Cloning, sequencing, and expression of *H.a.YNRI* and *H.a.YNII*, encoding nitrate and nitrite reductases in the yeast *Hansenula anomala*

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Abstract

A single *Hansenula anomala* genomic DNA fragment containing the genes *H.a.YNRI* (yeast nitrate reductase) and *H.a.YNII* (yeast nitrite reductase) encoding nitrate and nitrite reductase, respectively, was isolated from a λ EMBL3 genomic DNA library. As probe, a 3.2 kb DNA fragment isolated from a λ gt11 *H. anomala* genomic DNA library screened with antiserum anti-NR from *H. anomala* was used. *H.a.YNRI* and *H.a.YNII* genes are separated by 473 bp and encode putative proteins of 870 and 1077 amino acids, respectively, with great similarity to nitrate and nitrite reductases from other organisms. Northern blot analysis revealed that both genes are highly expressed in nitrate, very low in nitrate plus ammonium, and no expression was detected in ammonium or nitrogen-free media. Levels of nitrate reductase and nitrite reductase were very low or undetectable by Western blot analysis in nitrogen-free and ammonium media, whereas both proteins were present in nitrate and ammonium plus nitrate media. The nucleotide sequence Accession No. is AF123281.

Keywords: *Hansenula anomala*; nitrate and nitrite reductases encoding genes; nitrate

Introduction

Among the yeast genera able to assimilate nitrate, *Hansenula*, *Leucosporidium*, *Pachysolen*, *Rhodospiridium*, *Rodhotorula*, *Sporidiobolus*, *Williopsis*, *Brettanomyces*, *Candida*, etc. (Barnett *et al.*, 1984), *Hansenula anomala*, along with *Hansenula polymorpha* and *Candida utilis*, are the few yeast species in which the nitrate assimilation pathway has been studied to some extent (Hipking, 1989; Siverio *et al.*, 1993; Avila *et al.*, 1998; Sengupta *et al.*, 1996, 1997). In yeast, the nitrate assimilation pathway follows that described for plants and filamentous fungi. Once nitrate enters the cells, it is reduced to ammonium by the consecutive action of NR (nitrate reductase) and NiR (nitrite reductase). In the yeast *H. polymorpha*, the genes *YNT1*, *YNII* and *YNRI*, encoding a nitrate transporter, nitrite reductase and nitrate reductase, respectively, are

clustered and coordinately induced by nitrate and repressed by the reduced nitrogen sources. In addition, the cluster contains a Zn(II)₂ Cys₆ transcriptional factor (*YNA1*) involved in the nitrate induction of the nitrate assimilation genes (Avila *et al.*, 1995; Brito *et al.*, 1996; Pérez *et al.*, 1997; Avila *et al.*, 1998).

H. anomala NR has been studied in our group (Gonzalez and Siverio, 1992; Gonzalez *et al.*, 1994; Siverio *et al.*, 1993) and several results have attracted our attention: (a) the levels of enzyme are only partially repressed by ammonium when nitrate is present; (b) the high levels of the enzyme in comparison with those exhibited by *H. polymorpha*; (c) the reversible inactivation of the enzyme by heat-shock and nitrite; and (d) the binding of inactive NR to the mitochondria. All these facts led us to consider the isolation of the NR encoding gene from *H. anomala* as a preliminary step to

further studies on the molecular mechanisms involved in the regulation of NR in *H. anomala*. As a result of this work, the clustering of the *H.a.YNRI* and *H.a.YNII* genes is reported. Furthermore, the levels of expression in different nitrogen sources of these genes and the proteins they encode are shown.

Materials and methods

Yeast strains and growth conditions

H. anomala CECT1112 (Colección Española de Cultivos Tipo) was used in all the experiments. Cells were grown at 30 °C with shaking in liquid medium containing 0.17% (w/v) Yeast Nitrogen Base without amino acids and (NH₄)₂SO₄ (Difco), 2% (w/v) glucose and the indicated nitrogen sources (5 mM NaNO₃ or 5 mM NH₄Cl).

Nucleic acid isolation

Yeast DNA was isolated (Rothstein, 1985), harvesting the cells in the early exponential phase of growth. Lambda DNA was isolated as described in (Sambrook *et al.* 1989). Total yeast RNA was isolated by the hot acidic phenol method (Collart and Oliviero, 1995).

