (100 μg ml⁻¹). C. albicans strains were maintained on yeast extract peptone dextrose (YPD) media. For agar plates, 2.5 % (w/v) Bacto Agar was added to the medium. All strains were stored as frozen stocks with 15 % (v/v)

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glycerol at −80 °C. Before each experiment, cells were freshly revived on YEPD plates from this stock.

3'-Rapid amplification of cDNA ends (3'-RACE). The nucleotide sequence of the oligonucleotide primers used for the reverse transcription-3'-RACE-nested PCR were selected based on the published sequences of CDR1 (GenBank accession no. X77589) (Prasad et al., 1995). Total RNA isolated from each isolate using the TRIzol reagent (as per the manufacturer’s specifications) was enriched with poly(A)+ (polyadenylated) mRNA using the Oligotex mRNA Mini kit protocol (Qiagen) and used subsequently for performing the reverse transcription-3'-RACE reaction as described elsewhere (Gerads & Ernst, 1998). For a typical 3'-RACE reaction, cDNA synthesized from −0.1 μg poly(A)+ RNA was placed in a 0.5 ml reaction tube with 1 μM oligo(dT)18 anchor primer stock, and the volume was adjusted to 11 μl with diethylpyrocarbonate (DEPC)-treated water (Fig. 1a, step 1). The mixture was incubated for 10 min at 70 °C and chilled on ice for 1 min, after which the remainder of the reaction mixture was added from a master mix to the reaction tube in order for each reaction to contain a 1 mM concentration of each of dATP, dCTP, dGTP and dTTP, and 40 U RNase inhibitor (MBI, Fermentas) in a buffer consisting of 50 mM Tris/HCl (pH 8.3), 50 mM KCl, 4 mM MgCl2 and 10 mM DTT. After brief mixing, the reaction was incubated for 10 min at 37 °C followed by addition of

Fig. 1. Determination of termination sites of CDR1 mRNA. (a) The 3'-RACE strategy employed for the detection of CDR1 3' UTR length is detailed in Methods. Briefly, cDNA synthesized from poly(A)+-enriched mRNA samples from clinical C. albicans isolates was PCR-amplified using CDR1-specific forward and reverse anchor primers. Following electrophoresis, the amplified PCR products were visualized by staining with ethidium bromide (indicated by a long thick arrow), cloned and sequenced. To confirm the RT-PCR specificity, another round of 3' UTR-specific nested PCR amplification of CDR1 of each isolate was performed. Following electrophoresis, the amplified PCR products were further visualized by staining with ethidium bromide (indicated by a short thick arrow), cloned and sequenced. The locations of DNA size markers (bp) are marked on the left-hand side of the gel. C. albicans isolates are labelled above the gels. (b) Schematic representations of the mapped 3' UTRs of CDR1. The open box and wavy line represent the CDR1-coding and poly(A) tail-harbouring 3' end regions respectively, while thick straight lines with numbers on top indicate the mapped CDR1 3' UTR length of each isolate.
40 U M-MuLV reverse transcriptase (MBI, Fermentas). Finally, the reaction was incubated at 37 °C for 60 min and then stopped by heating at 70 °C for 10 min followed by chilling on ice for 1 min. The synthesized cDNA was used for amplification of the CDR1 mRNA-specific 3’ end of each isolate (Fig. 1a, step 2), using the corresponding appropriate dilution of cDNA as template (determined empirically for CDR1 to give a product in the linear range, generally 1:4) and 1 μM of each CDR1-specific forward primer CT-CDR1-F-RM (corresponding to positions 4271–4293 in the CDR1 genomic sequence) and reverse PCR anchor primer as described in Supplementary Table S1. PCR parameters were initial denaturation of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s, elongation at 72 °C for 40 s and final extension at 72 °C for 10 min. As a positive control, CDR1-specific forward primer CT-CDR1-F-RM and reverse primer CT-CDR1-R-RM (corresponding to positions 4475–4503 in the CDR1 genomic sequence) were also used (data not shown). The negative control (without reverse transcriptase) established that the PCR products generated in the RT-PCR were not due to genomic DNA contamination (data not shown). The RT-PCR product of each isolate was electrophoresed on a 1.2 % agarose gel in 1 x TAE. The gel-purified 3’-RACE product (Qiagen PCR Cleanup kit) of each isolate was cloned directly in the pGEMT-Easy vector using a T/A cloning kit (Promega) as per the manufacturer’s recommendations and sequenced (Fig. 1a, step 3). The corresponding clone of each isolate was sequenced and subsequently used as template for a second nested PCR amplification of CDR1 (Fig. 1a, step 4) with forward and reverse primers (UTR-F-PacI and UTR-R-MluI, Supplementary Table S1) specific to each isolate. PCR parameters were: initial denaturation of 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 30 s and final extension at 72 °C for 10 min. The resulting PCR-amplified fragments (now exactly specific to mapped 3’ UTR CDR1 of each isolate) were purified (Qiagen PCR Cleanup kit) and further subcloned in pGEMT-Easy (Fig. 1a, step 5a), generating CDR1 3’ UTR-specific clones of each isolate, as described in Supplementary Table S2. All the cloned PCR-amplified products were confirmed by appropriate restriction digestion analysis and sequenced further to determine the exact and precise length of the CDR1 3’ UTR (Fig. 1a, step 5b).

**Custom service nucleotide sequencing.** Multiple 3’ UTR-specific clones of each isolate were sequenced directly by extension from both sense and antisense strands using T7 promoter/T7 promoter primer and SP6 promoter/SP6 promoter primer (Supplementary Table S1) by exploiting BigDye Terminator chemistry and an automated DNA sequencer (ABI Prism 3100). Reproducibility of the sequencing was confirmed by processing all samples at least twice.

