The EMBO Journal vol.6 no.7 pp.2131–2137, 1987

Deficiency in mRNA splicing in a cytochrome *c* mutant of *Neurospora crassa*: importance of carboxy terminus for import of apocytochrome *c* into mitochondria

Rosemary A.Stuart, Walter Neupert and Maximilian Tropschug

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, 8000 München 2, FRG

Communicated by W. Neupert

Molecular cloning and characterization of cytochrome *c* cDNA clones of *Neurospora crassa* wild-type (74A) and a cytochrome *c*-deficient mutant (*cyc1-1*) are described. Southern blot analysis of genomic DNA indicates that only one cytochrome *c* gene exists in the *N. crassa* genome. The cDNA sequence of the wild-type cytochrome *c* confirmed the previously determined protein sequence. Sequence analysis of the *cyc1-1* cDNA for cytochrome *c* revealed the presence of a larger open reading frame, owing to the presence of an unspliced intron in the 3′ end of the coding region. Splicing of this intron is obviously prevented due to the presence of two base exchanges in the highly conserved intron consensus sequences. Consequently, *cyc1-1* synthesizes apocytochrome *c* with an altered carboxy terminus, 19 amino acids longer than the wild-type cytochrome *c*, with the final 27 amino acids being of an unrelated sequence. This alteration in the carboxy terminus renders the apocytochrome *c* incompetent for binding to mitochondria and, consequently, import into mitochondria. Thus, unlike other mitochondrial precursor proteins, where it has been demonstrated that the amino terminus alone is sufficient to target the protein to the mitochondria, an intact carboxy terminus is required for efficient import of apocytochrome *c* into mitochondria. This is independent confirmation for the view that the import pathway of cytochrome *c* is unique with respect to all other mitochondrial proteins studied to date.

Key words: cytochrome *c*/intron splicing/mitochondria/protein transport

Introduction

The import pathway of cytochrome *c* into mitochondria differs in many respects from those of other mitochondrial precursor proteins (for most recent model see Nicholson *et al.*, 1987). Apocytochrome *c*, the precursor of holocytochrome *c*, is synthesized on free cytoplasmic ribosomes, released into the cytosol and imported into mitochondria in a post-translational manner. Unlike many other precursor proteins, however, apocytochrome *c* is not synthesized with an amino-terminal prepiece and thus is not proteolytically processed upon import into mitochondria (for reviews see Harmey and Neupert, 1985; Zimmermann, 1986; Nicholson and Neupert, 1987). Apocytochrome *c*, a basic protein, can penetrate spontaneously into artificial lipid bilayers (Rietveld *et al.*, 1985) and thus it is probably able to insert spontaneously (at least partially) into the outer mitochondrial membrane. A binding protein is thought to specifically interact with the partially inserted apocytochrome *c* with high affinity, and thus serves to trap the apoprotein in the outer membrane (Hennig *et al.*, 1983). The enzyme cytochrome *c* heme lyase (Hennig and Neupert, 1981; Taniuchi *et al.*, 1983; Nicholson *et al.*, 1987), catalyses the covalent attachment of heme to cysteines 18 and 21 of the apocytochrome *c*, which appear to be exposed to the intermembrane space as a result of action of the cytochrome *c* binding protein. The covalent addition of the heme to apocytochrome *c* causes a re-folding of the polypeptide chain which is thought to be the driving force for the translocation of the polypeptide across the outer membrane. Cytochrome *c* import, unlike that of most other mitochondrial precursor proteins, does not depend on the presence of a membrane potential across the inner membrane (Zimmermann *et al.*, 1981; Pfanner and Neupert, 1985).

