Subunit VII of ubiquinol:cytochrome-*c* oxidoreductase from *Neurospora crassa* is functional in yeast and has an N-terminal extension that is not essential for mitochondrial targeting

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cDNA clones encoding subunit VII of the *Neurospora crassa* *bc*₁ complex (ubiquinol:cytochrome-*c* oxidoreductase), which is homologous with subunit VIII of the complex from yeast (encoded by *QCR8*), were identified on the basis of functional complementation of a yeast *QCR8* deletion strain. The clones contain an open reading frame encoding a protein with a calculated molecular mass of 11.8 kDa. The N-terminal eight residues of the amino acid sequence deduced from the cDNA clones are absent from the mature protein, as revealed by direct sequencing of the isolated protein. To investigate the potential role of the N-terminal octapeptide in mitochondrial targeting, constructs were made encoding the precursor and the mature form of subunit VII from *Neurospora*. Incubation of isolated mitochondria with the two proteins revealed that the N-terminal extension of the precursor is removed on import. However, the

presequence does not encode information for targeting, as the proteins encoded by both constructs can be imported into isolated mitochondria with equal efficiency. In contrast, the octapeptide seems to have functional importance: the defect in the yeast *qcr8*-null mutant is not complemented on transformation with the construct encoding mature subunit VII from *N. crassa* in a single-copy plasmid. We therefore speculate that the N-terminal extension plays a role in intramitochondrial sorting of *N. crassa* subunit VII. This is supported by the fact that the subunit VII precursor is processed by a protease other than the general mitochondrial processing peptidase. Interestingly, the presequence of *N. crassa* subunit VII has an amino acid composition similar to the octapeptides cleaved off by the mitochondrial intermediate peptidase.

INTRODUCTION

Ubiquinol:cytochrome-*c* oxidoreductase (complex III or *bc*₁ complex; ubiquinol:ferricytochrome-*c* oxidoreductase, EC 1.10.2.2.) is an essential component of the respiratory chains of mitochondria and bacteria. It transfers electrons from ubiquinol to cytochrome *c*, coupled to proton translocation across the mitochondrial inner membrane or the prokaryotic plasma membrane [1]. Three subunits: cytochrome *b*, cytochrome *c*₁ and the Rieske iron-sulphur protein (RIP) are essential and sufficient for the reaction mechanism of the *bc*₁ complex. Some prokaryotes, like *Paracoccus denitrificans*, have a ubiquinol:cytochrome-*c* oxidoreductase consisting of only these three subunits [2]. The equivalent complex in mitochondria consists of these three plus six to eight additional subunits [1], whose function is still not clearly understood.

One of these additional subunits is the product of the *QCR8* gene (*QCR8p*) in *Saccharomyces cerevisiae*. The protein has a calculated molecular mass of 11 kDa [3] and is homologous with the 9.5 kDa subunit of the respiratory enzyme complex from bovine heart [4]. *QCR8p* is thought to be in close association with cytochrome *b* and subunit VII, and has been proposed to form, together with these two subunits, a subcomplex as a first

step in the assembly of the enzyme [5]. Disruption of the *QCR8* gene [6] results in the loss of *bc*₁ complex activity and prevents growth on non-fermentable carbon sources. The *QCR8*-null mutant displays decreased levels of apo-cytochrome *b*, RIP and subunit VII; holocytochrome *b* is not detectable. *QCR8p* has recently been shown to contribute to the quinol oxidation centre of the ubiquinol:cytochrome-*c* oxidoreductase [3], which is consistent with the identification of its bovine heart homologue as part of the binding site of azido-quinone analogues [7,8].

Computer screening of databases has led to the identification of homologues of *QCR8p* in a wide variety of organisms. The protein family displays a low degree of sequence conservation, but sequence alignments have allowed the identification of a number of conserved residues and the formulation of a characteristic signature [9]. To assess the functional equivalence of the different evolutionary variants we have followed an approach involving the construction of site-directed mutants, based on the consensus sequence, the construction of hybrid complexes containing *QCR8p* homologues in a *S. cerevisiae* background and a study of their enzymic properties. Here we apply this approach to the study of the *QCR8p* homologue from *Neurospora crassa*.