**Sequence alignments, in silico analysis and computerized secondary structure predictions of CDR1 3’ UTR.** Multiple sequence alignment of mapped CDR1 3’ UTR for both matched pairs of isolates was done using the CLUSTAL W (version 1.83) program (Thompson et al., 1994). In silico analysis of the 3’ UTR for prediction of the putative regulatory site was carried out by the UTRscan (UTRResources) program (Pesole et al., 2002; Pesole & Liuni, 1999). We used the mfold (version 3.0) algorithm created by Zuker et al. (1999) for in silico computer predictions of the secondary structure of the 3’ UTR by a minimization of free energy-based method. The folding temperature was fixed at 37 °C and ionic conditions were 1 M NaCl without constraints, and no limit in the maximum distance between paired bases.

**Reporter plasmid construction.** Plasmid pCPL51 (Manoharlal et al., 2008), harbouring lacZ, was used for cloning of the mapped CDR1 3’ UTR in transcriptional fusion with a heterologous lacZ reporter. For this purpose, sequences at the junctions of lacZ-

**Yeasts transformation.** C. albicans was transformed by the standard electroporation protocol (Reuss et al., 2004). Briefly, 5 μL (~1 μg) of the specific enzyme-digested and gel-purified linearized DNA fragments was mixed with 40 μL electrocompetent cells and electroporated using a Bio-Rad GenePulser XL (0.2 cm cuvette, 1.5 kV). Following electroporation, transformants were washed with 1 ml 1 M sorbitol, resuspended in 1 ml YEPM medium and incubated for 3–4 h with shaking at 30 °C prior to plating on YEPM plates containing 200 μg nourseothricin ml⁻¹ and grown at 30 °C. Nourseothricin-resistant (Nou⁸) transformants were picked after 1 day of growth and restreaked on YEPM plates containing 100 μg nourseothricin ml⁻¹.

**β-Galactosidase reporter assay.** β-Galactosidase assays were performed using duplicate samples of cells from three independent experiments, as described by Uhl & Johnson (2001). β-Galactosidase activity was determined by the standard equation and expressed in Miller units (mg protein)⁻¹. Miller units are arbitrary units: β-Galactosidase activity (Miller units) = (420 x 1000) / (OD₆₅₀ x t x v) where t is the time of reaction (min) and v is the culture volume (ml).

**Polyadenylation test (PAT) analysis of poly(A) tail length.** A new and improved variation of the 3’-RACE PAT involving G-tailing of mRNA was employed to show both the presence of the poly(A) tail and its length, as described elsewhere (Kusov et al., 2001).

**Guanylation of mRNA.** The purified poly(A)⁺-enriched mRNA (as described above) was used directly for polyguanylation using yeast poly(A) polymerase (PAP) (catalogue number E74225Y, Amersham Pharmacia Biotech/US Biochemicals) as per the manufacturer’s recommendations. To abolish the higher-ordered secondary structure at the 3’ end of the mRNA, samples (0.1 μg) were heated at 65 °C for 5 min and immediately placed on ice. They were incubated for 1 h at 37 °C with 600 U PAP and 0.5 mM GTP in a 25 μl reaction mixture containing 20 mM Tris/HCl (pH 7.0), 50 mM KCl, 0.7 mM MnCl₂, 0.2 mM EDTA, 100 μg BSA ml⁻¹ and 10 % (v/v) glycerol. An additional 300 U PAP was further added and incubation was continued for an additional 1 h. The reaction was terminated by heat treatment at 65 °C for 10 min, chilled on ice and kept at ~80 °C till further use.

**RT-PCR.** Polyguanylated mRNA (as described above) was subjected directly to RT-PCR analysis using hot start conditions (65 °C, 30 s). The first-strand cDNA synthesis (reverse-transcription, 42 °C for 1 h) was primed by oligo(dC)₆₅₀-anchor primer (1 μM). The reverse-transcription reaction was stopped by denaturation at 70 °C for 10 min; the synthesized cDNA product (1:4 dilution) was used for

\[T_{ACT}^{T} \text{ and } T_{ACT}^{CaS} \text{ were mutated sequentially by introducing }\]

Padc and MluI sites at these corresponding junctions (lacZ-T_{ACT} and lacZ-T_{ACT}^{CaS}) using lacZ-F-PacI-RM/lacZ-R-PacI-RM and lacZ-F-MluI-RM/lacZ-R-MluI-RM primers (Supplementary Table S1) with a QuickChange site-directed mutagenesis system (Stratagene) to generate the plasmids pCPL53-RM (with Padc site only) and pCPL54-RM (with both Padc and MluI sites introduced), respectively. Mapped CDR1 3’ UTR fragments of each isolate were excised from their corresponding 3’ UTR-harbouring pGEMT-Easy vector clones (Supplementary Table S2) by digestion at Padc and MluI sites (sites introduced in primers during the synthesis), and further subcloned in Padc/MluI-digested pCPL54-RM to generate pCPL54-Gu4/Gu5-3’ UTR-RM and pCPL54-DSY294/DSY296-3’ UTR-RM (Supplementary Table S2). All constructs were confirmed by appropriate restriction digestion analysis. The flanking CDR1 sequences in all these plasmids served for genomic integration of the P_{CaSAT1} lacZ-3’ UTR reporter fusion cassettes at the native CDR1 locus, and the dominant CaSAT1 marker (Reuss et al., 2004) was used to select nourseothricin-resistant (Nou⁸) transformants.
PCR amplification with 1 μM of each CDR1-specific forward primer (CT-CDR1-F/UTR-F-Pad1) and reverse anchor primer as described in Supplementary Table S1. PCR parameters were: initial denaturation of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were gel-purified and used for cloning into the T/A cloning vector as recommended by the manufacturer (Promega). Approximately 8–10 random clones from at least three independent RT-PCRs for each isolate were sequenced.