It has recently been demonstrated via gene fusion experiments that the amino-terminal prepieces of mitochondrial precursor proteins contain sufficient information required for targeting and intra-mitochondrial sorting (for examples see Douglas *et al.*, 1984; Hurt *et al.*, 1984, 1985). Mitochondrial prepieces share several common characteristics, namely a high proportion of positively charged amino acids, an absence of negatively charged amino acids with a few exceptions, a high content of hydroxylated amino acids and they display the ability to form amphiphilic α-helical structures. The problem is posed as to how precursors which do not contain removable prepieces are targeted to mitochondria. Studies involving two proteins from mitochondrial outer membrane, which do not undergo proteolytic processing upon import, namely a 70-kd protein (Riezman, 1983; Hase *et al.*, 1984), and the mitochondrial porin (Mihara and Sato, 1985; R.Kleene, M.Tropschug and W.Neupert, unpublished results), have suggested that the targeting information is also contained in their amino termini. Does apocytochrome *c* contain such an internal amino-terminal targeting sequence also? We have studied

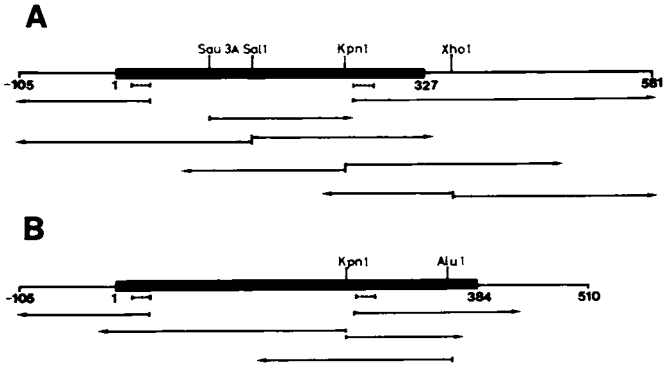

Fig. 1. Sequencing strategy of wild-type (**A**) and mutant (**B**) cDNA inserts. The filled boxes indicate the coding regions for the apocytochrome *c* proteins, the first nucleotide of which being indicated by the number 1. The dashed lines correspond to the areas of hybridization of the synthetic oligonucleotide primers used in the sequencing analysis. The direction and extent of sequence determinations are shown by horizontal arrows.