Construction of *H. anomala* genomic libraries

Genomic DNA fragments of around 5 kb, obtained by the mechanical digestion of *H. anomala* genomic DNA, were used to construct an expression library in λ gt11, as described in (Young and Davis 1991). Lambda EMBL3 *H. anomala* genomic DNA library was prepared as described (Avila *et al.*, 1995).

Northern blot analysis

20 μ g of total RNA were subjected to electrophoresis in an agarose formaldehyde denaturing gel and transferred to a positively charged nylon membrane (Roche). The 1.6 kbp *EcoRV*–*SacI*, 0.8 kbp *EcoRI*–*EcoRI* and 1 kbp *HindIII*–*XhoI* DNA fragments, corresponding to *H.a.YNRI*, *H.a.YNII* and *Saccharomyces cerevisiae* *S.c.ACT1*, respectively, digoxigenin-labelled, were used as probe. The membrane was reprobed subsequently with *YNRI*, *YNII* and *S.c.ACT1*. Stripping was carried out by washing with 0.1% SDS, as indicated in *The DIG System User's Guide for Filter Hybridization*

(Roche). Northern blot analysis with *H.a.YNRI*, *H.a.YNII* probes was carried out according to (Engler-Blum *et al.* 1993). For the heterologous *S.c.ACT1* probe, prehybridization and hybridization were done in high SDS concentration hybridization buffer at 46 °C. The filter was subsequently washed twice at room temperature in 2 $\times$ SSC (1 $\times$ SSC=150 mM NaCl and 15 mM nitrate) plus 0.1% SDS and twice at 60 °C in 0.5 $\times$ SSC+0.1% SDS, as indicated in *The DIG System User's Guide for Filter Hybridization*. RNA–DNA hybrids were detected on membranes with CDP-Start (Roche).

PAGE and Western blot analysis

Extracts were made as previously described (Gonzalez and Siverio, 1992). SDS–PAGEs were loaded with 20 μ g protein per lane. Proteins were transferred to PVDF membranes and immunodetection was carried out using either a 1:3000 dilution of an antiserum raised against purified *H. anomala* NR (Gonzalez and Siverio, 1992) or a 1:2000 dilution of an antiserum raised against a peptide of *H. polymorpha* NiR (Perdomo *et al.*, unpublished). Anti-rabbit IgG coupled to peroxidase was used as the second antibody, and it was detected with ECL Plus (Amersham Pharmacia Biotech, Uppsala, Sweden), following the manufacturer's instructions.

DNA sequencing

Bluescript phagemid plasmids from Stratagene (La Jolla, CA, USA) were used. Unidirectional deletions were carried out using the Double-stranded Nested Deletion Kit (Amersham Pharmacia Biotech). DNA was sequenced on double strands by the dideoxi-chain termination method (Sanger *et al.*, 1977) using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham Pharmacia Biotech).

Nucleotide sequence Accession No.

The Genbank/EMBL Accession No. for the sequence reported in this paper is AF123281.

Results and discussion

To clone the NR gene, the λ gt11 *H. anomala* genomic DNA library was screened with an antiserum against purified NR previously prepared (Gonzalez and Siverio, 1992). The screening of 10⁴

phages yielded five identical positive clones with a size of 3.2 kb. One of these clones was sequenced and shown to code partially for proteins homologous to NR at one end and to NiR at the other (Figure 1A). This fragment was used as a probe to screen a λ EMBL3 *H. anomala* genomic library. From 10^4 phages screened, 30 were found to be positive. One of these phages (λ PG15) was characterized and shown to contain a 13 kb insert. After digestion of this phage with *Eco*RI, part of the insert was released as five *Eco*RI–*Eco*RI fragments (0.8, 0.9, 1.8, 3.2 and 4.8 kb) that were cloned into the pBluescript KS[−] phagemid to be sequenced. The sequenced region rendered 7580 bp, containing two ORFs of 2610 (*H.a.YNR1*) and 3231 (*H.a.YNII*) bp (Figures 1B, 2) encoding putative polypeptides of 870 and 1077 amino acids with extensive sequence identity to the NRs and NiRs from other species, identity being 57% and 60% with the corresponding proteins encoded for *YNR1* and *YNII*, respectively, in *H. polymorpha*. The calculated molecular masses of these proteins are 98.9 kDa for NR and 120.4 kDa for NiR, which are very similar to those reported for other NRs (Solomonson and Barber, 1990) and NiRs (Siegel and Wilkerson, 1989). *H.a.YNR1* and *H.a.YNII* genes are separated by 473 bp and transcribed in the same direction. The *H. anomala* region containing *H.a.YNR1* and *H.a.YNII* presents two major differences with its counterpart in *H. polymorpha*: (a) *H.a.YNR1* is upstream from *H.a.YNII*, whereas in *H. polymorpha* these genes are in the opposite orientation; (b) the region between *H.a.YNR1* and *H.a.YNII* does not encode any significant ORF,

