***Neocallimastix frontalis* enolase gene, *enol*: first report of an intron in an anaerobic fungus**

R. Durand, M. Fischer, C. Rascle and M. Fèvre

Author for correspondence: R. Durand. Tel: +33 72 44 85 42. Fax: +33 72 43 11 81.

Laboratoire de Biologie
Cellulaire Fongique,
Centre de Génétique
Moléculaire et Cellulaire,
CNRS UMR 106, Bât. 405,
Université Lyon I, 69622
Villeurbanne Cedex,
France

A DNA clone containing a putative enolase gene was isolated from a genomic DNA library of the anaerobic fungus *Neocallimastix frontalis*. It was deduced from sequence comparisons that the enolase gene was interrupted by a large 331 bp intron. The enolase gene, termed *enol*, has an ORF of 1308 bp and encodes a predicted 436 amino acid protein. The deduced amino acid sequence shows high identity (71·5–71%) to those of enolases from the yeasts *Saccharomyces cerevisiae* and *Candida albicans*. The G + C content of the enolase coding sequence (43·8 mol%) is considerably higher than the G + C content of the intervening sequence (14·2 mol%) or the 5' and 3' non-translated flanking sequences (15·2 and 4·7 mol%, respectively). The codon usage of the *N. frontalis* enolase gene was very biased as has been found for the highly expressed genes of yeast and filamentous fungi. The gene has all the canonical features (polyadenylation signal, intron splicing boundaries) of genes isolated from aerobic filamentous fungi. Only one enolase gene could be detected in *N. frontalis* genomic DNA by Southern analysis with a homologous probe. RNA analysis detected a single enolase transcript of about 1·6 kb. When mycelium was grown on glucose, levels of enolase mRNA were markedly increased by comparison with enolase mRNA levels in mycelium grown on cellulose, suggesting that expression of the *N. frontalis* enolase gene was transcriptionally regulated by the carbon source.

Keywords: enolase, anaerobic fungus, rumen, *Neocallimastix frontalis*

INTRODUCTION

Although the main diet of herbivorous mammals is plant material, they are totally dependent on the micro-organisms in their digestive tracts to break complex polysaccharides into small molecules prior to absorption. Rumen bacteria, protozoa and fungi are the main contributors to the process of digestion. The anaerobic fungus *Neocallimastix frontalis*, first isolated from the sheep rumen by Orpin (1975), has been intensively studied for its large production of a wide range of glycoside and polysaccharide hydrolases (Hébraud & Fèvre, 1988). In axenic culture, *N. frontalis* can utilize several monosaccharides and plant cell-wall polymers as carbon sources (Li & Heath, 1993). The fermentation of glucose by *N. frontalis* proceeds via the Embden–Meyerhof–Parnas pathway (Marvin-Sikkema *et al.*, 1993). Enzyme activities leading to the formation of succinate, lactate, ethanol and

formate are associated with the cytoplasmic fraction, while the enzymes leading to the formation of the main fermentation products H₂, CO₂, acetate and the production of ATP are localized in microbodies identified as hydrogenosomes.

N. frontalis has been used as a model to clone the genes expressed during the hydrolysis of complex cell-wall polysaccharides. A differential screening of a cDNA library led to the isolation of several clones which were highly expressed during growth on cellulose. Several of these clones have been characterized by restriction analysis, partial sequencing and Northern blotting (Reymond *et al.*, 1991). Three cDNAs have been identified as clones encoding enzymes of the glucose fermentation pathway: phosphoenolpyruvate carboxykinase (Reymond *et al.*, 1991), hydrogenosomal β -succinyl-CoA synthetase (unpublished results) and enolase (this study).

