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Molecular cloning and functional characterisation of a glucose transporter, *CaHGT1*, of *Candida albicans*

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Abstract

We have cloned the first glucose transporter *CaHGT1* (***C**andida **a**lbicans* **h**igh-affinity **g**lucose **t**ransporter) of a pathogenic yeast, *Candida albicans*. The DNA sequence (GenBank accession number Y16834) analysis revealed an ORF encoding a novel protein of 545 amino acids with a predicted molecular mass of 60.67 kDa. The putative protein with 12 transmembrane domains has 51% identity with *Kluyveromyces lactis* high-affinity glucose transporter, *HGT1*. The protein signatures which are conserved and distinctive of the sugar transporters belonging to the major facilitator superfamily (MFS) were also found in CaHgt1p. When heterologously expressed, the ORF functionally complemented a mutant strain of *Saccharomyces cerevisiae* RE700A which was deleted in seven hexose transporter genes and thus was unable to grow or transport glucose. The expression of *CaHGT1* in *C. albicans* showed a transcript of 1.6 kb which was enhanced in response to the human steroid hormone progesterone. Interestingly, the transcript levels were also enhanced in the presence of drugs, e.g. cycloheximide, chloramphenicol and benomyl. The results suggest that *CaHGT1*, which encodes a MFS protein, could be linked to the drug resistance phenomenon in *C. albicans*.

Keywords: Major facilitator superfamily; Glucose transporter; *Candida albicans*; *CaHGT1*

1. Introduction

Candida albicans is an opportunistic, infectious pathogenic yeast, which particularly infects immuno-compromised patients such as AIDS and organ transplant patients [1,2]. There is considerable interest to understand the mechanisms of regulation of growth and morphogenesis of this yeast, which is affected by several factors including carbon sources [2,3]. There is evidence to suggest that *C. albicans* accumulates a number of sugars, such as L-sorbose, D-xylose, D- and L-arabinose, against concentration gradients [4,3]. However, unlike other yeasts, work on sugar transport and transporters of *C. albicans* is still at an incipient stage and an in-depth investigation is required to gain insight into the mechanisms underlying transport of sugars.

The emergence of antifungal resistance in *C. albicans* has become a major threat to the usefulness of chemotherapy in treating the infection. To date several genes have been identified whose expression is linked to azole resistance [5–7]. How these genes are regulated is being vigorously pursued, and transcription factors like FCR (fluconazole resistance) *1,2,3* and *CAP1* (*C. albicans AP-1*) regulating MDR genes of *C. albicans* have been identified [8]. The well studied yeast, *Saccharomyces cerevisiae*, whose genome is completely sequenced, revealed several genes involved in pleiotropic drug resistance (PDR) along with several transcription factors which regulate their expression [9,10]. Interestingly, two putative sugar transporters, i.e. *HXT9* and *HXT11*, are regulated by transcription

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factors, *PDR1* and *PDR3*, which are known to regulate *PDR* genes of *S. cerevisiae* [11]. The facilitative glucose transporter *GLUT1* has also been shown to be involved in the development of drug resistance in mammalian cells [12].

This paper describes the molecular cloning of the first glucose transporter gene, *CaHGT1* (***C**andida **a**lbicans* **h**igh-affinity **g**lucose **t**ransporter) of *C. albicans*. Using heterologous expression in *S. cerevisiae* mutant strain RE700A, the functionality of *CaHGT1* is demonstrated. It encodes a high-affinity glucose transporter. In addition, an enhancement of *CaHGT1* transcript is shown in response to the human steroid hormone progesterone and drugs. The results suggest that *CaHGT1*, which encodes a major facilitator superfamily (MFS) protein, could be linked to the drug resistance phenomenon in *C. albicans*.