The *bc*₁ complex from *N. crassa* consists of nine subunits [10,11]: cytochrome *b*, cytochrome *c*₁, RIP, the two so-called

Abbreviations used: *bc*₁ complex, ubiquinol:cytochrome-*c* oxidoreductase; MIP, mitochondrial intermediate peptidase; MPP, mitochondrial processing peptidase; *QCR8*, gene for subunit VIII of ubiquinol:cytochrome-*c* oxidoreductase; *QCR8p*, product of *QCR8*; RIP, Rieske iron-sulphur protein.

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'core' proteins and four subunits with molecular masses of 14 kDa or less. All cytoplasmically made subunits except the 14 kDa polypeptide are synthesized as precursors with higher molecular masses and are proteolytically processed after their transfer into mitochondria [11,12]. Here we report the characterization of subunit VII of the protein complex from *N. crassa*, which is homologous with the subunit VIII protein of the *bc*₁ complex from *S. cerevisiae*. cDNA clones were isolated on the basis of functional complementation of the *S. cerevisiae* *qcr8*-null mutant. Results are presented on the analysis of heterologous *bc*₁ complexes consisting of nine *S. cerevisiae* subunits supplemented with subunit VII from *N. crassa* and on the import pathway of this subunit into mitochondria.

MATERIALS AND METHODS

Strains and growth media

Escherichia coli strain DH5 α [*recA*, (*r*_k⁻, *m*_k⁺) λ ⁻] was used for the propagation of recombinant DNA constructs. *E. coli* transformants were grown in YT medium [1% (w/v) yeast extract/1.6% (w/v) bactotryptone/0.5% (w/v) NaCl] containing 100 μ g/ml ampicillin.

Isolation of clones by functional complementation

The yeast/*E. coli* expression vector pYPGE15 [13] containing a cDNA library of *N. crassa* was recovered out of the λ PG15 cloning vehicle as described in [13], by infection of a Cre-expressing *E. coli* strain BNN132 {JM107[*AKC(kan^r, cre)*]. Both λ PG15 and BNN132 were purchased from the Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City, U.S.A.).

The *S. cerevisiae* (subunit VIII)-null mutant DLL80 (α , *his3*, *ura3*, *LEU::qcr8*) [14] was transformed as described in [15]. Transformants were selected on minimal media containing 0.67% yeast nitrogen base without amino acids (Difco)/2% (w/v) glucose/2% (w/v) agar, supplemented with histidine (20 mg/ml) or uracil (20 mg/ml). The respiratory capacity of the transformants was tested on solid media containing 1% (w/v) yeast extract, 1% (w/v) bactopeptone, 2% (v/v) glycerol and 2% (v/v) ethanol.

DNA manipulations

Isolation of plasmid DNA from yeast cells was performed as described in [16] and from bacterial cells as described in [17]. Positive clones were transferred to pBluescript SK (Stratagene) and the DNA sequence on both strands was determined as described in [18]. Restriction and other enzymes used in DNA manipulation were purchased from Boehringer, Biolabs and Sigma and used as recommended by the manufacturers. Radioactive chemicals were obtained from Amersham. All other chemicals were of the highest purity available.

Construction of plasmids

An 840 bp *HindIII*–*SalI* fragment carrying the *S. cerevisiae* *QCR8* was cloned from plasmid pUC18-H11S [3] into the multiple cloning site of the centromeric *E. coli*/*S. cerevisiae* shuttle vector pRS316 [19], giving pRS316HQ8S. From the last plasmid an 870 bp *BamHI*–*SalI* fragment was subcloned into the pRS313 vector [19], giving pRS313BQ8S. Site-directed mutagenesis was performed to create an *NcoI* site at the first codon of the *S. cerevisiae* *QCR8*, resulting in the pRS313NQ8 construct. Making use of a natural *Clal* site 27 bp after the stop codon, it

A

B

N-terminal sequences:

N.c.QCR8 -ori - p MRPTQTMLGGGGGAPI...
N.c.QCR8 -1st - p MGPTQTMLGGGGGAPI...
N.c.QCR8 -2nd - p MVGGGGGAPI...