Enolase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11) is a ubiquitous enzyme that catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the only dehydration step of the glycolytic

The EMBL/GenBank accession number for the nucleotide sequence reported in this paper is X80474.

pathway. Enolase genes have been cloned from different sources: the yeasts *Saccharomyces cerevisiae* (Holland *et al.*, 1981) and *Candida albicans* (Brett Mason *et al.*, 1993), the human malaria parasite *Plasmodium falciparum* (Read *et al.*, 1994), higher plants such as tomato and *Arabidopsis* (Van der Straeten *et al.*, 1991), and several animals such as rat and chicken. In avian and mammalian tissues, three types of enolase isoenzyme (α , β and γ) have been described, and the active form of the enzyme is a homodimer or a heterodimer as a consequence of the expression of genes in a tissue- and development-specific manner (Segil *et al.*, 1988). The enzyme from *S. cerevisiae* is by far the best characterized and its crystal structure has been determined (Lebioda *et al.*, 1989). Two structural genes, *ENO1* and *ENO2*, are expressed differentially in yeast cells grown on glucose and in cells grown on gluconeogenic carbon sources. *ENO2* is induced more than 20-fold in cells grown on glucose, whereas *ENO1* expression is similar in cells grown on glucose or on gluconeogenic carbon sources (Cohen *et al.*, 1986). Complex *cis*-acting regulatory sequences that bind distinct *trans*-acting factors have also been identified (Cohen *et al.*, 1987; Carmen & Holland, 1994).

In spite of the isolation of various cDNAs, very little information is available on gene organization in rumen anaerobic fungi. A 3 kb genomic clone carrying the endoglucanase gene *celB* from the related species *Neocalimastix patriciarum* was analysed by Zhou *et al.* (1994) and found to contain no intron. This paper describes the characterization of a genomic clone of the *N. frontalis* enolase gene. The primary structure of the enolase sequence of this filamentous fungus is compared with the yeast, plant and animal counterparts. To our knowledge, this is the first gene containing an intron isolated from an anaerobic fungus, so it was of interest to compare its organization to those of aerobic fungi. The overall organization of the enolase gene (intervening sequence, intron boundaries, polyadenylation sequence, codon usage) was found to be very similar to the organization of other genes from filamentous aerobic fungi.

METHODS

Strains, plasmids, phage and media. *N. frontalis* MCH3, isolated from sheep (Laboratoire de Microbiologie CRZV, INRA, Theix, France) was grown anaerobically at 39 °C for 2–5 d in culture flasks containing 500 ml synthetic liquid medium (Lowe *et al.*, 1987) supplemented with 1.5% (w/v) glucose or 0.6% (w/v) Avicel as carbon sources. *S. cerevisiae* strain MD4014C (a *his 3-11 his 3-15 leu 2-3 leu 2-112 trp1 ura2 2 μ* ⁺) provided by J. L. Souciet (IBMC Strasbourg, France) was grown at 30 °C in liquid medium: 10 g yeast extract l⁻¹, 20 g bacto-peptone l⁻¹, 20 g glucose l⁻¹. *Escherichia coli* strain C600 hfl [e14(*mcrA*)*supE44 thi-1 thr1 leuB6 lacY1 tonA21 hflA150::Tn10*] was used for the propagation of the λ 1149 cDNA bank and maintained on BBL medium (Maniatis *et al.*, 1982).

E. coli strain XL1-Blue from Stratagene [*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac(F'proAB lacI^qZAM15 Tn10 tet^r)*] was used for the construction of the genomic library and the Lambda Zap cDNA bank, and the propagation and ampli-

fication of hybrid pBluescript plasmids. All the XL-1 Blue cultures were on NZY medium (Maniatis *et al.*, 1982).

Construction of a genomic bank of *N. frontalis*. Mycelium of *N. frontalis* was harvested by centrifugation, frozen in liquid nitrogen and freeze-dried. The dried mycelium was ground in a mortar in the presence of liquid nitrogen and resuspended in 10 ml extraction buffer (0.2 M Tris/HCl, pH 7.6, 0.025 M NaCl, 0.025 M EDTA, 0.5% SDS, 400 mg ml⁻¹ proteinase K) per g dry material. After 30 min incubation at 65 °C, the contaminating proteins were extracted with chloroform and the DNA was precipitated from the upper aqueous phase by 0.7 vol. isopropanol. The DNA pellet was resuspended in 4 ml TE (Maniatis *et al.*, 1982) and precipitated in the presence of 11.25% (w/v) PEG 6000. The DNA pellet was collected by centrifugation (10 min, 12000 g) resuspended in TE and precipitated by 5 vols ethanol in the presence of 1 vol. 5 M ammonium acetate at -20 °C; after centrifugation (30 min, 12000 g) the DNA was resuspended in H₂O.