2. Materials and methods

2.1. Strains and media

C. albicans ATCC 10261 was grown on YEPD medium (2% Bacto peptone, 1% yeast extract and 2% D-glucose). The glucose transport-deficient mutant of *S. cerevisiae* RE700A (*MAT***a** *ura3-52 hxt1*Δ::*HIS3*::Δ*hxt4 hxt5*::*LEU2 hxt2*Δ::*HIS3 hxt3*Δ::*LEU2*::Δ*hxt6 hxt7*::*HIS3*) [13] was maintained on YEP medium containing 2% maltose. RE700A was transformed with the expression vector pYEX-BX containing *CaHGT1* downstream of the *CUP*1 promoter. The transformant was selected on minimal YNB medium consisting of 0.67% yeast nitrogen base and 2% carbon source (maltose or D-glucose) supplemented with appropriate amino acids without uracil. Plasmid pYEX-BX was induced by adding 0.5 mM copper sulfate. Transformation of *S. cerevisiae* RE700A and plasmid recovery from the transformants was carried out as described elsewhere [14].

2.2. PCR and DIG labeling

CaHGT1 was cloned by amplifying *C. albicans* genomic DNA through PCR using primers: forward primer: 5′-ATGATGTTAGGTTTTGATATTTCTTCAATG-3′, reverse primer: 5′-CCCAATAATCAATTGAGCACGATTTTGAAC-3′, designed from an unpublished partial sequence of the putative *C. albicans* sugar transporter (http://www.alces.med.umn.edu). The PCR product (260 bp) was ligated as a blunt-end fragment (Sure Clone Ligation Kit, Pharmacia, Freiburg, Germany) to *Sma*I-digested pUC18 (Pharmacia). This was sequenced and used as a probe for Southern hybridisation. All the probes used in the Southern blotting were labeled with digoxigenin-11-dUTP (DIG) using the random primer method with the DIG DNA Labeling kit from Boehringer Mannheim.

2.3. Subcloning and sequencing

Genomic DNA *Hin*dIII fragments were ligated to *Hin*dIII-digested pUC18 and *Hin*cII fragments from the fosmids were ligated to *Sma*I-digested pUC18. The constructs were used to transform *Escherichia coli* XL1 Blue cells. Plasmids recovered from the resulting transformed colonies were screened by the method of Southern hybridisation. Subclones were made using the restriction enzyme sites delineated by restriction mapping. These positive subclones were sequenced by the dideoxy chain termination method using double-stranded plasmid DNA as template [15].

A *Bam*HI site was placed upstream of the putative *CaHGT1* ATG start codon (5′-ATAATCTCAA**GGATC**-**C**ATGTCGTCCAA) and a *Pst*I site was placed downstream of the stop codon UAA (3′-CCTAAATGGAATT-TAG**CTGCAG**CTTCAATTCTTCA) by PCR amplification of the clone pAV-*CaHGT1* containing the full ORF of *CaHGT1*. The resulting PCR product was double-digested with *Bam*HI and *Pst*I and then ligated to the yeast expression vector pYEX-BX double-digested with *Bam*HI and *Pst*I downstream of the *CUP*1 promoter.

2.4. RNA extraction and Northern blotting

Total RNA isolation from *C. albicans* cells and Northern analyses were as described earlier [5].

2.5. Glucose uptake and accumulation assays

The cells were grown to mid-exponential phase (15–16 h), harvested, washed with distilled water and incubated at 30°C for 2 h in fresh medium containing 0.1% D-glucose. Consumption of D-glucose from the medium was assayed enzymatically as described by Bergmeyer [16].

2.6. Nucleotide sequence accession number

The *CaHGT1* nucleotide sequence has been assigned GenBank accession number Y16834.