while in *H. polymorpha* the *YNA1* gene encoding a Zn(II)₂Cys₆ transcriptional factor involved in nitrate induction is found in this region. In addition, in *H. polymorpha* the intergenic regions in the nitrate assimilation cluster containing *YNT1*, *YNII*, *YNA1*, *YNR1* (Avila *et al.*, 1998) are shorter than that found between *H.a.YNR1* and *H.a.YNII*.

TATA sequences are found in the 5' non-coding region of *H.a.YNR1* and *H.a.YNII* at −45 and −307 bp, respectively, from ATG. TATA elements far away from ATG have been shown to have a role in transcription initiation in *S. cerevisiae* (Nagawa and Fink, 1985). Thus, TATA sequences in this promoter could still have a role in the initiation of transcription. The 5' non-coding region of *H.a.YNR1* and *H.a.YNII* presents no homology with the corresponding *H. polymorpha* region. Nevertheless, potential sites to bind GATA proteins (Chiang and Marzluf, 1994) involved in nitrate derepression are found in *H. polymorpha* and *H. anomala*. The binding site described for NIRA and NIT4 (Fu *et al.*, 1999; Punt *et al.*, 1999) were not found in either *H. anomala* or *H. polymorpha* 5' non-coding regions.

Analysis of *H.a.YNR1* and *H.a.YNII* expression by Northern blot (Figure 3A) showed a high mRNA level in nitrate medium and a very low level of transcription in ammonium plus nitrate medium, indicating that ammonium does not repress *H.a.YNR1* and *H.a.YNII* completely in the presence of nitrate. *H.a.YNR1* and *H.a.YNII* transcripts were undetectable in ammonium as well as in nitrogen-free medium. This contrasts with that found in other organisms such as *H. polymorpha*, where the genes *YNT1*, *YNR1* and *YNII* (Bríto *et al.*, 1996; Avila *et al.*, 1998; Pérez *et al.*, 1997) are only expressed in nitrate. Therefore, in *H. polymorpha*, unlike *H. anomala*, the presence of ammonium, even plus nitrate, or the absence of nitrate seems to completely abolish the expression of nitrate assimilation structural genes. Similar results were found in *N. crassa* (Marzluf, 1981).

Western blot analysis (Figure 3B) reveals that levels of NR and NiR proteins are the highest in nitrate, about half of that with ammonium plus nitrate, and very low or undetectable in ammonium and nitrogen-free. These results are in good agreement with our previous results on the influence of the nitrogen source on the level of NR activity and protein (Gonzalez and Siverio, 1992). Even though

Figure 1. Genomic DNA region bearing *H.a.YNR1* and *H.a.YNII* genes and the probe used for cloning. (A) Probe isolated from the λ gt11 library, containing part of *HaYNR1* (dashed box) and *HaYNII* (dotted box). (B) Restriction map of phage λ PG15 insert showing the region sequenced and ORFs found