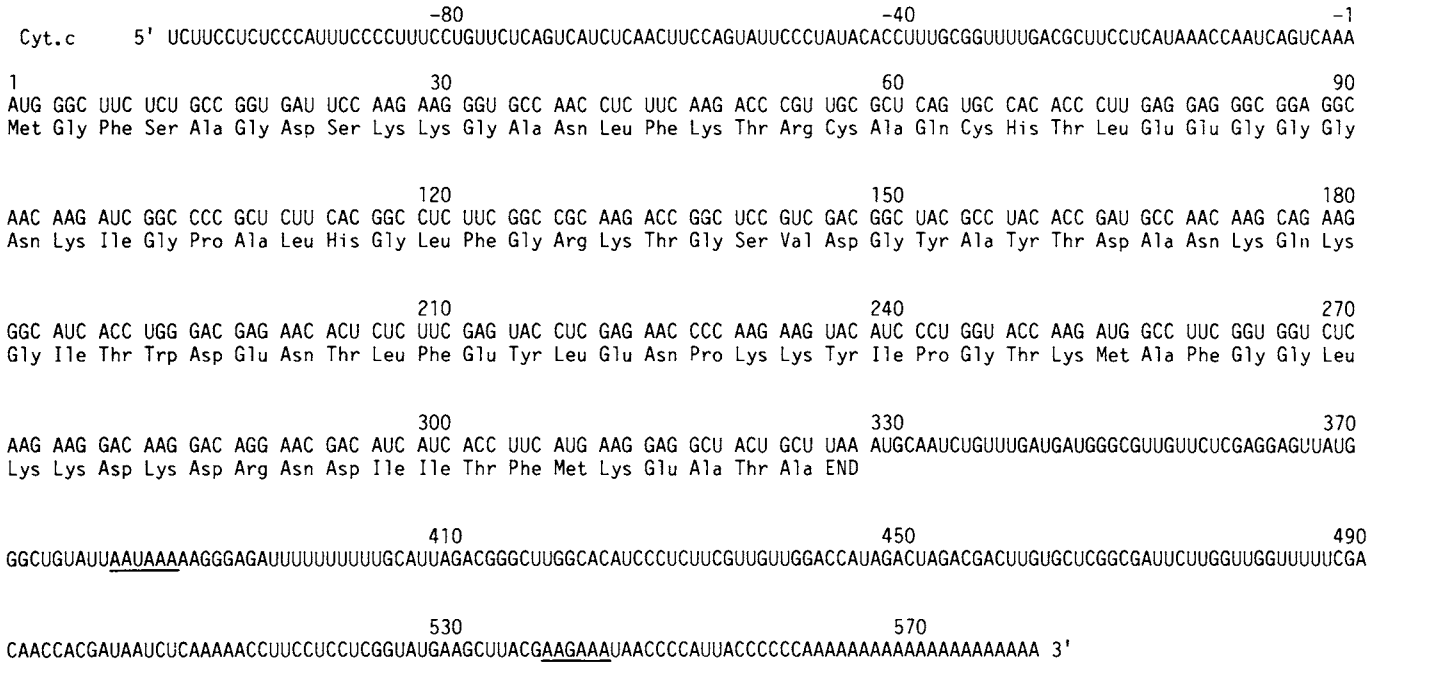

Fig. 2. Nucleotide sequence of *N. crassa* cytochrome *c* mRNA and deduced amino acid sequence. Nucleotide residues are numbered in the 5′−3′ direction, beginning with the first residue of the initiation codon. Putative polyadenylation signals are underlined.

a cytochrome *c* mutant of *Neurospora crassa* known as *cyc1-1*. Phenotypically, this mutant has a slightly reduced growth rate when compared with that of the wild-type form, and spectral analysis has indicated that holocytochrome *c* levels are reduced to 10−20% of wild-type level whereas levels of other cytochrome components of the respiratory chain are unaltered (H.Bertrand, personal communication). This mutation does not affect the biogenesis of cytochrome *c* at the level of transcription or translation of the cytochrome *c* mRNA (B.Hennig and W.Neupert, unpublished data). Apocytochrome *c* is, in fact, synthesized in the cell, albeit in an altered form which is incompetent for import into mitochondria. To investigate the specific details of this alteration in the apocytochrome *c* protein, sequence studies of wild-type and mutant cytochrome *c* cDNA clones were performed. The similarities and differences of the wild-type cytochrome *c* mRNA, compared with those of the mutant, are discussed with reference to their relevance on the targeting of apocytochrome *c* to mitochondria.

Results

Isolation of cytochrome c cDNAs and genomic DNA

A cDNA library prepared from *N. crassa* (74A, wild-type) poly(A)$^+$ mRNA was cloned into the *Pst*I site of pBR322 by dG/dC tailing. Recombinant clones were screened using a short *N. crassa* cDNA insert specific for cytochrome *c* as a hybridization probe. Of the cytochrome *c* recombinant clones that were isolated, the largest insert identified, A5, was 680 bp. Northern blot analysis, where both poly(A)$^+$ mRNA and the 680 bp insert were resolved on agarose−formaldehyde gels, confirmed that insert A5 represents a full-length cDNA for cytochrome *c* (results not shown).

A *cyc1-1* mutant cDNA library was then screened using the A5 cDNA insert. The longest insert detected (610 bp) did not represent a full-length cDNA as shown by Northern blot analysis (results not presented). Sequence analysis confirmed, however, that the complete coding region for cytochrome *c* was contained in this insert.

The 680-bp insert of the wild-type cDNA was also used to screen a *N. crassa* wild-type genomic library. Approximately 20 000 clones were screened and 21 positive clones were identified and isolated for further analysis.

Nucleotide sequence of wild-type cytochrome c cDNA

Overlapping fragments of the cytochrome *c* cDNA were sequenced in pUC19 as outlined in Figure 1A. The 3′ and 5′ noncoding regions were also sequenced using specific synthetic primers.

The A5 cDNA insert contains 683 bp derived from the cytochrome *c* mRNA including a poly(A) tail of 21 nucleotides. It consists of a noncoding 5′ region of 105 nucleotides followed by an open reading frame of 327 bp corresponding to a protein of 108 amino acids (Figure 2). The protein sequence deduced from the cDNA sequence is in agreement with the previously determined protein sequence for cytochrome *c* from *N. crassa* (Heller and Smith, 1966; Lederer and Simon, 1974). The 5′ noncoding region contains a significantly low content of guanosine residues, only 8.5% of total residues. The sequence GTCAAA, preceding the ATG initiation codon, displays similarities with many other sequences of *N. crassa* mRNAs at this position, e.g. porin, a 20-kd protein from mitochondria, β-subunit of the ATP synthase complex (our unpublished results) and the ADP/ATP carrier protein (Arends and Sebald, 1984).