**Construction of gene disruption cassettes.** Two different PAP1 disruption cassettes were designed in this study. Two cassettes, C1 and C2, in plasmids pRM3 and pRM4 were designed using the URA-blaster system (Fonzi & Irwin, 1993). C1 bears the deletion of a portion of 602 bp between nucleotides +500 and +1102 with respect to the first ATG codon of PAPI-a. C2 was designed to delete a region of 636 bp between nucleotides +750 and +1386 with respect to the first ATG codon. To construct these two deletion cassettes (C1 and C2), the entire PAPI-a ORF and a portion of the PAPI-a ORF (corresponding to nucleotides +259 to +1677 with respect to the first ATG codon) were first amplified from genomic DNA of DSY294 and DSY296, respectively, using the cloning primer pairs PAPI-a-F-BamHI-RM/PAPI-a-R-Xhol-RM and PAPI-alpha-F-BamHI-RM/PAPI-alpha-R-Xhol-RM, respectively (Supplementary Table S1). The resulting PCR fragments were cloned into pBluescript-KS (+) to yield pRM1 and pRM2, respectively (Supplementary Table S2). For disruption with cassettes C1 and C2, deletions were created from pRM1 and pRM2 by PCR using deletion primer pairs PAPI-a-F-BglII-RM/PAPI-a-R-PstI-RM and PAPI-alpha-F-BglII-RM/PAPI-alpha-R-PstI-RM, respectively (Supplementary Table S1). The obtained PCR fragment was digested with PstI and BglII, and the 3.7 kb PstI/BglII URA3-blaster fragment from pMB7 (Fonzi & Irwin, 1993) was inserted to obtain deletion constructs pRM3 and pRM4, respectively (Supplementary Table S2). The linearized fragments C1 and C2 obtained by digestion of deletion constructs (pRM3 and pRM4) with ApaI/SacI, were used for transformation in C. albicans. It is of note that Δura3 mutants of the clinical isolates DSY294 and DSY296, i.e. DSY3040 and DSY3041 (Coste et al., 2006), respectively, were used for PAPI disruptions and subsequent experiments. After generation of heterozygous mutants for each allele, the ura3 marker was regenerated by plating Ura- colonies on selective medium containing 5-fluoroorotic acid (5-FOA), as described previously (Sanglard et al., 1995). Our recent study on the molecular characterization of CDR1 and its products (mRNA/protein) in these clinical isolates suggested that the MDR phenotype is governed by enhanced transcription activation and the mRNA stability of the CDR1 transcript (Manoharal et al., 2008). In this study, we have explored the molecular basis of the mRNA stability of CDR1 in AR isolates.

**RESULTS**

We have analysed two pairs of matched clinical AS and AR C. albicans strains isolated from recurrent episodes of oropharyngeal candidiasis (OPC) in two different human immunodeficiency virus-positive (HIV+) AIDS patients, in whom MDR in AR isolates is predominantly linked to overexpression of CDR1 (Franz et al., 1999; Sanglard et al., 1995). Our recent study on the molecular characterization of CDR1 and its products (mRNA/protein) in these clinical isolates suggested that the MDR phenotype is governed by enhanced transcription activation and the mRNA stability of the CDR1 transcript (Manoharal et al., 2008). In this study, we have explored the molecular basis of the mRNA stability of CDR1 in AR isolates.

**The 3′ UTR of CDR1 mRNA displays length heterogeneity**

As an initial step to study the post-transcriptional mechanisms involved in enhanced CDR1 mRNA stability, transcription termination sites of CDR1 in AS and AR isolates were determined by the 3′-RACE method (Gerards & Ernst, 1998). For this, RT-PCR products of CDR1 of each isolate were generated (Fig. 1a, step 1 and 2) using the CDR1-specific forward primer CT-CDR1-F-RM and oligo(dT)18AP-RM anchor primer (Supplementary Table S1), cloned directly into the pGEMT-Easy vector and sequenced (Fig. 1a, step 3). To further confirm the RT-PCR specificity of CDR1 (because of close homology of CDR1 with CDR2), a subsequent second round of nested PCR of corresponding CDR1 3′ UTR-harbouring clones (Fig. 1a, step 3) was performed with CDR1 3′ UTR-specific primers for each isolate (Fig. 1a, step 4) (Supplementary Table S1). 3′ UTR products specific to CDR1 for each isolate were amplified and further subcloned into pGEMT-Easy (Fig. 1a, step 5a). Direct sequencing (Fig. 1a, step 5b) of multiple tested drugs (see Fig. 5 for drug concentrations used). Yeast cultures were grown overnight in YEPD and diluted to a density of 1.5 × 10^7 cells ml^-1, and 10-fold serial dilutions were performed to a final dilution step containing 1.5 × 10^3 cells ml^-1. Five microlitres of each dilution were spotted onto YEPD plates with or without drugs. Plates were incubated for 48 h at 30 °C.