The low quantity of DNA recovered (1 mg per 20 g fresh mycelium) and the average size of the isolated DNA (20–30 kb) excluded a gradient size separation of restriction fragments. It was decided to use an insertion phage λ Zap II (Stratagene) to construct the genomic library. Genomic DNA (30 μ g) was digested to completion by an overnight incubation with 10 U *Xba*I (Boehringer) and purified by phenol/chloroform extraction and ethanol precipitation. The genomic fragments were ligated in the λ Zap arms and the recombinant phages were packaged *in vitro* according to the manufacturer's instructions.

Cloning and sequencing of the enolase gene. The λ Zap and λ 1149 cDNA libraries that were previously constructed by Raymond *et al.* (1991) and the genomic library were screened by colony hybridization with the [α -³²P]dCTP-labelled 1.3 kb *Eco*R1 incomplete enolase cDNA as a probe. This cDNA clone was previously isolated from a cDNA library (Raymond *et al.*, 1991) and was identified by sequencing as a truncated cDNA enolase clone. The genomic inserts of positive plaques were isolated by *in vivo* excision using the helper phage R408 (Stratagene). After localization of the *enol* gene on the genomic clone by Southern hybridization, using the incomplete cDNA clone as a probe, subclones of this genomic fragment were constructed by conventional methods (Maniatis *et al.*, 1982).

Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) on different templates: double-stranded DNA, single-stranded DNA produced from the pBluescript plasmid (Stratagene), and deleted clones produced by unidirectional *Exo*III digestion (Erase-a-Base, Promega) according to the manufacturer's instructions. When necessary, synthetic oligonucleotides were used to confirm the sequence. Sequence analyses were made by the Bisanse computer programs (Dessen *et al.*, 1990).

Southern analysis. *S. cerevisiae* chromosomal DNA was isolated as described by Johnston (1988). *N. frontalis* and *S. cerevisiae* DNAs were digested to completion with the appropriate endonucleases and the resulting fragments were fractionated by electrophoresis on a 0.8% agarose gel and transferred to nitrocellulose membranes (Schleicher & Schuell). DNA probes were labelled with [α -³²P]dCTP (111 TBq mmol⁻¹, Amersham) by use of a random-primed labelling kit (Pharmacia). The DNA on the blots was hybridized with DNA probes at 42 °C in the presence of 50% (v/v) formamide as recommended by the manufacturer.

After hybridization the membranes were washed twice for 20 min each time with 2 $\times$ SSC, 0.1% SDS and 0.2 $\times$ SSC, 0.1%

SDS. The washing temperature was 65 °C for the homologous probing (*N. frontalis* DNA blot) and 55 °C for the heterologous probing (*S. cerevisiae* DNA blot).

Northern analysis of *N. frontalis* RNA. Total RNA was isolated as described by Chirgwin *et al.* (1979). RNA (30 µg) were fractionated on a 1.2% (w/v) agarose gel in denaturing conditions (Maniatis *et al.*, 1982), and transferred to nitrocellulose membrane using the Northern procedure (Maniatis *et al.*, 1982). DNA probes were labelled with [α -³²P]dCTP by the random primer-labelling method (Feinberg & Vogelstein, 1983) using a Pharmacia kit. Hybridization was performed according to standard procedures (Maniatis *et al.*, 1982): hybridization was done at 42 °C in the presence of 50% formamide, 5 × SSC, 1 × Denhardt's solution, 20 mM sodium phosphate buffer, pH 6.8, 0.2% SDS and 200 µg yeast tRNA ml⁻¹; membranes were washed twice, for 20 min each time, at 42 °C with 2 × SSC, 0.1% SDS and 0.2 × SSC 0.1% SDS, and exposed to autoradiographic film. The membranes were then washed in 0.1 × SSC, 0.1% SDS at 95 °C and re-hybridized with an rRNA genomic probe (R.D., unpublished results). The resultant autoradiograms were analysed by densitometry using the Gel Reader program (Appligene).