Several interesting features were observed in the 3′ noncoding region of the cytochrome *c* mRNA. A number of uridine-rich regions can be seen, an observation previously reported for other eukaryotic mRNAs (Boss *et al.*, 1981). The sequence AAUAAA, occurring ~20 nucleotides preceding the poly(A) tail in several mRNAs, has been proposed to play an important role in polyadenylation (Proudfoot and Brownlee, 1976). In the cytochrome *c* mRNA, however, the sequence AAGAAA occurs 17 nucleotides upstream from the polyadenylation site. Thus it is assumed that this sequence represents the polyadenylation signal in *N. crassa* cytochrome *c* mRNA.

An alignment study of the *N. crassa* cytochrome *c* mRNA sequence with that of iso-1 cytochrome *c* from *Saccharomyces cerevisiae* (Boss *et al.*, 1981), reveals a high degree of homology

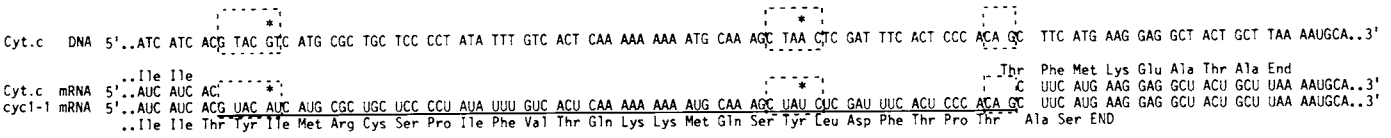

Fig. 3. The mutant cytochrome *c* mRNA contains an unspliced intron. The nucleotide sequence of the 3′ coding region of the wild-type cytochrome *c* gene is displayed in the upper half of the figure and is aligned with the corresponding partial nucleotide sequences of the wild-type and mutant cytochrome *c* mRNAs, below. The amino acid sequence deduced from the wild-type mRNA is displayed above the nucleotide sequence, the first codon presented corresponds to codon 99 of the complete cytochrome *c* mRNA sequence. The nucleotide sequence and deduced amino acid sequence from the mutant mRNA is presented below that of the wild-type. The intervening sequence of the mutant mRNA is indicated by an underlined area, and asterisks indicate the two base exchanges in the intervening nucleotide sequence, which is otherwise identical to the intron sequence present in the wild-type gene. The boxed-in areas indicate the 5′ and 3′ intron consensus sequences and the branch sequence in the DNA and mutant mRNA sequences.

(65%), comparing the coding regions of the two mRNAs (results not shown). The same degree of homology was observed at the level of the amino acid sequence.

An intervening sequence is present in the mutant cytochrome c mRNA

The 610-bp mutant cytochrome *c* cDNA was digested with restriction enzymes and the resulting fragments were sequenced in pUC19 (Figure 1B). Synthetic cytochrome *c* specific primers were also used to sequence the 5′ and 3′ regions of the insert.

The mutant sequence is identical to the wild-type sequence up to nucleotide position 302 (not shown here). Beyond this point the mutant mRNA assumes a new sequence resulting in a total coding region of 384 nucleotides, in contrast to the 327 nucleotides of the wild-type coding region. Owing to this change in the 3′ area of the coding region the mutant mRNA codes for a cytochrome *c* protein with an altered and extended carboxy terminus (19 amino acids) (Figure 3). Genomic cytochrome *c* clones from wild-type *N. crassa* were also isolated and sequenced to investigate if an intron exists at this position in the wild-type gene. A cytochrome *c* primer, hybridizing to codons 85–91 of the wild-type cDNA, was used to sequence the corresponding region of the gene. The resulting partial gene sequence is represented in an alignment study with the 3′ regions of the wild-type and mutant mRNA sequence (Figure 3). Indeed, the cytochrome *c* gene contains an intron at a position corresponding to codon 101 of the wild-type cDNA sequence.