TCATCATGCT	TTTGTAATGCT	GATGTGTGCA	CCCAACAGCAC	CAATAACATG	ATGGATCTCT	TGCTTCAGAT	CAACAGCATG	CAACAGCTTC	ATCTTTAGCG	100	
TGCCACAGCT	ATCATATTTC	ATGATCAACGA	CATTGACAGC	CCCAATACCT	TATATAAAAT	AGGTGTTAAT	CAGAGCAGTG	CCTCATGCTA	CTCTCCGAGT	200	
CCTCTTGAAT	CTTCCGTGAT	CGTGTTCGCG	AAAAATAAAT	ATTTCTTTGA	CTGTGGAATA	CGTAAACAC	CGTGATGAT	GGCCATCGGA	AAATAGTAGT	300	
TCATCATGCT	TTTCTATGAC	AGGCAGATGT	TTGTGCTGAT	TGTGGCTATT	TGTGTATGGA	GTCTGACATT	TGCTTGATGT	CTGAGTGCTG	CTCTTTTAA	400	
AGCTGTGTAT	TCCCAGAATG	ATTCAAGTGA	TGGATTTCATG	ACACATCAAA	CATCATC	ATG AGC TCA	GAA TCC CAA	AGC AGT GTT	TTT AGT GAG	493	
ACG TCC AGT	ATT CAG ATC	AGT GAG ACT	GAT GTG GAG	GCT GAA C	GAG CTT GAA	LTC AAC	TTT CCT TTA	AGA AAA GAT	GCT CCA AAG	TCA AAA GTG	577
CTG GAG TTG	GAT ATA ACC	AAA GAT TAT	AGA GTT AGA	GCT CCA AGA	GAC CCT CPT	CGT CTG	TTG AGA TTG	ATG TGT TCT	TCT CAT CCT	TTC AN	661
LE L G	D I N T	K D Y R	V P R D	CT R L	CTG T	TTG L	AGA TTG	AGT TGT	TCT CAT	CCT TFC AN	68
TGT GAA GCT	CCA TTG ACT	ACA TTG TTT	GAG GAT GGC	TTT ATA TCT	TCA TCA GAT	TTA CAT	TTT GTT	AGA AAT CAT	GGG CPT GTT		745
CCT CAE	P L T T	L F E D	G D G G	F I T S	G V E G	L T S D	L H F V	R V R	N H G C	P V T	96
CCT CAT GTT	GAA GAT GAA	GAA ATT	ATG AAT TGG	ACA TFC TCT	GTG GAA GGT	TTA GTT	GAT GAA CAA	TTT GAA ATG	AAG TTA TCT		829
P H V E	D E E E	I M N W	T T T	T F S V	E G L L	V D V D	E P F E	M K L S		124	
GAT ATG ATC	CAA GAA TTT	CCT CAA TAC	ACT AAT TGG	ACA ATC ACT	TTA TGT TCT	GCA GGA AAT	CGT CGT AGG	GAA CAA AAT	CAA GTT	913	
D M I Q	E E F P	Q Y T N	P I T N	C I T L	C T G C	A G N R	R R K E	Q N Q V		152	
AAA AAG GGG	AAA GGT TTT	AAT TGG GGA	GCT GCT GGT	GTT TCA ACT	TCT TTA TGG	ACT GGT	GCA TTT TTA	TGG GAT	GTT ATA TCA	997	
K K G G	K K G F	F N W G	G A A G	A G A T	G T T G T	T G G A A G	G T G T	G A T C T	G C A A A G	G T	1083
AAA GCT AAA	CCT TCT AAA	AAG CAA AGA	ATA GTT GTT	TGG ATG GAA	GCT TGT GAT	GAT CCT	GCA AAA GGT	GCT TAT GGT	ACA TGT GTT	1083	
CCT TTA CCT	ATG GTT AAA	GAC CCT GAA	AGA ATA ATA	ATG ATG CTG	TGT TAT AAA	CAA AAT	GGT ATC TAT	TTG GAA CCT	GAT CAT GGT	1165	
CTT L P M	V K D P	E A R N	I M L C	T Y K Q	N G I Y	L T Y L	E P D H	G C V		236	
AAA CCC CTA	AGA ATC GTT	ATA CCA GGT	GTT ATT GGT	GGT AGA TCT	GTT AAA TGG	TTA AAG	AAA TTA	GTG GTT	AGT GAA CCA	1249	
K A P L	R I V I	T P G V	I T G G	G R S Y	V K W K	L V L V	K K K L	V G V S	D A A P	264	
AGT GAT AAT	TGG TAT CAT	TCT TTT GAT	AAT AGA GTT	TTA CCA ACT	ATG TGT ACA	CCT GAA	ATG GCT GCA	TCT GAT	GAA TCA	1333	
S D N W	Y H F F	D N R V	L P T M	V T P E	M A S D	E S W				292	
TGK AAA GAT	GAA AGA TAT	GCA ATT	TAT GAT	TTG AAT	CTA CAA	AGT ATT	ACG GTT	TAT CCT	GAA AAT	GAT GAA ACT	1417