3. Results and discussion

3.1. Isolation of the C. albicans CaHGT1 Gene

A partial unpublished DNA sequence (http://www.alces.med.umn.edu) of a putative glucose transporter *HGT1* of *C. albicans* was used to design two oligonucleotide primers. Under conditions of high stringency, these primers were used for PCR amplification with *C. albicans* ATCC 10261 genomic DNA. The purified PCR product of expected size (260 bp) was cloned into the pUC18 vector. Two positive clones were sequenced and found to be identical, both displayed 85% sequence identity with the high-

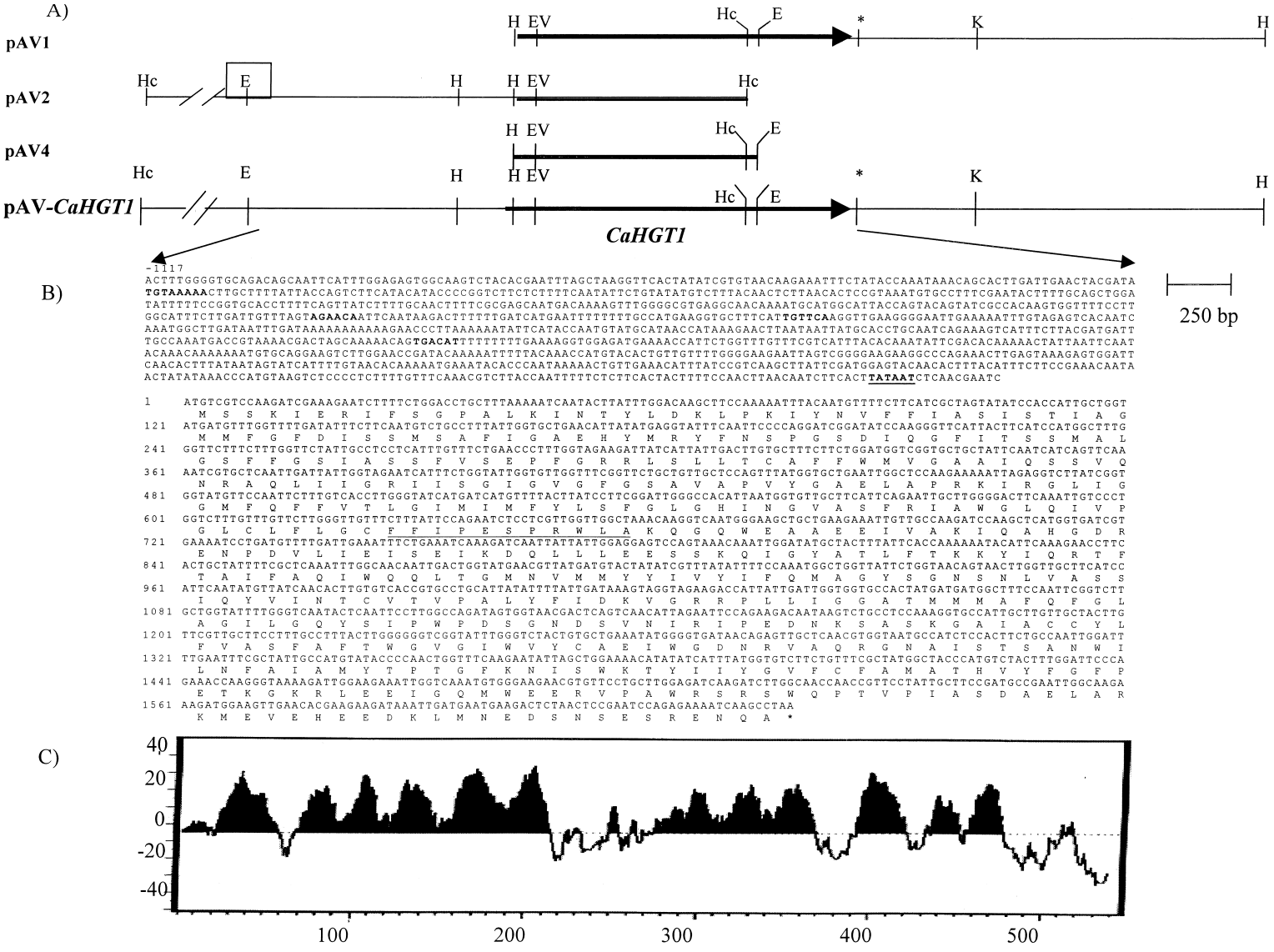

Fig. 1. The sequence and hydrophobicity of *CaHGT1*. A: Restriction map of *CaHGT1* of *C. albicans*. The bold arrow indicates the open reading frame. Plasmid pAV1 represents the truncated genomic clone, pAV2 shows the chromosomal fragment obtained after screening the fosmid library. Plasmid pAV4 represents the subclone used as a probe in Southern analysis. pAV-*CaHGT1* represents the plasmid containing the complete ORF made after ligating the *Eco*RV-*Kpn*I fragment of pAV1 to pAV2 digested by *Eco*RV-*Kpn*I. E: *Eco*RI; ERV: *Eco*RV; H: *Hin*dIII; Hc: *Hin*cII; K: *Kpn*I; *: stop codon. B: Nucleotide and deduced amino acid sequence of *CaHGT1*. The TATA box is shown in bold and underlined. Various putative elements are shown in bold and the stop codon is indicated by *. C: Hydrophobicity plot of *CaHGT1* of *C. albicans*. The plot was constructed from the hydrophobicity values determined at each residue by the method of Eisenberg et al. [20] using a window size of 15 residues.

affinity glucose transporter *HGT1* of *Kluyveromyces lactis*. The insert from one of these clones was used as a probe for genomic DNA Southern hybridisation. A ~3-kb positive band was cloned in pUC18 (pAV1) and used for further characterisation. DNA sequence analysis of pAV1 revealed an incomplete ORF in the cloned 3-kb insert with homology to *HGT1* of *K. lactis* and hexose transporter genes (*HXT*s) of *S. cerevisiae* [17,18] and was found to be localised on chromosome I. In order to clone the complete ORF, the corresponding fosmid library of *C. albicans* [19], containing chromosome I fragments, was screened using the 1-kb *Eco*RI-*Hin*dIII subfragment of pAV1 (pAV4, Fig. 1A) as a probe. Southern analysis gave two positive bands of 4.2 kb