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 ZEMBL3 genomic DNA library. As probe, a 3.2 kb DNA fragment isolated from a Agt11 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, Rhodosporidium, 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 (YNAI) 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 λgt11, 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 μg 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–EcoRl and 1 kbp HindIII–XhoI DNA fragments, corresponding to *H.a.YNRI*, *H.a.YNII* and *Saccharomyces cerevisiae* *S.c.ACTI*, respectively, digoxigenin-labelled, were used as probe. The membrane was probed subsequently with *YNRI*, *YNII* and *S.c.ACTI*. 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.ACTI* 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 × SSC (1 × SSC = 150 mM NaCl and 15 mM nitrate) plus 0.1% SDS and twice at 60°C in 0.5 × 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 μg protein per lane. Proteins were transferred to PVDF membranes and immunodetection was carried out using either a 1:3000 dilution of an antiserum against purified *H. anomala* NR (Gonzalez and Siverio, 1992) or a 1:2000 dilution of an antiserum 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 λgt11 *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 \( \lambda \)EMBL3 \( H. \) anomala genomic library. From 10\(^6\) phages screened, 30 were found to be positive. One of these phages (\( \lambda \)PG15) was characterized and shown to contain a 13 kb insert. After digestion of this phage with EcoRI, part of the insert was released as five EcoRI–EcoRI 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 \( YNI1 \), 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)\(_2\)Cys\(_6\) 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 \( YNT1 \), \( YNI1 \), \( YNA1 \), \( YNR1 \) (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 \( YNI1 \) (Brito 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).
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H. a. YNR1 and H. a. YNI1 expression is several times higher in nitrate than in ammonium plus nitrate, the levels of NR and NiR proteins are only about twice as much in nitrate as in ammonium plus nitrate. This apparent discrepancy could be due to the detection systems for Northern and Southern blots, as well as to the fact that low levels of H. a. YNR1 and H. a. YNI1 transcripts account for the NR and NiR protein levels reached.

Preliminary Southern blot studies of this gene cluster in the genome (data not shown) are not consistent with the existence of only one copy of the cluster per genome. Further experiments are being carried out to check this possibility.

The cloning of H. a. YNR1 and H. a. YNI1 will allow us to progress further in the study of the complex regulation system of H. anomala NR, as well as of the nitrate assimilation regulation in this yeast.

**Figure 2.** Nucleotide sequence of the H. anomala region containing H.a.YNR1 and H.a.YNI1 genes and the deduced amino acid sequence. H.a.YNR1 and H.a.YNI1 comprise the nucleotides 457–3067 and 3538–6769, respectively. The FAD (44–441) haem (592–616) and molybdopterin co-factor (616–870) domains located in the NR (Avila et al., 1995) have been highlighted, whereas in the NiR those involved in the binding of the tetranuclear iron–sulphur centre and sirohaem (CX 5CXnGCX3C) and in the FAD and NADPH binding (GXGXXG) (Brito et al., 1996) are highlighted. In the 5′ non-coding region, TATA boxes and the GATA sites are also indicated.


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References


*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 S.c.ACT1, which was used as internal control. The position of the 112 kDa marker is shown for each Western blot.

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