RESULTS

Cloning and sequencing of *N. frontalis* enolase gene

A 1.3 kb cDNA clone, pPR6.1, previously isolated by Raymond *et al.* (1991), was sequenced and identified by sequence comparisons in the databases as an enolase clone. The deduced amino acid sequence of the cDNA clone had 69% identity with the amino acid sequence from position 110 to 432 of ENO1 from *S. cerevisiae* (Holland *et al.*, 1981) and the 5' part of the *N. frontalis* cDNA clone was missing. Several screenings of the two cDNA banks (λ 1149, λ Zap) did not lead to the isolation of a complete enolase cDNA clone. The partial cDNA clone pPR6.1 was used as a probe to screen a λ Zap genomic DNA library; from 44·10³ plaques, four positive plaques were identified which had the same restriction pattern. One clone pRDX6.5 contained a 6.5 kb DNA

insert and was used for further analysis. A number of overlapping restriction fragments from this clone hybridized to the cDNA pPR6.1 (data not shown) indicating that the genomic clone contained the enolase gene and large flanking sequences (Fig. 1). The nucleotide sequence of the 2.8 kb *Dra*I–*Hind*III fragment of the pRDX6.5 was determined (Fig. 2). The identification of the clone was confirmed by sequence analysis and comparisons with the NBRF database. The deduced amino acid sequence was identified as encoding an enolase on the basis of its high homology with the *S. cerevisiae* ENO1 and ENO2 sequences. By comparing the nucleotide and amino acid sequence with those from *S. cerevisiae*, one intron was assumed to be present in the coding region.

A large sequence (331 bp) from position +161 to +512 bp (relative to the first start codon) had intron-splicing boundaries matching the consensus sequences found in filamentous fungal genes (Gurr *et al.*, 1987). The splicing signals were GTAAGT and TAG for the 5' and 3' intron boundaries, respectively, compared with the GTANGT and PyAG fungal consensus sequences. An internal sequence, TACTTAAA (+306 to +313 bp), presumed to be involved in lariat formation, showed some similarity with the sequence PyGCTAACN of other fungal genes (Gurr *et al.*, 1987). Assuming this to be an intron, the deduced amino acid sequence of the coding region showed a high overall identity to those of *S. cerevisiae* ENO1 and ENO2 (71.5 and 71%, respectively). Therefore, we concluded that this gene does indeed encode an *N. frontalis* enolase; this gene was termed *enol*.

At the 3' end of the DNA sequence from bases +1742 to +1747, there is a putative AATAAA polyadenylation signal typical of higher eukaryotic genes (Fig. 2).

The G + C content of the coding sequence of enolase is 43.8 mol%, while the 5' and 3' flanking sequences and the intervening sequence have a much lower G + C content of 4.7, 15 and 14 mol%, respectively. This base composition is strongly divergent from that of an aerobic fungus as indicated by the data of statistical analysis of *Neurospora* genes, revealing that coding and non-coding sequences have a G + C content of 59 and 46 mol%, respectively (Edelmann & Staben, 1994).

The 1308 bp ORF encoded a polypeptide of 436 amino acids with a predicted molecular mass of 47 kDa. The deduced amino acid sequence of the pPR6.1 cDNA showed 100% identity to the corresponding part of the enolase gene (data not shown). The codon usage of the *N. frontalis* *enol* gene was highly biased, with only 36 codons used of the possible 61 sense codons (Table 1). The unused codons always end in A or G. If a purine is present in the third position, A is used in preference to G with the exception of lysine (100% AAG).

Comparisons of amino acid sequences

The alignment of the predicted enolase proteins from a representative range of organisms is shown in Fig. 3, and

Fig. 1. Restriction map of the *N. frontalis* enolase clone and sequencing strategies. The coding region of the *enol* gene and the partial enolase cDNA are indicated by shaded bars. Arrows indicate the positions and directions of sequencing. C, *Clal*; D, *Dra*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; X, *Xba*I.