**Thiolutin chase assay.** To measure the CDR1 mRNA half-life, a potent in vivo transcriptional inhibitor of C. albicans, thiolutin, at an optimized concentration (40 μg) was used as described previously (Manoharal et al., 2008). Briefly, 100 ml cells was grown at 30 °C to OD_600 1.0. Aliquots of cells were taken at the indicated times after transcriptional shutoff by thiolutin. Total RNA was isolated using the Ambion Ribopure-Yeast RNA isolation kit (catalogue no. 1926) as per the manufacturer’s instructions. Equal RNA loading was assessed by staining the agarose gel with ethidium bromide prior to blotting. For Northern blots, ~25 μg total RNA from the above samples was hybridized with a single probe derived from CDR1-specific primers (primer pairs KM1 and KM2, Supplementary Table S1) that was used throughout this study. Hybridization signal intensity was quantified with a phosphorimager scanner (FLA-5000, FLA5000 Fuji phosphorimager), normalized to the band intensity at time ti and plotted as a line graph.
3' UTR-specific clones of AS and AR isolates confirmed and revealed exact transcription termination sites of CDR1 (data not shown). For the matched isolates Gu4/Gu5 and DSY294/DSY296, 3' UTR length was mapped to 137/163 bases and 162/137 bases, respectively (positions are relative to the UAA stop codon) (Fig. 1b).

**CDR1 3' UTR sequences reveal polymorphism and potential mRNA destabilizing signals**

Since differences in sequence at the 3' UTR could be involved in polyadenylation site selection (Edwalds-Gilbert et al., 1997) and mRNA stability (Russell et al., 1998), we did a comparative *in silico* analysis of mapped 3' UTR fragments of CDR1 of AS and AR isolates and analysed their relevant sequence features. 3' UTR sequence alignment of CDR1 revealed polymorphism at positions −7U (insertion), U19C and C64U (substitution) [Fig. 2a, open box, where the base(s) before and after the number is the sequence from Gu4 and Gu5 isolates, respectively] and an additional extended 26 and 25 base AU (adenylate/uridylate)-rich stretch at the 3' end for only Gu5 (AR) and DSY294 (AS) isolates, respectively (Fig. 2a). All nucleotide positions are relative to the UAA translational stop codon (considered as +1).

*Fig. 2.* Sequence analysis of CDR1 3' UTR. (a) Alignment of CDR1 3' UTRs from the two matched pairs of clinical isolates obtained using the CLUSTAL W program (Thompson et al., 1994). Conserved residues are indicated by asterisks. Mismatched residues are represented by open boxes. Gaps (marked with dashes) have been introduced to maximize the alignments. Continuous and discontinuous underlining denotes the polypyrimidine (Py) and cytoplasmic polyadenylation element (CPE) tract, respectively. The eukaryotic poly(A) signal is indicated by shaded boxes. Elliptical boxes denote the AU-rich elements (AAUAAA). Numbers at the right are relative to the UAA translational stop codon (considered as position +1). (b) Schematic representation of the predicted regulatory motifs in the 3' UTR, indicated by boxes.
The CDR1 3’ UTR is ~78% AU, represents several putative consensus sequences for cytoplasmic RNA-binding protein(s) (Fig. 2b), and has perfect AU-rich elements (AURE) (Fig. 2a, elliptical boxes). The AURE motifs are characterized by the presence of the consensus sequence (AUUUA) and have been reported to be involved in destabilization of their corresponding mRNAs (Trzaska & Dastych, 2005). The presence of one perfect (UAGU) and two degenerate (A/CAUAAA, AAUAAA/U) yeast polyadenylation signals (Fig. 2a, shaded boxes), suggests that mRNA variants influence the CDR1 mRNA steady-state levels, as has been reported for other cells (Edwalds-Gilbert et al., 1997; Higgins, 1991; Hsu et al., 1990). A poly(U)-rich and a cytoplasmic polyadenylation element (CPE) tract were also predicted (Fig. 2a, continuous and discontinuous underlining, respectively).

3’ UTR swapping does not affect β-galactosidase reporter activity

The sequence and structure analyses of the 3’ UTR of CDR1 suggested that the polymorphism (Fig. 2a) as well as differential secondary structure (Supplementary Fig. S1) of AS and AR isolates contribute to the observed enhanced mRNA stability. To obtain direct experimental evidence, we tested whether the CDR1 3’ UTR from AS isolates has any mRNA destabilizing effect in AR isolates and vice versa. For this purpose, expression vectors harbouring P_CDR1-lacZ-CDR1 3’ UTR chimeric transcriptional reporter fusions were constructed (Fig. 3a, see Methods). The linearized transformation cassette of each isolate was confirmed by Southern hybridization (data not shown). Two representative NouK transformants of each construct was confirmed by Southern hybridization corresponding matched isolates. Single-copy integration of each parental strain were used for further analysis. The resulting reporter strains were designated Gu4L2-CUN (P_CDR1-lacZ-3’UTR); Gu4L2-CUS (P_CDR1-lacZ-3’UTR); Gu5L2-CUN (P_CDR1-lacZ-3’UTR); Gu5L2-CUS (P_CDR1-lacZ-3’UTR); DSY294L2-CUN (P_CDR1-lacZ-3’UTR); DSY294L2-CUS (P_CDR1-lacZ-3’UTR); and DSY296L2-CUN (P_CDR1-lacZ-3’UTR) and DSY296L2-CUS (P_CDR1-lacZ-3’UTR). The superscripts ‘N’ and ‘S’ in the designation constructs stand for native and swapped CDR1 3’ UTR reporter transformants, respectively.