A closer look at the mutant mRNA sequence indicates that the original 3′ end of the coding region of the wild-type mRNA is interrupted by an intervening sequence starting after nucleotide 302 of the wild-type sequence. This intervening sequence extends for 74 nucleotides after which the wild-type sequence is reassumed, now out of frame, at a position corresponding to the 2nd last codon of the mutant coding region. A termination codon, normally out of frame within the original wild-type coding region, is brought into frame due to the presence of this intervening sequence and serves to terminate translation of the mutant cytochrome *c*.

The intervening sequence displays all the characteristics of an unspliced intron: the sequence GUACAU exists at the 5′ insertion junction and resembles the conserved 5′ intron consensus sequence GTAXGT previously reported for *Neurospora* genes (Arends and Sebald, 1984). The conserved 3′ intron consensus sequence CTPuAC . . . 9–15 nt . . . PyAC (Arends and Sebald, 1984) would also appear to be present since a similar sequence CUAUC is situated 14 nucleotides upstream from a CAG triplet present at the 3′ insert junction. Thus the classic 5′ and 3′ intron consensus sequences which are present in the wild-type gene sequence are not found in the mutant unspliced intron. In contrast, in the mutant mRNA these sequences contain two base exchanges at positions which are indicated by asterisks in Figure 3.

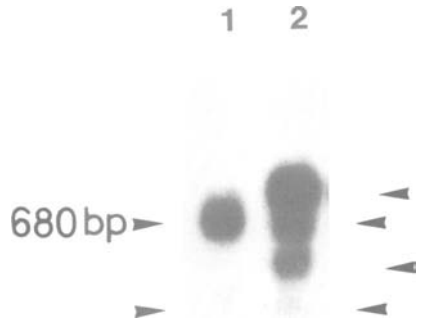

Fig. 4. Northern blot analysis of poly(A)$^+$ mRNA from wild-type and *cyc1-1 N. crassa*. Poly(A)$^+$ mRNA (10 μg) from wild-type **(lane 1)** and from *cyc1-1* **(lane 2)** *N. crassa* was resolved on 2.5% agarose–formaldehyde gels, blotted onto Biodyne A filters and hybridized to a nick-translated cDNA insert, representing the full-length wild-type cytochrome *c* mRNA.

Analysis of the wild-type and mutant N. crassa cytochrome c mRNAs

To obtain information about the cytochrome *c* transcripts, Northern blot analysis was performed with wild-type and *cyc1-1* poly(A)$^+$ RNA, using the A5 cDNA insert as a labelled probe. This cytochrome *c* fragment hybridized to two mRNA species of the wild-type mRNA (Figure 4, lane 1). The larger and more abundant transcript displayed the same mobility as the full-length cDNA insert (results not shown). Southern blot analysis of both the wild-type and mutant genomic DNA has shown that *N. crassa* contains only one gene for cytochrome *c* (see below). Thus the second, smaller and less abundant cytochrome *c* mRNA must represent a second transcript of this single cytochrome *c* gene. The presence of a sequence AAUAAA in the 3′ noncoding region (nucleotide position 380) could represent a second polyadenylation signal and thus result in a smaller mRNA transcript with a correspondingly shortened 3′ terminus.

The nick-translated cytochrome *c*-specific probe hybridized to four mRNA species of the mutant mRNA. The two less abundant of these transcripts displayed the same mobilities as those detected in the wild-type mRNA and presumably represent normal wild-type transcripts (Figure 4, lane 2). The other two remaining transcripts are larger by the same proportion in comparison with the large and small transcripts of the wild-type mRNA, a finding which would be in agreement with the fact that these mRNAs contain an unspliced intervening sequence. The source of the wild-type-sized transcripts present in the mutant mRNA is still not clear. These transcripts may be due to correct splicing of the mutant mRNAs with low efficiency.