The enolase sequences most similar to *N. frontalis* enolase are those of the yeasts *S. cerevisiae* and *C. albicans* (70–71 % identity). The dendrogram deduced from alignment of the enolase sequences (Fig. 4) demonstrated the presence of three families corresponding to fungal (yeast, *Candida*),

F TTT	0.0	S TCT	3.2	Y TAT	0.0	C TGT	0.5
F TTC	3.4	S TCC	1.6	Y TAC	2.8	C TGC	0.2
L TTA	1.8	S TCA	0.2	* TAA	0.2	* TGA	0.0
L TTG	0.0	S TCG	0.0	* TAG	0.0	W TGG	0.9
L CTT	5.7	P CCT	0.0	H CAT	0.0	R CGT	2.5
L CTC	0.9	P CCC	0.0	H CAC	1.4	R CGC	0.0
L CTA	0.0	P CCA	3.9	Q CAA	3.2	R CGA	0.0
L CTG	0.0	P CCG	0.0	Q CAG	0.0	R CGG	0.0
I ATT	4.4	T ACT	3.4	N AAT	0.7	S AGT	0.7
I ATC	1.1	T ACC	0.7	N AAC	4.8	S AGC	0.0
I ATA	0.0	T ACA	0.0	K AAA	0.0	R AGA	0.5
M ATG	2.1	T ACG	0.0	K AAG	8.5	R AGG	0.0
V GTT	6.0	A GCT	7.3	D GAT	5.0	G GGT	9.2
V GTC	1.4	A GCC	3.0	D GAC	1.6	G GGC	0.2
V GTA	0.0	A GCA	0.2	E GAA	6.7	G GGA	0.2
V GTG	0.0	A GCG	0.0	E GAG	0.0	G GGG	0.0

The effects of different carbon sources of the culture medium on expression of the *N. frontalis enol* gene were investigated by growing the fungus in the presence of glucose, crystalline cellulose (Avicel) or methylglucose (known as high-level inducers of secreted polysac-

<i>Arabidopsis</i>	MAT---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	66
Tomato	MAT---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	66
Maize	MA-VT-ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	67
<i>Drosophila</i>	MT---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
Chicken	S---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	64
Human β	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
Human γ	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
<i>N. frontalis</i>	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
<i>S. cerevisiae</i> 1	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
<i>S. cerevisiae</i> 2	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	65
<i>C. albicans</i>	MA---ITVWARGITDSRGMPTEVDLH-TAKGFRRAAVPSGASTGIEALLEDGG--SDYLGKGVKA	66

<i>Arabidopsis</i>	VGVNNTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	134
Tomato	VGVNNTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	134
Maize	VGVNNTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	135
<i>Drosophila</i>	VGVNNTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
Chicken	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	128
Human β	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
Human γ	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
<i>N. frontalis</i>	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
<i>S. cerevisiae</i> 1	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
<i>S. cerevisiae</i> 2	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	129
<i>C. albicans</i>	VEHINTIIPALI--GKDPDTGQTAIDNFMVHLELDTQNEHMGCKRKLGNALIAVSLAVKAGAVVGP	130

<i>Arabidopsis</i>	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	202
Tomato	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	202
Maize	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	203
<i>Drosophila</i>	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	197
Chicken	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	196
Human β	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	197
Human γ	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	197
<i>N. frontalis</i>	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	198
<i>S. cerevisiae</i> 1	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	199
<i>S. cerevisiae</i> 2	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	199
<i>C. albicans</i>	LYKHIAQLA--GNK--LVPVPAFVINGGSHAGKLANQFPHI LPVGAASFKEAHKIGAEVYTHLKV	200

<i>Arabidopsis</i>	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	271
Tomato	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	271
Maize	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	273
<i>Drosophila</i>	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	265
Chicken	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	263
Human β	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	264
Human γ	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	264
<i>N. frontalis</i>	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	266
<i>S. cerevisiae</i> 1	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	267
<i>S. cerevisiae</i> 2	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	267
<i>C. albicans</i>	KRYGQDANTVDEGGFAPNIGENKGLLELTA:EKAGYTGKVIIGDVAASEFYTK--DKYDILNFKEN	268