Figure 1 Gene constructs used in the complementation assays and the N-terminal sequences of the *N. crassa* *QCR8p* homologues

(A) The yeast *QCR8* gene was cloned with its own promoter and terminator region. An *NcoI* site was introduced at the ATG of the *S. cerevisiae* *QCR8*, allowing the *NcoI*–*Clal* fragment containing only the gene to be removed and replaced by a homologous counterpart. The introduction of an *NcoI* and a *Clal* site by PCR at the ATG and after the stop codon respectively made it possible to clone the *N. crassa* *QCR8*. Two different constructs were made: one with the *NcoI* site on the first ATG (–2) and another with the *NcoI* site on the second ATG (17). Note that the codon after the ATG has to start with a G owing to the creation of the *NcoI* site, consequently changing the amino acid residue at position 2. (B) The three different subunits VII used in this study had different N-termini: *N.c.QCR8*-ori-p is the original protein derived from the cDNA library; *N.c.QCR8*-1st-p is the protein derived from the *N.c.QCR8*-1st construct, and *N.c.QCR8*-2nd-p is the protein derived from the *N.c.QCR8*-2nd gene construct.

was possible to replace the *NcoI*–*Clal* fragment carrying the *S. cerevisiae* *QCR8* in pRS313NQ8 with homologous genes from other organisms (*N. crassa* in this paper or *Schizosaccharomyces pombe* in [20]) as shown in Figure 1. The heterologous genes placed under the control of the *S. cerevisiae* *QCR8* promoter were subcloned as a *HindIII*–*SalI* fragment into the multicopy vector YEplac195 [21]. It should be kept in mind that for the creation of a *NcoI* site the first base after the ATG has to be a G, possibly resulting in a change in the second amino acid.

Doubling times, isolation of mitochondria, spectral analysis and *bc*₁ complex assay

Doubling times were determined by inoculating 50 ml lactate medium [0.5% yeast extract/0.2% (w/v) magnesium sulphate/0.6% (w/v) ammonium phosphate/2% sodium lactate (70% w/v)/1.3% lactic acid (75% w/v) (pH 4.5)] with yeast cells from an overnight culture to give a starting *D*₆₀₀ of about 0.1. The growth proceeded at 28 °C to the stationary phase. At different time points, samples were taken to measure the attenuation at 600 nm on a Zeiss spectrophotometer. Mitochondria were isolated from cells grown in lactate medium as described earlier [22]. Spectral measurements were performed at room temperature in an Aminco dual-wavelength spectrophotometer model DW2.

Concentrations of cytochromes were determined with the following molar absorption coefficients and wavelength pairs for the reduced minus oxidized proteins: $\epsilon_{605-630} = 24.0 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for cytochrome *aa*₃ [23], $\epsilon_{550-540} = 20.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for cytochromes *c* + *c*₁ [24] and $\epsilon_{562-575} = 28.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for cytochrome *b* [24]. The ubiquinol:cytochrome-*c* oxidoreductase assay was performed spectrophotometrically at 30 °C by measuring the reduction of 18 μM horse-heart ferricytochrome *c* at 550–540 nm by 25 μM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2). The buffer used contained 2 mM EDTA, 0.5 mM potassium cyanide and 20 mM potassium phosphate, pH 7.4, to obtain maximal activity with horse-heart cytochrome *c* as acceptor [25]. Spectral and kinetic data were analysed with Popspec software on an Atari 1040ST computer coupled to the spectrophotometer via the Poptronics 4 channel analogue-to-digital converter.

Isolation of the *bc*₁ complex from *N. crassa* and N-terminal sequencing of subunits VI and VII

Ubiquinol:cytochrome-*c* oxidoreductase from *N. crassa* was purified as outlined in [26]. The subunits of the isolated enzyme were separated by Tricine/SDS/PAGE [27] and blotted on Immobilon membranes (Millipore, Bedford, MA, U.S.A.). Proteins were stained with Ponceau S and both the 11.5 and 14 kDa subunits were cut out and analysed directly by cyclic Edman degradation with an Applied Biosystems pulsed-liquid-phase sequencer.

Import and processing of *N. crassa* subunit VII into isolated mitochondria *in vitro*

Mitochondria from *N. crassa* were isolated in accordance with the protocol published in [28]. Transcription *in vitro* was performed with a transcription kit (Stratagene, Heidelberg, Germany) in accordance with the supplier's instructions; translation *in vitro* was performed in the presence of [³⁵S]methionine with reticulocyte lysate (Promega, Madison, WI, U.S.A.). Import reactions were performed as described in [29]. Import of proteins into mitochondria was demonstrated by treatment of the organelles with proteinase K (0.5 mg/ml) for 20 minutes at 10 °C. Proteins were analysed by Tricine/SDS/PAGE [27] and autoradiography. Processing of radiolabelled translation products *in vitro* was performed in a buffer containing 20 mM Tris/HCl, pH 8.0, 25 mM NaCl, 0.6% Triton X-100, 0.1 mM MnCl_2 and 0.1 mM MgCl_2 . The processing peptidase from potato was isolated as reported previously [30,31]. Processing reactions were performed for 1 h at 25 °C and processing products were analysed by SDS/PAGE and autoradiography.