Expression of the lacZ reporter gene in various strains was qualitatively assessed by comparing the intensity of the blue colour produced by cells grown on agar plates containing the indicator dye X-Gal (Fig. 3b), and was quantified by measuring the β-galactosidase reporter activity in liquid assays (Fig. 3c). Two observations emerged from these results. First, the β-galactosidase activity observed in native as well as swapped 3’ UTR transformants of each AR isolate was always higher than their corresponding AS reporter transformants (3.22-fold for Gu5L2-CUN versus Gu4L2-CUN, 2.33-fold for Gu5L2-CUS versus Gu4L2-CUS; 4.44-fold for DSY296L2-CUN versus DSY294L2-CUN and 3.86-fold for DSY296L2-CUS versus DSY294L2-CUS). Second, the reporter activity of the 3’ UTR native transformants of either AS or AR isolates was comparable with that of their corresponding swapped reporter fusion transformants (Gu4L2-CUN versus Gu4L2-CUS, Gu5L2-CUN versus Gu5L2-CUS, DSY294L2-CUN versus DSY294L2-CUS, and DSY296L2-CUN versus DSY294L2-CUS). Thus, swapping of the CDR1 3’ UTR of AS and AR isolates did not affect the reporter activity. In another set of experiments, we observed comparable lacZ transcript half-lives in native as well as swapped 3’ UTRs of CDR1 in both AS and AR isolates (Supplementary Fig. S2). The influence of the 3’ UTR of CDR1 was measured by employing a heterologous lacZ reporter. However, the possibility that the CDR1 3’ UTR might have a cis effect on transcriptional or post-transcriptional control of its transcript cannot be ruled out.

CDR1 mRNA poly(A) tail length is longer in AR isolates

Since poly(A) tail removal is a critical step in the 3’-exonuclease-mediated mRNA decay pathway (Higgins, 1991; McCarthy, 1998; Ross, 1996), we checked the polyadenylation status of CDR1 mRNA between AS and AR isolates. For this, we employed a recently described PCR-based PAT assay, where the 3’ end of the mRNA is polyguanylated using yeast poly(A) polymerase, as described earlier (Kusov et al., 2001). With this step, a poly(A)–oligo(G) junction is generated, which serves as specific target for the amplification of the 3’ end of the transcriptome with the universal reverse oligo(dC)_n anchor primer and a gene-specific forward primer. RT-PCR products were cloned and sequenced for the accurate measurement of poly(A) tail length. Chromatogram analysis of cloned PAT-PCR products of each AS and AR isolate confirmed precisely that the CDR1 poly(A) tail length was shorter, with 24 ± 2 A residues in AS isolates, in contrast to 35 ± 2 A residues in AR isolates (Table 1; a maximum variation of ± 2 bases was observed between different clones). It should be mentioned that a minimum of 10 random clones were subjected to sequencing to determine the precise poly(A) tail length.

Poly(A) polymerase 1 (PAP1) is heterozygous (PAP1-a/PAP1-a) in AS isolates but homozygous (PAP1-a/PAP1-a) in AR isolates

In eukaryotes, poly(A) tail synthesis at the 3’ end of mRNAs in the nucleus is catalysed by the canonical poly(A) polymerase (PAP), which belongs to the DNA polymerase-like nucleotidyl transferase superfamily (Holm & Sander, 1995). C. albicans poly(A) polymerase (PAP1) is located within the MTL locus on chromosome 5 (Coste et al., 2006). To analyse the PAP1 allelic status in AS and AR isolates, oligonucleotides were designed to specifically amplify the two PAP1 alleles PAP1-a and PAP1-a (Supplementary Table S1). For this, multiplex PCR was performed with genomic DNA of each of the isolates, as
Fig. 3. Schematic depiction of lacZ reporter fusion integrants and qualitative and quantitative assay of β-galactosidase reporter activity in AS and AR isolates. (a) Structure of the DNA cassettes used to integrate the P<sub>CDR1</sub>-lacZ-3'UTRN (native) and P<sub>CDR1</sub>-lacZ-3'UTRS (swapped) reporter fusions into the CDR1 locus of the clinical C. albicans isolates (centre). The CDR1- and lacZ-coding regions are represented by white and blue arrows, respectively, the SAT1 marker by a grey arrow, and the 3' UTR of CDR1 by the filled circle. CDR1 upstream and downstream regions are represented by solid lines; the CDR1 promoter (P<sub>CDR1</sub>) is symbolized by the bent arrow. The straight arrow indicates the direction of transcription. The probe used to verify the correct integration is indicated by a thick line. Only relevant restriction sites are shown. (b) Transformants harbouring the chromosomally integrated P<sub>CDR1</sub>-lacZ-3'UTRN (native, left) and P<sub>CDR1</sub>-lacZ-3'UTRS (swapped, right) constructs and their corresponding parental strains (without lacZ) were streaked on minimal medium plates containing X-Gal and photographed after 3 days of growth at 30 °C. The positions of the individual strains on the plates are shown in the scheme (centre). (c) β-Galactosidase quantitative reporter activities of each transformant were determined as described in Methods. The reported quantities are mean ± SD (indicated by error bars) of three independent experiments with duplicate measurements of two independent clones. Empty and filled bars indicate P<sub>CDR1</sub>-lacZ-3'UTRN (native) and P<sub>CDR1</sub>-lacZ-3'UTRS (swapped) reporter fusion transformants in both AS and AR backgrounds.
Table 1. Representative CDR1 transcript poly(A) tail lengths and corresponding transcript half-lives in clinical AS and AR isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>CDR1 transcript poly(A) tail length</th>
<th>CDR1 transcript half-life* [t1/2 (min)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gu4</td>
<td>26 ± 2</td>
<td>~60</td>
</tr>
<tr>
<td>Gu5</td>
<td>35 ± 2</td>
<td>&gt;180</td>
</tr>
<tr>
<td>DSY294</td>
<td>24 ± 2</td>
<td>~60</td>
</tr>
<tr>
<td>DSY296</td>
<td>36 ± 2</td>
<td>&gt;180</td>
</tr>
</tbody>
</table>

*The CDR1 transcript half-lives of the mentioned matched pairs of AS and AR isolates were deduced previously (Manoharal et al., 2008).

detailed in Methods. We observed from PCR amplicon analyses that while PAP1-a and PAP1-α alleles were generated from both the AS isolates, only the PAP1-α allele could be recovered from AR isolates (Fig. 4).