GAT TCT TCA	AAT GAA GAT	GAA TTA TAC	AAT GTT AAA	GGT TTT GCT	TAT AAT	GGT GGT	GGT AAA	AGA GTT	GGT AGA	GTA GAA ACT	1501
D S S N	E D E D	E L Y N	V K G F	A Y A N	N G G G	G K R V	G G R V	E I		348	
TCA TTA GAT	GGT GGG AAA	ACT TGG AAA	CTA TGT GAA	ATA GAT TAT	CA GAA GAT	TCA TAT	CGT GAT	GCA GGC	TAT ATT	GAA TTA	1585
S L D D	G G K T	L W K L	T C E I	D Y P C	E D S Y	R D A G	Y I E L			376	
TAT GTT GGG	ACT ATA AAT	GTT TGT GAT	AGA R M S	TCT TGT T C	T T T TGT	TGG TGT	TTT TGG	AAT TTA CAA	ACT PAA	AGG GAA TTT	1669
Y G G C	T I N V	D R M S	C L C W	C T G C	F F W N	L Q I P	K R E L			404	
AAA GTT GCA	AAA GAT CTA	GTT GTT AGA	GCT ATG GAT	ATC TCA ATG	ACT GTT CAA	CAA AAT	GAT GAA	ATT AGA	TTT GAA	CAT CTA TTA	1753
K C A K	D L V V	R A M D	I S M T	V T Q P	R N M Y	W N V T	S			432	
ATG TTA AAT	AAT TGG TGG	TAT AGA ATT	GCT ATC CAA	CGT TCA CAA	AAT GAT GAA	ATT AGA	TTT GAA	CAT CTA	TTA GCT	1837	
M L N N	N W Y R	I A I Q	P G S Q	N D E I	R F E H	P A C T	L A A			460	
AAC AAA CCT	GGT GGT TGG	ATG AGT AGA	GTT AAA AAG	GAA GGT GGT	GAT TTA AAT	CCA AAT	TAT GGT	GAA AGA	GTT GCA AAT	1921	
N K P G	G G W M	D R V K	K E G G	G D I L	N P N Y	G E R V	A N			488	
GGG GAA CAA	GAA GAA GAA	CGT AAA CCA	TAT GTT GAT	GAA GAA TTA	CAA ATG ATT	TTA AAC	CCA GAA	ATC AAC	ATC ATC	2005	
G E Q E	E E E R	K P Y V	D E E L	Q M I L	N P D K	V N I I	I I			516	
ACA AAA GAA	CAA TTA GCT	CAA CAT TCA	AAT GAA CAG	GAC CCA TGG	TTT ATT GTT	AAA GGT	CAT GTT TTT	GAT GGT	GCT CCA	2089	
T K E Q	L A Q H	S N E D</

Figure 3. Influence of the nitrogen source on levels of *H.a.YNR1* and *H.a.YNI1* transcripts and NR and NiR proteins. Northern (A) and Western (B) analysis of cells grown in ammonium and transferred for 90 min to the indicated nitrogen sources (5 mM in all cases) are shown. In the Northern blot analysis the membrane was subsequently reprobed with *H.a.YNR1*, *H.a.YNI1* and *ScACT1*, which was used as internal control. The position of the 112 kDa marker is shown for each Western blot

Acknowledgements

We thank R. Serrano (Valencia, Spain) for much advice and for permitting us to carry out the construction and screening of the λ gt11 library in his laboratory, C. Gancedo (Madrid) for fruitful discussions, L. Rodriguez and T. Ruiz (Microbiology, La Laguna) for their help in Northern blot analysis and Neil Abrey for checking the English style. P.G. and G.P. were recipients of fellowships from Universidad de Los Andes, Mérida, Venezuela, and Gobierno de Canarias. This work was supported by grants from Dirección General de Investigación Científica y Técnica (P97-1484) and Gobierno de Canarias (PI1998/027) to J.M.S.

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