and 2 kb from fosmids 11D6 and 18B4. The 4.2-kb *Hin*cII fragment was further subcloned into pUC18 at the *Sma*I site (pAV2) (Fig. 1A). DNA sequence analysis of pAV2 revealed that pAV1 and pAV2 had 100% identity in the overlapping region (Fig. 1A). In order to reconstruct the full ORF, the *Eco*RV-*Kpn*I fragment of pAV1 was ligated to pAV2 digested with *Eco*RV-*Kpn*I (*Kpn*I of MCS of pUC18) resulting in pAV-*CaHGT1* which, on sequence analysis, revealed a complete ORF comprising 545 amino acids (Fig. 1B). The protein has a predicted molecular mass of 60.67 kDa.

3.2. Sequence analysis of the CaHGT1 gene

Substantial sequence similarity was observed between the cloned ORF and the members of glucose transporter families of *S. cerevisiae* and *Schizosaccharomyces pombe* [17,18]. The greatest similarity (51% identity) was observed with *HGT1*, a high-affinity glucose transporter of *K. lactis* and hence the gene was designated *CaHGT1*. Hydrophobicity values determined at each residue by the method of Eisenberg et al. using a window size of 15 residues [20] showed the presence of 12 putative transmembrane (TM) domains (Fig. 1C), a characteristic feature of the major facilitator superfamily [21]. The alignment of amino acid sequences of *CaHGT1* (accession number Y16834) of *C. albicans* with *HGT1* (accession number U22525) of *K. lactis*; *GHT1* (accession number X91218), *GHT2* (accession number AF017180) and *GHT3* (accession number AF051139) of *S. pombe*; *HXT1* (accession number U00060) and *HXT2* (accession number M33270) of *S. cerevisiae; GLUT1* (accession number S80434), *GLUT2* (accession number L09674) and *GLUT3* (accession number M20681) of *Homo sapiens* and *SNF3* (accession number J03246) glucose sensor of *S. cerevisiae* [24], generated using the CLUSTALW program (data not shown), revealed a conserved signature (IDKVGRRPLLIGG) typical of sugar facilitators (MFS) [21]. The sequence similarities among glucose transporters from yeast to human justify a close relationship of CaHgt1p with the high-affinity glucose transporter Hgt1p of *K. lactis*. In a dendrogram (data not shown), *CaHGT1* and *HGT1* of *K. lactis* form a distinct family of transporters which is less related to the group of Hxtp of *S. cerevisiae* than to the cluster of hexose transporters represented by Ghtp members of *S. pombe* [23,24]