RESULTS

Cloning of *N. crassa* *QCR8*

The *N. crassa* cDNA library was transformed into the *S. cerevisiae* *QCR8*-null mutant DLL80. Of the approx. 10000 transformants that were replica-plated on ethanol/glycerol medium, 11 complemented the respiratory deficiency, thus permitting growth. Plasmid DNA was isolated and amplified in *E. coli*. After re-transformation to DLL80, only eight clones retained the ability to restore growth on ethanol/glycerol medium. *Eco*RI-*Xho*I fragments (see [13]) from these clones were isolated, subcloned into pBluescript SK and sequenced. Three clones with small differences in length were found. The largest clone contained an insert of 584 bp and was sequenced on both strands (Figure 2). The insert contained an open reading frame of 321 bp

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-127 GCTCTAGAACTAGTGGATCCCCCGGCTGCAGGA
-93 ATTCGGCAGGAGGAACCTTACGACGACTGCGT
-59 CCTACCCAGACCCGATTGATACCCCCATAGCG
-25 CGTTTCGCACACACATATTTCAAG
1 ATG CGT CCT ACT CAG ACC ATG CTC
M R P T Q T M L
25 GGT GGC GGC GGC GGT GCC CCC ATC
G G G G G A P I
49 GGC AAG CAC AAC CAC TAC CTT GGT
G K H N H Y L G
73 GGC TGG GGT AAC TTT GGT GGC ATG
G W G N F G G M
97 AAG CAG AGG GGC ATC ATC TCC TAC
K Q R G I I S Y
121 GGT ATC AGC CCT AAC CGC CAG AAC
G I S P N R Q N
145 CCC CTC GCC GGC ACT GCC CAC GAC
P L A G T A H D
169 GCC GTT TTC AAC ACC TTC AGA CGT
A V F N T F R R
193 GTC AGC TCC CAG TTC CTC TAC TGG
V S S Q F L Y W
217 GCC CCC TCC CTT GTT GCC GGC TAC
A P S L V A G Y
241 TAC ATC ATG AAC TGG GCT ATC GAG
Y I M N W A I E
265 CGG AAC CAC TAC CTC AAC TCG AAG
R N H Y L N S K
289 GCC GGT CGT GCT GAG TTC GCT GGC
A G R A E F A G
313 CAG GAG GAG TAA
Q E E *
325 GCGATGGACTTTACGATACCTTGGGACCGGAACA
359 GAGTGCAGTTCCCGAGAGAGAGAACTAGTACTCA
393 ATAGACTAACCTGTATATACCATCTTTATTCC
427 ACGCGGTTCGAAAAAATAAAAAAAAAA

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Figure 2 cDNA sequence and deduced amino acid sequence of the *QCR8* gene encoding subunit VII of the *bc*₁ complex from *N. crassa*

The stop codon is indicated with an asterisk.

coding for a protein of at most 107 amino acid residues with a calculated molecular mass of 11.8 kDa. The presence of an in-frame stop codon upstream of the 5'-most ATG codon suggests that the reading frame is complete and that it encodes the entire protein. A second ATG codon, located six codons downstream of the first, represents a second potential translational start site. The 3' non-coding region comprises 127 bp and is followed by a short poly(A) tail. Alignment of the deduced amino acid sequence for the subunit of the *bc*₁ complex from *N. crassa* with the primary structures of the corresponding proteins from other organisms (Figure 3) revealed a sequence identity between 20 and

Figure 3 Multiple sequence alignment of *QCR8p* homologues

Sequences were aligned with the GCG PileUp program (gap weight 3.00, gap length weight 0.10) [59]. An asterisk under a column indicates that the highlighted residues in that column are conserved in all sequences; highlighted residues in columns without an asterisk appear in at least 50% of sequences. Gaps are indicated by dots. Sequence origins: 1, *Solanum tuberosum* [34]; 2, *Oryza sativa* ([9], and GenBank accession number D48989); 3, *Arabidopsis thaliana* [9]; 4, bovine heart [33]; 5, human (GenBank accession number T47406); 6, *S. cerevisiae* [6]; 7, *K. lactis* [32]; 8, *Schizosaccharomyces pombe* [20]; and 9, *N. crassa* (this study).