**PAP1-a disruption decreases drug susceptibilities of AS isolates**

It has been demonstrated earlier that while both the PAP1 alleles (Δpap1-a/PAP1-α and PAP1-a/Δpap1-α) are independently functional in heterozygous deletion mutants, they are collectively essential, since a Δpap1-a/Tet-PAP1-α conditional mutant is not viable under Tet-repressing conditions (Jiang et al., 2008). To get an insight into the functional relevance of the observed PAP1 allelic status in AS and AR clinical isolates, we used ura3 Δ mutants DSY3040 (derived from DSY294) and DSY3041 (derived from DSY296) (Coste et al., 2006). For this, heterozygous mutants of PAP1-a (Δpap1-a/PAP1-α) and of PAP1-α (PAP1-a/Δpap1-α) in AS, and a single PAP1-α allele disruptive (Δpap1-α/PAP1-α) in AR, were constructed, as detailed in Methods. These heterozygous PAP1 mutants were tested for their drug susceptibilities, wherein drug spot assays were performed on YEPD plates containing fixed drug concentrations (Fig. 5a, b). The heterozygous PAP1-a mutant (Δpap1-a/PAP1-α) of AS isolates revealed reduced susceptibility to tested drugs as compared with the parental strain DSY3040 (Fig. 5a). Notably, the observed reduced susceptibility of the heterozygous PAP1-a mutant (Δpap1-a/PAP1-α) of the AS isolate to the tested drugs could be reversed if complemented with the PAP1-a allele (Δpap1-α/PAP1-α + PAP1-a). On the other hand, the heterozygous disruption of PAP1-α and its complementation in both AS and AR isolates resulted in drug sensitivities comparable with those of the respective parental strains, DSY3040 and DSY3041 (Fig. 5a, b).

**PAP1-a disruption (Δpap1-a/PAP1-α) results in hyperadenylation of CDR1 transcripts in AS isolates**

We checked further whether heterozygous disruption of PAP1-a and PAP1-α in AS and AR isolates could affect the polyadenylation of CDR1 transcripts. For this, we carried out PAT analysis (as described in Methods) with the heterozygous PAP1-a mutant (Δpap1-a/PAP1-α). This analysis revealed that PAP1-a deletion in DSY3040 (24 ± 2 A residues) leads to hyperadenylation (35 ± 2 A residues) of CDR1 transcripts (Table 2). Notably, the complementation of the heterozygous PAP1-a mutant with PAP1-a (Δpap1-a/PAP1-α + PAP1-a) restored the polyadenylation status of CDR1 transcripts to basal level (Table 2). In contrast, the disruption of PAP1-α (PAP1-a/Δpap1-α) as well as its subsequent complementation (PAP1-a/Δpap1-α + PAP1-α) in AS did not affect the polyadenylation of the CDR1 transcript (Table 2). With regard to the PAP1-α allele in the AR isolate, neither its heterozygous disruption (Δpap1-α/PAP1-α) nor its complementation (Δpap1-α/PAP1-α + PAP1-α) influenced the polyadenylation status of CDR1 transcripts (Table 2).

**PAP1-a disruption results in increased stability of CDR1 transcripts in AS isolates**

Since, in eukaryotes, most mature mRNAs having a poly(A) tail at their 3′ end participate in mRNA stability (Higgins, 1991; McCarthy, 1998; Ross, 1996), we evaluated whether the observed decrease in drug susceptibility and increase in polyadenylation of CDR1 transcripts in a heterozygous PAP1-a mutant (Δpap1-a/PAP1-α) within the AS background correlates with enhanced stability of its mRNA. Using a thiolutin chase assay, we revealed increased half-life of CDR1 transcripts (t1/2 ~120 min) in a Δpap1-a/PAP1-α mutant (RMY4) in contrast to its parental counterpart, DSY3040 (t1/2 ~60 min). The increased CDR1 transcript stability of RMY4 could be reversed to parental levels if complemented with the PAP1-a allele (Δpap1-a/PAP1-α + PAP1-a) (Fig. 6a, b). It is of note that the CDR1 mRNA decay rate of the heterozygous PAP1-a mutant...
remained intermediate ($t_{1/2} \sim 120\ min$) to that of AS ($t_{1/2} \sim 60\ min$) and AR isolates ($t_{1/2} \sim 180\ min$) (Fig. 6a, b), indicating that along with PAP1-α, some additional unknown mechanism(s) also contribute to enhanced CDR1 transcript stability in AR isolates. Notably, heterozygous PAP1-α mutants of AS (Δpap1-α/PAP1-α) and AR isolates (Δpap1-α/PAP1-α), and their complemented counterparts, PAP1-α/Δpap1-α + PAP1-α and Δpap1-α/PAP1-α + PAP1-α, respectively, did not affect the stability of CDR1 transcripts (Fig. 6a, b).

**DISCUSSION**

In many AR clinical isolates of *C. albicans*, a positive correlation between resistance and CDR1 overexpression has been reported by several independent research groups (Franz et al., 1998, 1999; Lopez-Ribot et al., 1998; White, 1997; White et al., 1998). Various other studies have dissected the cis-acting elements in the CDR1 promoter region (De Micheli et al., 2002; Gaur et al., 2004; Karmani et al., 2004; Puri et al., 1999) and identified its transcription factor(s) (Chen et al., 2004; Coste et al., 2006; Znaidi et al., 2008), which contribute to its differential mRNA expression in AS and AR isolates. We have recently observed that along with increased transcriptional activation, enhanced mRNA stability can also contribute to the sustained CDR1 overexpression in AR isolates (Manoharlal et al., 2008). In the present study, we evaluated the cause(s) of the increased CDR1 mRNA stability in AR isolates.