<i>Arabidopsis</i>	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	341
Tomato	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	341
Maize	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	343
<i>Drosophila</i>	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	333
Chicken	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	331
Human β	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	332
Human γ	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	332
<i>N. frontalis</i>	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	333
<i>S. cerevisiae</i> 1	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	335
<i>S. cerevisiae</i> 2	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	335
<i>C. albicans</i>	NNGSQIKSGDQALDKYKSFVSEPIVSIIDFPDQDMEHANTTEGTEVQIVGDDLVTPHRIATAI	338

<i>Arabidopsis</i>	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	411
Tomato	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	411
Maize	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	413
<i>Drosophila</i>	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	403
Chicken	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	401
Human β	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	402
Human γ	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	402
<i>N. frontalis</i>	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	406
<i>S. cerevisiae</i> 1	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	405
<i>S. cerevisiae</i> 2	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	405
<i>C. albicans</i>	AEKSCALLLRVQIGSVTESIAVWKSRAAGVWVSHRSGEDEDITADLVGLTQGIITGAPCRSE	408

<i>Arabidopsis</i>	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	444
Tomato	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	444
Maize	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	446
<i>Drosophila</i>	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	433
Chicken	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	433
Human β	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	434
Human γ	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	434
<i>N. frontalis</i>	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	436
<i>S. cerevisiae</i> 1	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	437
<i>S. cerevisiae</i> 2	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	437
<i>C. albicans</i>	RLAKYQHLRIIEELGSEATYAGVNF--RPVPEP--Y	440

Table 2. Percentage amino acid identity between different eukaryotic enolases

	1	2	3	4	5	6	7	8	9	10	11
1 <i>N. frontalis</i>		70	71	70	70	70	70	68	73	70	70
2 <i>C. albicans</i>			78	76	63	64	63	62	66	65	65
3 <i>S. cerevisiae</i> 1				95	61	61	60	61	65	64	62
4 <i>S. cerevisiae</i> 2					60	61	59	59	63	63	61
5 Tomato							87	90	66	70	71
6 Maize								86	65	67	69
7 <i>Arabidopsis</i>									66	69	70
8 Chicken										70	85
9 <i>Drosophila</i>											72
10 Human β											83
11 Human γ											

The NBRF accession numbers for enolases are indicated in brackets. 1, *N. frontalis*; 2, *C. albicans* (A45241); 3, *S. cerevisiae* ENO1 (NOBY); 4, *S. cerevisiae* ENO2 (NOBY2); 5, tomato (JQ1185); 6, maize (S16257); 7, *Arabidopsis thaliana* (JQ1187); 8, chicken (A23850); 9, *Drosophila melanogaster* (S07586); 10, human β form (S06756); 11, human γ form (NOHUG).

Fig 4. Phylogenetic relationships between eukaryotic enolases. The dendrogram was established from the multiple alignment seen in Fig. 3 using the parsimony method (Hein, 1990) from the TREEALIGN program of Bisanice (Dessen et al., 1990).

Fig 3. Amino acid alignment of *N. frontalis* enolase with eukaryotic enolases. Asterisks indicate identical residues in the 11 sequences. Dashes indicate gaps created by the TREEALIGN program of Bisanice (Dessen et al., 1990).

charidases), and maltose or lactose as carbon sources. The size of the *enol* transcript predicted from the knowledge of the coding sequence is about 1600 nt. Equal amounts of total cellular RNAs from the different cultures were analysed by Northern blotting using a probe for the *enol* coding sequence. The *N. frontalis* enolase mRNA was detected as an abundant single transcript of 1.6 kb (Fig. 6). The expression levels of *enol* were quite similar when the fungus was grown on glucose, methylglucose, maltose or lactose. However, the amount of *enol* mRNA from mycelium grown on crystalline cellulose (Avicel) was tenfold lower. In the presence of cellulose, the fungal biomass was quite similar to the biomass obtained on

other carbon sources. Furthermore, the levels of rRNA detected with the 16S rRNA probe were similar in the different culture conditions (data not shown).