Nucleotide sequence analysis revealed the presence of TATA-like motif at position −107 with correlated CAP signal, as assessed using the EUKPROM algorithm (PC Gene, IntelliGenetics). The 5′ upstream region of the initiation codon was found to be A+T-rich and harbouring a number of regulatory elements. Most striking was the presence of a putative progesterone/glucocorticoid response element (PRE, GRE: AGAACA) at −718 and a putative estrogen response element (ERE: TGACAT) at −453 with respect to ATG [22]. A putative pheromone response element (PhRE: TGTAAAAA) was also found at position −991. The putative elements of *PDR1* and *PDR3*, which are present in *HXT9* and *HXT11* of *S. cerevisiae*, were absent in *CaHGT1* promoter.

3.3. CaHGT1 could complement the glucose transport defect when expressed in S. cerevisiae

In order to check the functionality of the cloned putative glucose transporter, the glucose uptake-deficient strain RE700A of *S. cerevisiae*, which is devoid of *HXT1, 2, 3, 4, 5, 6* and *7* [13], was used for heterologous expression of the *CaHGT1* gene. The *CaHGT1* ORF was placed under the copper-inducible promoter (*CUP1*) in the expression vector pYEX-BX with *URA3* as the auxotrophic selectable marker. The expression construct pYEX-*CaHGT1* was used to transform RE700A, yielding several colonies on medium containing 2% maltose without uracil. These colonies were then tested for growth on glucose. The transformants showed growth on glucose while RE700A and its transformant containing empty vector pYEX-BX did not show any growth on glucose (Fig. 2A). In order to confirm that the phenotype was plasmid-based, one of the transformants, AVY701, was grown under non-selective conditions (2% maltose+uracil) for several generations and then plated onto glucose-containing medium without uracil where several colonies failed to grow (data not shown). This confirmed that the growth of AVY701 on glucose was conferred by the plasmid pYEX-*CaHGT1*.

The glucose transport and specificity were determined enzymatically by following its consumption from the medium (Fig. 2B). A steady consumption of glucose was demonstrated by the transformant AVY701 which was comparable even in the presence of a threefold excess of D-galactose. This confirmed that the observed D-glucose consumption was mediated by a specific heterologously expressed *C. albicans* glucose transporter, CaHgt1p. The apparent K_T value in the range of 1 mM was obtained from a Lineweaver-Burk plot (data not shown). Based on K_T values of other hexose transporters [25,26], it would mean that the *CaHGT1* gene product encodes a high-affinity glucose transporter.