Figure 4 Analysis of the growth characteristics of the *S. cerevisiae qcr8*-null mutant (DLL80) transformed with the *N. crassa QCR8* homologue on a single-copy vector

The DLL80 mutant was transformed with the *S. cerevisiae QCR8* gene (ScVIII), the *N.c.QCR8*-1st gene construct (Nc1stVII) and the *N.c.QCR8*-2nd gene construct (Nc2ndVII), all on a single-copy plasmid. Cells were first grown on medium containing glucose and were subsequently replicated on ethanol/glycerol medium.

48%. The addition of the *N. crassa* and human sequences to the list of homologues results in some differences in the alignment compared with [9]. However, the most striking feature of this alignment is the presence in *N. crassa* of a short N-terminal extension that is absent from all its counterparts. As there is a second ATG codon located six codons downstream of the potential initiator codon, the translation start remains to be established.

Construction of a heterologous *bc*₁ complex containing the *N. crassa QCR8p* homologue, and complementation studies in *S. cerevisiae* DLL80

To quantify the extent of functional complementation of DLL80 by the *N. crassa QCR8* gene, the protein-coding sequence was cloned under control of the *S. cerevisiae QCR8* promoter in the centromeric *E. coli/S. cerevisiae* shuttle vector pRS313 [19]. To elucidate the start of translation, two different constructs were made by the introduction of an *Nco*I site either at the first ATG codon (*N.c.QCR8*-1st) or at the second ATG codon (*N.c.QCR8*-2nd) (see Figure 1). As a consequence of the creation of an *Nco*I site the second residue was not Arg or Leu but Gly or Val respectively. The disruption of the *S. cerevisiae QCR8* gene could be partly suppressed by the *N.c.QCR8*-1st gene construct as shown in Figure 4, whereas the *N.c.QCR8*-2nd construct could not complement the *QCR8*-null strain to a detectable level, unless overexpressed in a multicopy vector (YEplac195). Consequently the N-terminal extension of the *N. crassa* homologue of the yeast subunit VIII of the *bc*₁ complex is important for functional complementation and therefore most probably forms part of the translation product *in vivo*. Translation initiation presumably starts at the first ATG codon. However, the extension is most probably absent in the mature protein, as most subunits of the *bc*₁ complex from *N. crassa* were shown to be processed on

Figure 5 Purification of the *bc*₁ complex from *N. crassa* and determination of the N-terminal sequences of the 14 and 11.5 kDa proteins

Isolated *bc*₁ complex from *N. crassa* was analysed by SDS/PAGE and the subunits of the protein complex were revealed by staining with Coomassie Blue. The molecular masses of standard proteins are shown at the left, in kDa. The subunits of the *N. crassa bc*₁ complex are core I (apparent molecular mass 50 kDa), core II (46 kDa), cytochromes *b* and *c*₁ (32 and 30 kDa) RIP (22 kDa) and four small proteins (14, 11.5, 11 and 6 kDa). The N-terminal sequences of the 14 and 11.5 kDa subunits are shown at the right.

import into isolated mitochondria [12]. Possibly the N-terminus of the protein comprises information to direct the polypeptide to its proper site of location.

N-terminal sequence of the mature subunit VII from *N. crassa*

To define mitochondrial targeting information, *N. crassa* subunits VI–IX were isolated and directly sequenced. Isolated *bc*₁ complex from *N. crassa* comprises four small subunits with apparent molecular masses of 14, 11.5, 11.0 and 6 kDa (Figure 5). N-terminal amino acid sequencing of these proteins revealed that the primary structure of the 11.5 kDa protein (subunit VII) corresponds to the sequence deduced from the *N. crassa QCR8* homologue (Figure 5). The mature subunit VII starts at position 9 of the sequence derived from the cDNA. We therefore concluded that the protein has a short presequence comprising eight amino acids. The calculated molecular mass of the mature subunit VII is 10.8 kDa, which corresponds well to the apparent molecular mass on analysis by SDS/PAGE. The 14 kDa protein of the *bc*₁ complex from *N. crassa* (subunit VI) exhibited sequence similarity to the yeast *QCR7* product (Figure 5).