DISCUSSION

In this paper we report the cloning and characterization of the first enolase gene (*enol*) from a filamentous fungus, *N. frontalis*. A cDNA clone was first isolated and identified by the high degree of similarity to the enolase genes from other organisms, but this 1.3 kb clone was found to be

Fig 5. Southern hybridization of *N. frontalis* enolase to restricted genomic DNAs. (a) *N. frontalis* genomic DNA was digested with *Bam*HI (1), *Xba*I (2), *Eco*RI (3), *Hind*III (4) or was undigested (5). The probe was the 2.4 kb *Xba*I–*Hind*III fragment overlapping the coding part and the 5' non-coding part of the genomic clone. (b) *S. cerevisiae* genomic DNA was digested with *Bam*HI (1), *Xba*I (2) or was undigested (3). The probe was the 1.3 kb partial enolase cDNA clone pPR6.1. The sizes of marker DNA fragments are indicated at the sides.

Fig 6. Analysis of *N. frontalis* enolase expression after growth on different carbon sources. (a) Total RNAs (30 µg per lane) from *N. frontalis* grown on glucose (1), Avicel (2), methylglucopyranoside (3), maltose (4) and lactose (5) were separated on agarose gel. (b) Identification of the *enol* gene transcript was obtained by probing total RNAs with the partial cDNA enolase clone pPR6.1. The numbers under each lane indicate the level of expression obtained by densitometric analysis after re-probing the blot with a rRNA probe. The ratio of the signal for *enol* mRNA to that for rRNA was taken as 100% for glucose-grown mycelium.

incomplete: the part of the sequence encoding the N-terminal amino acids of the expected protein was missing (Reymond *et al.*, 1992). A large number of positive clones

were isolated from several rounds of hybridization of cDNA libraries, but all the analysed clones were the same size. This feature may be common to enolase cDNAs, as clones isolated from other organisms appeared to be incomplete. In *C. albicans*, Franklin *et al.* (1990) have isolated a partial cDNA clone, and in *P. falciparum*, Read *et al.* (1994) have isolated a 1.3 kb enolase cDNA and used the 5' rapid amplification of cDNA ends (RACE) technique to isolate a complete cDNA clone. It is possible that in these different organisms the primary structure of the enolase genes leads to the formation of strong secondary structures in the mRNA, producing a pause in the reverse transcription reaction during cDNA synthesis.

Comparison of the predicted amino acid sequence of the *N. frontalis* enolase with enolase sequences from other eukaryotes shows a high degree of evolutionary conservation (Fig. 3). Homology has been retained throughout the protein and 187 amino acids arranged in short tracts are conserved in the eleven enolase sequences analysed. Enolase sequences from *Bacillus* and *P. falciparum*, sharing 53.5 and 63% homology, respectively, with *N. frontalis* enolase, were excluded from the alignment studies.

N. frontalis possesses a single enolase gene as confirmed by Southern blotting (Fig. 5), and the catalytically active enzyme must therefore be a homodimer of the enolase gene product. In higher eukaryotes the expression of enolase isoenzymes are specific for a cell type or a developmental stage of the organism (Segil *et al.*, 1988). In *S. cerevisiae*, the expression of *ENO1* ensures that glycolysis continues when the environmental conditions do not favour expression of *ENO2* (McAlister & Holland, 1982). However, *C. albicans* (Brett Mason *et al.*, 1993) and *N. frontalis* appear to have only a single enolase gene. These two organisms are adapted to special ecological niches, the human body and the rumen, where the environmental conditions are relatively constant and this may explain the pattern of expression of the enolase gene.