By employing a 3′-RACE method, we mapped and observed the variation in the length of the 3′ UTR of CDR1 in AS and AR isolates (Fig. 1a, b). Notably, the heterogeneous product lengths at the 3′ ends observed were not specific to AS or AR isolates, since each isolate exhibited a 3′ UTR length for CDR1 independent of theazole susceptibility as well as of the level of CDR1 expression. The existence of this heterogeneity at the 3′ end of CDR1 can be explained by the alternative usage of several polyadenylation signals, as has been well documented for other unrelated MDR genes (Edwards-Gilbert et al., 1997; Hsu et al., 1990). For example, although evolutionarily quite distant from yeast, mouse mdr1a (Hsu et al., 1990) and EhPgp5 mRNA of *Entamoeba histolytica* trophozoites responsible for the MDR phenotype (Lopez-Camarillo et al., 2003) also show length variations at their 3′ ends, which affects mRNA half-life. Notably, several other yeast genes also produce multiple transcripts with different 3′ ends as a result of a carbon source-regulated choice between alternative polyadenylation sites (Sparks & Dieckmann, 1998).

**Table 2.** Representative CDR1 transcript poly(A) tail lengths and corresponding transcript half-lives in PAP1 mutant and revertant clinical strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>CDR1 transcript poly(A) tail length</th>
<th>CDR1 transcript half-life [$t_{1/2}$ (min)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSY3040</td>
<td>24 ± 2</td>
<td>-60</td>
</tr>
<tr>
<td>RMY4</td>
<td>35 ± 2</td>
<td>-120</td>
</tr>
<tr>
<td>RMY7</td>
<td>24 ± 2</td>
<td>-60</td>
</tr>
<tr>
<td>RMY5</td>
<td>24 ± 2</td>
<td>-60</td>
</tr>
<tr>
<td>RMY10</td>
<td>24 ± 2</td>
<td>-60</td>
</tr>
<tr>
<td>DSY3041</td>
<td>36 ± 2</td>
<td>-180</td>
</tr>
<tr>
<td>RMY6</td>
<td>36 ± 2</td>
<td>-180</td>
</tr>
<tr>
<td>RMY12</td>
<td>36 ± 2</td>
<td>-180</td>
</tr>
</tbody>
</table>

Fig. 5. Drug susceptibility testing of PAP1 mutants and revertant clinical strains. Drug susceptibility assays were carried out on YEPD medium containing 2 µg fluconazole ml⁻¹ (for AS) and 5 µg fluconazole ml⁻¹ (for AR), 15 µg terbinafine ml⁻¹ and 300 µg cycloheximide ml⁻¹. Spotting assays were performed with serial dilutions of overnight cultures onto YEPD medium containing the indicated drugs. Plates were incubated for 48 h at 30 °C. Graduation filled bars indicate 10-fold serial dilutions of culture, as described in Methods. Strains and genotypes: DSY3040, PAP1-α/PAP1-α; RMY4, Δpap1-α/PAP1-α; RMY7, Δpap1-α/PAP1-α + pap1-α; RMY5, PAP1-α/Δpap1-α; RMY10, PAP1-α/Δpap1-α + PAP1-α; DSY3041, PAP1-α/PAP1-α; RMY6, Δpap1-α/PAP1-α; RMY12, Δpap1-α/ PAP1-α + PAP1-α.

In silico analysis and secondary structure prediction of mapped CDR1 3′ UTR (~78% AU-rich) suggest the
Fig. 6. CDR1 mRNA decay assay. Exponentially growing cultures of *C. albicans* were incubated with the optimized thiolutin concentration (40 μg ml⁻¹) to inhibit ongoing *in vivo* transcription (as described in Methods). Total RNA was isolated at the times indicated thereafter and fractionated on a 1% (w/v) agarose/2.2 M formaldehyde denaturing gel. (a) The gel was stained with ethidium bromide before blotting to monitor equal loading of the RNA and subsequently blotted onto a charged nylon membrane. The blot was hybridized with [α-³²P]dATP-labelled CDR1-specific probe. Time points in minutes are indicated below the equally loaded RNA gel. (b) The hybridization signals were quantified using densitometry scanning in a phosphorimager scanner. The signal intensity at each time point was normalized to that of time t₀ (expressed as a percentage) and plotted as a line graph. t₁/₂, half-life. Strains and genotypes: DSY3040, *PAP1-a/PAP1-a*; RMY4, Δ*pap1-a/PAP1-a*; RMY7, Δ*pap1-a/PAP1-a* + *PAP1-a*; RMY5, *PAP1-a/Δpap1-α*; RMY10, *PAP1-a/Δpap1-α* + *PAP1-α*; DSY3041, *PAP1-α/PAP1-α*; RMY6, Δ*pap1-α/PAP1-α*; RMY12, Δ*pap1-α/PAP1-α* + *PAP1-α*. 

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existence of various regulatory RNA sequences and structural motifs in both AS and AR isolates (Fig. 2a, b and Supplementary Fig. S1). To experimentally test the proposed mRNA destabilizing activity of these predicted CDR1 3′ UTR regulatory motifs, the reporter activity of lacZ in transcriptional fusion with the CDR1 3′ UTR of either AS or AR isolates was examined (Fig. 3a). Interestingly, swapping of the CDR1 3′ UTR between AS and AR had no effect on lacZ reporter activities (Fig. 3b, c) or on its mRNA half-life (Supplementary Fig. S2), indirectly indicating that the CDR1 3′ UTR is not sufficient to affect either the transcription or the mRNA stability of CDR1. Notably, mammalian MDRI 3′ UTR (Prokipcak et al., 1999) and EhPgp5 3′ UTR (Lopez-Camarillo et al., 2003), which also harbour putative regulatory RNA motifs, do not behave as active destabilizing elements for their corresponding mRNAs.