Import of subunit VII into isolated mitochondria from *N. crassa*

The cleavable presequence of *N. crassa* subunit VII is extremely short and has an atypical amino acid composition for mitochondrial targeting sequences. To clarify the role of the presequence in mitochondrial targeting, import experiments were performed *in vitro* with either the original precursor (Figure 6B), the *N.c.QCR8*-1st product, which was identical with the original precursor except for a replacement of Arg by Gly at position 2 (Figure 6C), or the *N.c.QCR8*-2nd product, which very much resembled mature subunit VII (Figure 6D). The RIP from *N. crassa* was used as a control (Figure 6A). All of the transcription

Figure 6 Import of subunit VII from *N. crassa* into isolated mitochondria

The original subunit VII (*N.c.QCR8-ori*) and the *N.c.QCR8-1st* and *N.c.QCR8-2nd* products were synthesized by translation *in vitro* in the presence of [35 S]methionine (**B, C, D**). Radiolabelled *N. crassa* RIP was used as a control for the import experiments (**A**). The translation products were analysed by SDS/PAGE and fluorography. Lane 1, untreated translation products; lane 2, translation products after treatment with proteinase K; lane 3, translation products on incubation with isolated mitochondria; lane 4, translation products after incubation with isolated mitochondria and subsequent treatment with proteinase K; lane 5, translation products after incubation with isolated mitochondria and subsequent treatment with proteinase K in the presence of 1% (w/v) Triton X-100. The numbers at the left indicate the apparent molecular masses (in kDa) of the proteins as determined by comparison with the masses of standard proteins.

products could be translated, as shown in lane 1 of Figure 6. As expected, the original and the *N.c.QCR8-1st* products had apparent molecular masses of 12.5 kDa, whereas the *N.c.QCR8-2nd* product ran at 11.5 kDa. The sensitivity of the radiolabelled proteins towards proteinase K was monitored, as shown in lane 2. On incubation of the translation products with isolated mitochondria (lane 3) the original subunit VII (*N.c.QCR8-ori*) and the *N.c.QCR8-1st* products were shortened to the size of the *N.c.QCR8-2nd* product (11.5 kDa), which was not processed. Import of the radiolabelled proteins into mitochondria was proved by treatment of the organelles with proteinase K. The processed forms of the original subunit VII and the *N.c.QCR8-1st* products were protected against degradation by proteinase K (lane 4). Surprisingly the *N.c.QCR8-2nd* product, lacking the cleavable N-terminal extension, was also efficiently imported. On incubation of the organelles with proteinase K in the presence of Triton X-100, all radiolabelled proteins became susceptible to

Figure 7 Processing *in vitro* of subunit VII from *Neurospora*

The original subunit VII (lane 1), the *N.c.QCR8-1st* and *N.c.QCR8-2nd* products (lanes 2 and 3) and the *N. crassa* RIP (lane 4) were incubated with isolated MPP from potato. The proteins were analysed by SDS/PAGE and fluorography. The molecular masses of standard proteins are shown at the left, in kDa. Whereas the precursor for the RIP (25 kDa) is efficiently processed to its mature size (22 kDa), the three *QCR8* products are not shortened on incubation with the processing peptidase.

proteolytic degradation (lane 5). We therefore conclude that the N-terminal eight residues of subunit VII are removed on import of the precursor into mitochondria, and that information essential for mitochondrial targeting is not located in this extension but within the mature subunit.

Subunit VII from *N. crassa* is processed by a peptidase other than mitochondrial processing peptidase (MPP)

Cleavable mitochondrial targeting sequences are removed by the general MPP. If the N-terminal extension of *N. crassa* subunit VII is not a targeting signal, it should be processed by a protease other than MPP. Therefore the three subunit VII products and, as a control, the RIP from *N. crassa* were incubated with isolated MPP from potato (Figure 7). Although the RIP was efficiently processed (lane 4), the *N.c.QCR8-ori* and the *N.c.QCR8-1st* products were not processed by MPP (lanes 1 and 2).