Studies of DNA base composition from *N. frontalis* and other anaerobic fungi have demonstrated these organisms to have the most A + T-rich genome of any organisms identified so far (Billon-Grand *et al.*, 1991; Brownlee, 1994). Evidence was presented that the A + T-rich sequences are widely spread in the genome as repeated sequences and spacer regions of DNA flanking the highly repeated rRNA coding regions. From our present study on enolase gene organization and from the characterization of several cDNA clones of *N. frontalis*, phosphoenolpyruvate carboxykinase (Reymond *et al.*, 1992), β -succinyl-CoA synthetase (T. H. C. Brondijk, personal communication), xylanase 1 and xylanase 2 (R. D., unpublished results), it could be postulated that the coding sequences of the genes from *N. frontalis* are separated in the genome by long stretches of very A + T-rich flanking sequences (less than 15 mol% G + C). Moreau *et al.* (1982) reported a general pattern of organization of DNA in eukaryotic genomes whereby A + T-rich 'linker' sequences systematically punctuate regions that contain coding sequences and might be

involved in DNA functional organization at the chromosome level. Brownlee (1994) pointed out that the very A + T-rich genome of *N. frontalis* might be expected to affect the amino acid composition of the encoded proteins. Clearly the amino acid sequence comparisons with eukaryotic enolases (Fig. 3) demonstrate that this is not the case, as 269 residues (63 % of the total amino acids) are invariant or similar.

The codon usage observed in *N. frontalis enol* gene was biased since only 36 codons out of the 61 sense codons were used. This codon preference (Table 1) is very close to that of the enolase from *C. albicans* (Sundstrom & Aliaga, 1992), where 35 codons were used. The only minor differences were that the *N. frontalis* gene utilized AAT for Asn, GAT for Asp, CAT for His, TTT for Phe and TAT for Tyr more frequently (Table 1), whereas in *C. albicans*, the last T was replaced by a C in the same codons except for Leu (TTG). However, the *P. falciparum* enolase showed a lower codon usage bias as 46 codons were used. The codons always ended in A, reflecting the 36 mol % G + C content of the coding sequence. In the *p190* gene of *P. falciparum* the coding area had an A + T content of 74 mol % (Mackay, 1987). In the filamentous fungi *N. crassa* and *Aspergillus nidulans*, highly expressed genes tend on the whole to show more marked codon bias than those expressed at a low level (Gurr *et al.*, 1987). The high degree of codon bias for *N. frontalis enol* is consistent with it being a highly expressed gene using the most abundant isoacceptor tRNAs.

The *enol* gene presents other features characteristic of filamentous fungal genes. The enolase gene was interrupted by a 331 bp long intervening sequence. The introns of filamentous fungi are generally short, on average less than 100 bp (Gurr *et al.*, 1987), although in *Neurospora crassa* some are as large as 691 bp (Edelmann & Staben, 1994). The *enol* gene intron boundaries followed the consensus sequences GTANGT and PyAG determined for filamentous fungal genes (Gurr *et al.*, 1987). All these results show that the remarkably low overall G + C content in an anaerobic fungus does not affect the gene organization which is similar to that in aerobic fungi.

N. frontalis enolase mRNA was detected as an abundant transcript in mycelia grown on glucose or on the disaccharides maltose or lactose (Fig. 6). However, when the fungus was grown on glucose the level of the enolase transcript was tenfold higher than that detected in cellulose-grown mycelia. These results are reminiscent of the 20-fold glucose-mediated induction of the *S. cerevisiae* *ENO2* gene (McAlister & Holland, 1992). In *C. albicans*, the level of enolase transcripts was sixfold higher in glucose-grown cells than in cells grown on a gluconeogenic carbon source (Brett Mason *et al.*, 1993).

Concluding remarks

The cloning and characterization of the *enol* gene from a genomic clone containing 5' flanking sequences greater than 1.5 kb will allow the characterization of the enolase gene promoter. Promoters of highly expressed genes such as 3-phosphoglycerate kinase (Takaya *et al.*, 1994) have

been useful for the construction of transformation vectors. Preliminary experiments have shown that the transformation of oomycetes or *N. frontalis* are unsuccessful using regulatory sequences from higher fungi. The presence of homologous promoting sequences in the transformation vectors should allow the efficient transformation of anaerobic fungi.

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