The mRNA abundance of a typical gene is affected by a concerted interplay of key factors such as the activities of poly(A) polymerase, deadenylases and poly(A)-binding proteins (Zhao et al., 1999). The pre-mRNAs are polyadenylated in a reaction involving 3′-endonucleolytic cleavage followed by poly(A) tail synthesis (Zhao et al., 1999). The poly(A) tail is considered to be a strong modulator of mRNA stability, and its length is subjected to cellular control throughout the life span of the mRNA (Higgins, 1991; McCarthy, 1998; Ross, 1996). It is well known that longer poly(A) tails provide higher stability to mRNA and promote a more efficient translation (Higgins, 1991; McCarthy, 1998; Ross, 1996). In our study, PAT assay revealed that CDR1 mRNA has an ~30–35% longer poly(A) tail in AR strains than in their respective matched AS isolates (Table 1), suggesting that polyadenylation and deadenylation events occur at different rates that could affect CDR1 mRNA half-life. The relatively longer poly(A) tail of EhPgp5 mRNA has been shown to be associated with its enhanced half-life in Entamoeba histolytica trophozoites grown in the presence of the drug emetine (Lopez-Camarillo et al., 2003). Interestingly, this increased poly(A) tail length of EhPgp5 mRNA has been associated with the Entamoeba histolytica nuclear poly(A) polymerase (EhPAP) that catalyses the poly(A) tail synthesis of mRNA in the nucleus (García-Vivas et al., 2005).

C. albicans PAPI, which is responsible for polyadenylation of mRNA, is located within the MTL locus on chromosome 5, where TAC1 and its linkage with the MTL in determining TAC1 hyperactivity have been established (Coste et al., 2006). It has been recently shown that a hyperactive TAC1 allele is responsible for CDR1 as well as CDR2 upregulation in isolate DSY296 (Coste et al., 2006). The existence of an association among MTL, TAC1 and PAPI is an exciting possibility that needs to be explored. It is of note that Sanglard’s group has shown by comparative genome hybridization (CGH) and single nucleotide polymorphism (SNP) arrays that LOH of TAC1 in an AR clinical isolate (DSY296) can occur either by recombination between portions of chromosome 5 or by chromosome 5 duplication. LOH is not restricted to MTL but also extends to ~250 kb flanking regions that also comprise PAPI alleles within MTL (Coste et al., 2006). Consistent with published results, we recovered two distinct PAPI alleles (PAPI-a/PAPI-a) from AS (MTL-a/MTL-a) and a single type of PAPI allele (PAPI-a) in matched AR isolates (MTL-a/MTL-a) (Fig. 4). It is of note that these two isoforms of C. albicans PAPI, PAPI-a (ORF 19.3197) and PAPI-a (ORF 19.10713), share only ~70% amino acid identity (Hull & Johnson, 1999). Earlier evidence of differential susceptibilities of the two PAPI isoforms to a natural product, parnafungin, suggests a difference in their in vivo activity (Jiang et al., 2008). We established differences in PAPI-a and PAPI-a activities by constructing heterozygous mutants in both AS and AR backgrounds. Notably, the heterozygous PAPI-a mutant (Δpapi-a/a-papi-a) displayed enhanced drug resistance as compared with the AS isolate (Fig. 5a), which could be reversed by complementing the PAPI-a allele in this heterozygous mutant (Δpapi-a/a-papi-a) (Fig. 5a). Subsequent analysis of relative hyperadenylation (Table 2) and decreased decay rate of CDR1 mRNA (Fig. 6a, b) in this heterozygous PAPI-a mutant suggests that PAPI-a has a dominant negative effect on PAPI-a activity. It seems that PAPI-a disruption in AS isolates leads to de-repression of PAPI-a activity, thereby leading to hyperadenylation and increased stability of CDR1 transcripts. This observation is consistent with the unchanged drug susceptibilities of heterozygous PAPI-a/a mutant in both AS (Δpapi-a/a-papi-a) and AR (Δpapi-a/a-papi-a) isolates (Fig. 5a, b). This was accompanied by unaltered polyadenylation status (Table 2) as well as stability of CDR1 transcripts (Fig. 6a, b). However, it should be noted that in the heterozygous PAPI-a/a mutant of AS isolates, although CDR1 transcript polyadenylation status switches from AS to AR level (24 ± 2 to 35 ± 2 A residues), its mRNA decay rate remains intermediate (t1/2 ~120 min) to that of AS (t1/2 ~60 min) and AR isolates (t1/2 ~180 min) (Fig. 6a, b, Table 2). This implies that along with increased poly(A) tail length, some additional mechanism(s) also contribute to enhanced CDR1 transcript stability in AR isolates.

In conclusion, our results provide what we believe to be the first evidence that LOH at the PAPI locus contributes to hyperadenylation and subsequent increased CDR1 transcript stability, and ultimately enhanced drug resistance in AR isolates. We recently observed that all TAC1 target genes show relatively higher transcriptional rates and mRNA half-lives in AR isolates (our unpublished observations). However, whether the observed increase in poly(A) tail length in AR isolates is specific to CDR1 transcripts alone or represents a general feature of the mRNA population of Tac1p targets as well as other transcripts within the ORFome of C. albicans remains to be established. Certainly, further characterization of the functional role of PAPI should lead to a better understanding of mechanisms underlying post-transcriptional events and should also be useful for the development of...
antifungal strategies that can be exploited to combat MDR in *C. albicans*.

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