Analysis of the heterologous bc_1 complex containing the *N. crassa* subunit VII

Doubling times of DLL80 cells containing wild-type *QCR8* or cells transformed with the *N.c.QCR8-1st* construct in a single-copy plasmid are shown in Table 1. The doubling time of the strain containing the *N.c.QCR8-1st* construct was 1.8-fold that of the wild-type, whereas the strain complemented with the *N.c.QCR8-2nd* construct presented no detectable growth. Table 1 summarizes the results of the optical spectra and enzymic analysis of mitochondria from cells containing wild-type *QCR8* or *N.c.QCR8-1st*. The concentration of holo cytochrome *b* (measured as cytochrome *b* reducible by succinate in the presence of antimycin) in the *N.c.QCR8-1st* construct was 50% of that in the wild type. The concentrations of cytochromes *c* + *c*₁ and *aa*₃ were also substantially lower. This sensitivity of the cytochrome-*c* oxidase concentration during growth on a non-fermentable carbon source towards the level of assembly of the bc_1 complex is in contrast with the results obtained when cells are grown on glucose [6] or maltose [25]. The specific activity of the bc_1 complex was decreased to 40% of that of the wild-type. The turnover number, however, calculated on the basis of the amount of cytochrome *b* and the specific activity of the bc_1 complex, was the same as that of the wild-type enzyme, as additionally

Table 1 Doubling time of DLL80 cells transformed with different gene constructs, and enzymic activity and cytochrome content of mitochondria derived from them

The numbers of measurements on individual isolates are given in parentheses. See the Materials and methods section for experimental conditions. Abbreviation: *S.c.*, *Saccharomyces cerevisiae*.

Strain	Doubling time (min)	Specific activity (nmol/mg per s)	Cytochrome <i>c</i> + <i>c</i> ₁ (nmol/mg)	Cytochrome <i>b</i> (nmol/mg)	Cytochrome <i>aa</i> ₃ (nmol/mg)	Catalytic-centre activity (s ⁻¹)
pRS313 + <i>S.c.QCR8</i>	240 (2)	12.6 (2)	0.20	0.14	0.08	180
pRS313 + <i>N.c.QCR8</i> -1st	435 (2)	5.2 (2)	0.14	0.06	0.02	173
YEplac195 + <i>S.c.QCR8</i>	281 (2)	16.4 (2)	0.10	0.09	0.05	364
YEplac195 + <i>N.c.QCR8</i> -1st	400 (2)	12.8 (2)	0.10	0.07	0.04	366

confirmed by transformation of DLL80 cells with constructs in the multicopy vector YEplac 195.

DISCUSSION

The experiments described here report a characterization of the molecular structure, the complementation properties and the biogenesis of subunit VII of the *bc*₁ complex from *N. crassa*. The similarity between the *QCR8* products of *S. cerevisiae* and *N. crassa* is high enough to obtain partial complementation. In comparison, the *QCR8* product of *Kluyveromyces lactis* complements as well as *S. cerevisiae QCR8p* [32], whereas the products of the bovine and potato *QCR8* homologues do not complement at all (H. Boumans and G. Lobo-Hajdu, unpublished work). These findings suggest that neither the consensus sequence [9] nor the conserved structural features contains all the elements necessary for proper assembly. Assuming that the consensus sequence is required for the functionality of an assembled complex, we can postulate that, in addition to the overall structure, the sequence of the regions of interaction with the other subunits of the complex determines the level of assembly. These sequences have evolved together with the sequences of the interacting proteins, so that assembly correlates with evolutionary distance. A future task will be to establish those regions of interaction, especially those with cytochrome *b* as it is the subunit with stronger contact with both subunits VII and VIII in the yeast *bc*₁ complex.

In contrast with the corresponding protein from other organisms, the *N. crassa QCR8p* homologue is synthesized as a larger precursor that is processed on import into mitochondria [6,9,20,32–34]. Many nucleus-encoded mitochondrial proteins have cleavable presequences, which generally have a preponderance of basic and hydroxylated amino acids and are capable of forming amphiphilic helices [35,36]. Other proteins lack presequences, and targeting information is presumed to form part of the mature protein. In some cases it has been reported that a protein is made as a precursor in certain organisms but not in others. Examples are the adenine nucleotide translocase, the phosphate carrier and the 'hinge' protein of the *bc*₁ complex [37–39]. The biological reason for this situation remains to be established. *QCR8p* of the *bc*₁ complex seems to be a further example for this group of proteins. However, the presequence of *N. crassa QCR8p* is extremely short, has only one basic residue and lacks any serine residues. The shortest presequences that were shown to direct proteins into mitochondria have a length of 12 residues [40]. In two cases shorter presequences of eight amino acids were defined, but their targeting function remains to be established [41,42]. Targeting experiments *in vitro* with subunit VII from *N. crassa*, with or without presequence, using *N. crassa* mitochondria as target, revealed very similar import rates for the

two forms of this protein. Obviously the N-terminal extension of subunit VII does not contain information essential for mitochondrial targeting. So far all *QCR8p* homologues, including that from *N. crassa*, contain the targeting signal within the mature protein. It is difficult to predict the location of the targeting information within the mature proteins, but most probably it is not located at the mature N-terminus.