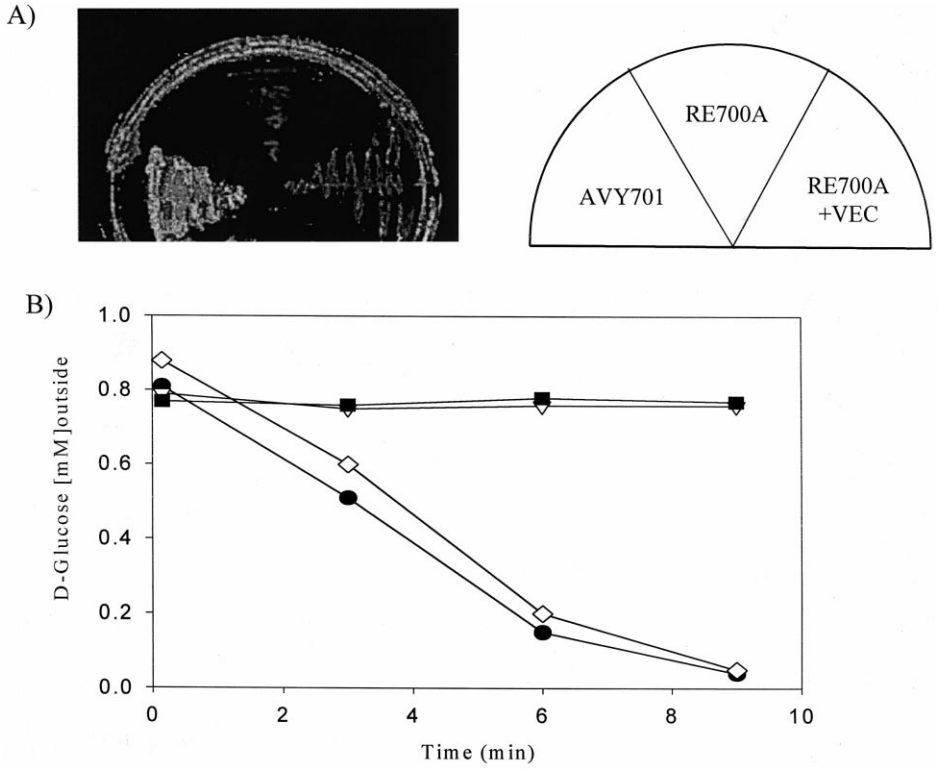

Fig. 2. Functional analysis of *CaHGT1*. A: Growth of AVY701. Comparison of growth of the transformant AVY701, RE700A and RE700A+vector-(pYEX-BX) on 2% glucose without uracil. B: Consumption of D-glucose by AVY701, RE700A and RE700A+vector. (▽) RE700A; (●) AVY701; (■) RE700A+vector; (◇) consumption of D-glucose by AVY701 in the presence of a threefold excess of D-galactose. The transformed strain AVY701 was grown in YNB medium containing 2% glucose without uracil; RE700A containing empty vector was grown in YNB containing 2% maltose without uracil; and RE700A was grown in YNB containing 2% maltose with uracil. After harvesting, the cells were washed with YNB and transferred to fresh YNB for 2 h. The enzymatic assay for D-glucose consumption was done as described by Bergmeyer [16].

3.4. *CaHGT1* expression can be induced by progesterone and drugs

Northern blot analysis revealed that *C. albicans* cells incubated with 1 mM progesterone for 15 min exhibited enhanced expression of *CaHGT1* (Fig. 3A, lane 3) as compared to the control (lane 1). β-Oestradiol, on the other hand, did not affect the expression of *CaHGT1* (lane 2). Interestingly, when *C. albicans* cells were preincubated with different drugs, viz. benomyl, chloramphenicol, cycloheximide, miconazole and 4-nitroquinoline-*N*-oxide, the expression of *CaHGT1* was enhanced upon incubation with only cycloheximide, benomyl and chloramphenicol. In comparison, the increase in expression was maximal with cycloheximide (Fig. 3B).

Earlier studies have suggested that the MFS protein and facilitative glucose transporter (Glut1p) of mammalian cells [12] could be involved in the development of drug resistance. Furthermore, *HXT9* and *HXT11* are regulated by transcription factors *PDR1* and *PDR3* which regulate the ABC transporters required for drug resistance in yeast. The induced expression of *CaHGT1* by drugs would suggest commonality among these MFS proteins. Whether CaHgt1p and several other putative glucose transporters of *C. albicans* (http://www.alces.med.umn.edu) are also regulated by ABC regulators remains to be established. It is tempting to speculate that the study of sugar transporter genes and their regulation would lead to a better understanding of the regulation of drug susceptibilities of *C. albicans*.