In this paper we have shown that the *N.c.QCR8p*, a nucleus-encoded mitochondrial protein, has an N-terminal presequence that is not required for mitochondrial import. What could be the function of this N-terminal extension? Its presence was shown to be required for detectable complementation of a yeast *QCR8* deletion mutant with the gene located in a single-copy vector. As the presequence is neither essential for mitochondrial targeting (also not in yeast because of the partial complementation obtained when the *N.c.QCR8*-2nd is present in a multicopy vector), nor a stimulator of mitochondrial import, as has been proposed for the TRM1 gene product [43], it might be a mitochondrial sorting sequence or be involved in functional activation of the protein, such as correct folding or binding to an assembly factor. Sorting sequences are necessary for some proteins to reach their correct intramitochondrial location, e.g. the mitochondrial inner membrane or the intermembrane space. In certain cases mitochondrially encoded proteins have sorting sequences, for instance subunits I and II of cytochrome-*c* oxidase from *N. crassa* [44,45]. Some nucleus-encoded mitochondrial proteins have presequences with sorting domains (bipartite signal sequences), e.g. cytochromes *c*₁ and *b*₂ [46,47]. These sorting sequences are processed by the inner membrane protease I/II (IMPI/II) [48,49]. Other proteins have a bipartite presequence that comprises an N-terminal mitochondrial targeting domain and additionally an octapeptide, which is removed by the mitochondrial intermediate protease (MIP) [50,51], e.g. the RIP from fungi or subunit VI of cytochrome-*c* oxidase from yeast [52–54]. The function of these octapeptides in protein sorting or activation is not fully understood [55].

An indication that the octapeptide of subunit VII is involved in sorting can be obtained from the fact that in the *bc*₁ complex of *N. crassa* all subunits that are located at the intermembrane space side of the inner mitochondrial membrane seem to contain a sorting sequence: cytochrome *c*₁ contains a presequence with a sorting domain, both the RIP and subunit VII contain an octapeptide, and subunit VIII also contains a presequence in the size range of an octapeptide [12]. Subunit VI, in contrast, a subunit that is located on the matrix side of the mitochondrial inner membrane, has no presequence, whereas the matrix-exposed core subunits 1 and 2 contain a presequence that comprises only matrix targeting information. This relationship for the subunits of the *bc*₁ complex of *N. crassa*, between the presence of a non-targeting presequence and location at the

outside of the inner membrane, strongly favours a sorting function for these presequences. Deletion of sorting domains generally leads to a false intramitochondrial localization of proteins. The intramitochondrial localization of the product of the *N.c.QCR8*-2nd construct in yeast cells grown on glucose was not investigated because we never found residual non-assembled 11.5 kDa subunit protein in cells with deficient assembly as the result of a mutation in this protein [56].

Our favoured hypothesis, that the N-terminal extension of *N. crassa* subunit VII is a sorting signal, is in line with the observation that the protein is processed on import by a protease other than the general mitochondrial processing peptidase, which cleaves off mitochondrial targeting sequences. MIP seems to be the best candidate for processing the subunit VII of *N. crassa* because of the length and the amino acid composition of the N-terminal extension. The consensus sequence for a MIP-cleavable extension is F/L/IXXS/T/GXXXX [57,58]. The cleaved presequence from the *N. crassa* subunit VIII is MRPTQTML, thus containing a Thr residue at position -5 relative to the mature N-terminus and a Met residue at position -8. We might suppose that Met is also allowed at position -8, because other hydrophobic residues such as Val are also found there [57]. Like other cleavable octapeptides the *N. crassa* subunit VII contains a Gln residue. MIP has been purified from rat liver [54] but not from *N. crassa* or any related organism. Whether or not the purified rat MIP will be able to cleave the *N. crassa* N-terminal extension remains to be determined. If the processing of the presequence from the *N. crassa* subunit VII does indeed occur, this will be the first known report of a protein processed by MIP without previous processing by MPP.

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