Mammalian steroid hormones from the microenvironment have been found to affect the growth and morphogenesis of the invading *C. albicans* [27]. In addition, oestrogen binding proteins (EBP) have been identified in *C. albicans* [28] which binds oestrogen with high affinity, stimulating the transition from yeast to the mycelial form [27]. The interaction of some of the azoles, such as ketoconazole, with the corticosteroid receptor and binding protein has also been suggested. We have earlier reported that human steroid hormones can up-regulate the expression of an ABC transporter, *CDR1* (*Candida* drug resistance) [5,14]. In this study we further show that progesterone can also transcriptionally activate the expression of the MFS transporter *CaHGT1*. The mechanism underlying steroid-mediated gene regulation in yeast is not clear. It is worth pointing out that steroid response elements do exist in *Candida* genes (http://www.alces.med.umn.edu), though unlike their mammalian counterparts, they do not exist as

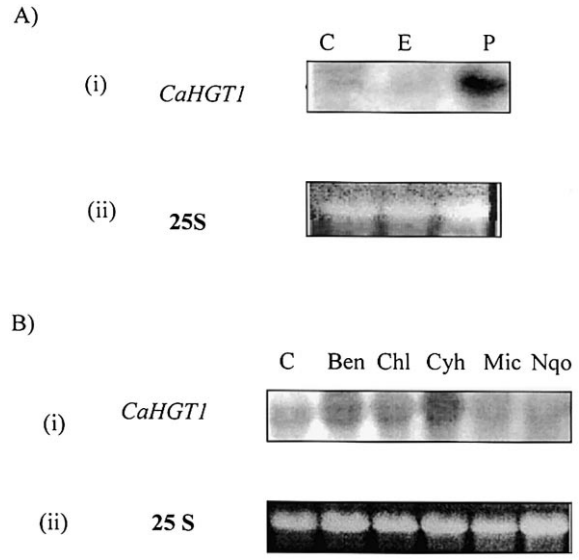

Fig. 3. Expression studies of *CaHGT1*. A: Expression of *CaHGT1* in response to human steroid hormones. *C. albicans* cells were grown to mid-exponential phase (10 h) in YEPD at 30°C and induced by 1 mM β-oestradiol or 1 mM progesterone for 15 min. Total RNA was isolated from the induced cells [5]. Lane 1: control RNA without any steroid induction; lane 2: RNA sample from β-oestradiol-treated cells; lane 3: RNA sample from progesterone-treated cells. The lower panel (ii) shows the gel load (25S rRNA) of panel i. B: Expression of *CaHGT1*, in response to drugs. *C. albicans* cells were grown to mid-exponential phase (10 h) in YEPD at 30°C and induced by different drugs, i.e. benomyl (Ben) 75 μg ml^{-1}, chloramphenicol (Chl) 1 mg ml^{-1}, cycloheximide (Cyh) 1 μg ml^{-1}, miconazole (Mic) 100 μg ml^{-1} and 4-nitroquinoline-*N*-oxide (Nqo) 10 μg ml^{1} for 30 min. Total RNA was isolated from the induced cells [10]. Lane 1: control RNA; lane 2: Ben-induced RNA; lane 3: Chl-induced RNA; lane 4: Cyh-induced RNA; lane 5: Mic-induced RNA; lane 6: Nqo-induced RNA. The lower panel (ii) shows the gel load (25S rRNA) of panel i.

palindromic repeats but only as one half of one. This, however, could suggest that *Candida* may harbour a unique steroid receptor cascade which remains unidentified. This aspect of gene regulation, mediated by human steroid hormones in *C. albicans*, is under investigation.

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