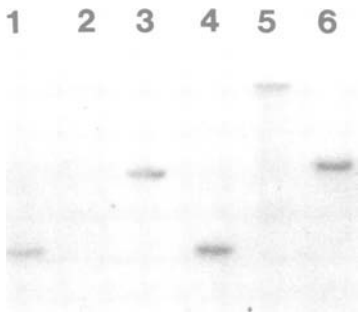

Fig. 5. Southern blot analysis of genomic DNA from wild-type and *cyc1-1* *N. crassa*. Each lane contains 10 μg of DNA isolated from wild-type **(lanes 1–3)** or *cyc1-1* **(lanes 4–6)** *N. crassa*. Samples were digested with *Hin*dIII **(lanes 1** and **4)**, *Eco*RI **(lanes 2** and **5)** or *Pst*I **(lanes 3** and **6)**, transferred to Biodyne A filters and hybridized to the nick-translated full-length cDNA insert for wild-type cytochrome *c*. The restriction enzymes used do not cut in the cytochrome *c* cDNA sequence.

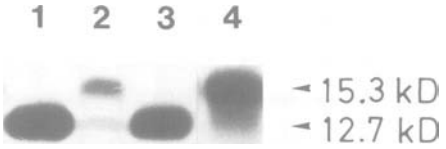

Fig. 6. *In vitro* synthesis of wild-type and mutant apocytochrome *c*. Poly(A)$^+$ mRNA, isolated from either wild-type **(lane 1)** or mutant **(lane 2)** *N. crassa*, was translated in rabbit reticulocyte lysates and apocytochrome *c* was immunoprecipitated. Plasmids (pDS5) containing either wild-type **(lane 3)** or mutant **(lane 4)** cDNA inserts for cytochrome *c* were transcribed with *E. coli* RNA polymerase in the presence of 7mGpppA. The resulting RNAs were translated in rabbit reticulocyte lysates and apocytochrome *c* was immunoprecipitated. The [^{35}S]methionine-labelled immunoprecipitates were separated by SDS–PAGE and visualized by autoradiography.

N. crassa contains only one cytochrome c gene per haploid genome

In order to investigate the complexity of cytochrome *c*-like sequences in *N. crassa* wild-type and *cyc1-1* mutant genomes, genomic DNA was digested with various restriction endonucleases and hybridized to a full-length, wild-type cytochrome *c* cDNA probe, A5 (Figure 5). In each lane only one hybridizing fragment was observed, indicating that only one gene for cytochrome *c* exists in both the wild-type and *cyc1-1* genomes.

Synthesis of wild-type and mutant cytochrome c in vitro

cDNA inserts containing the complete coding regions were cloned into the *Pst*I site of the pDS5 transcription vector (Stüber *et al.*, 1984) for both the wild-type and mutant cytochrome *c*. Transcription of the cytochrome *c* cDNAs was carried out in the presence of 7mGpppA and *Escherichia coli* RNA polymerase as described (Stüber *et al.*, 1984). The resulting mRNAs were translated in rabbit reticulocyte lysates in the presence of [^{35}S]methionine. Translation products were immunoprecipitated with cytochrome *c*-specific antiserum (Zimmermann *et al.*, 1979) and were analysed by SDS–PAGE and fluorography (Figure 6).

Transcription/translation of the wild-type cytochrome *c* cDNA yielded a product which was recognized by the cytochrome *c* antiserum (Figure 6, lane 3) and which displayed the same apparent mol. wt (12.7 kd) as apocytochrome *c* immunoprecipitated from reticulocyte lysate programmed with wild-type poly(A)$^+$ mRNA (Figure 6, lane 1). Translation of the mutant poly(A)$^+$ mRNA resulted in the synthesis of two apocytochrome *c* proteins (Figure 6, lane 2) having apparent mol. wts of 15.3 and 12.7 kd. The latter apocytochrome *c* displayed the same electrophoretic mobility as the wild-type apocytochrome *c*; it possibly represents the translation product of the mutant mRNA correctly spliced with low efficiency.

Transcription/translation of the mutant cDNA insert resulted in the synthesis of an apocytochrome *c* protein with an apparent mol. wt of 15.3 kd (Figure 6, lane 4), i.e. analogous to that of the large apocytochrome *c* protein observed in the mutant poly(A)$^+$ mRNA translation. The difference in the mol. wt observed between the wild-type and mutant apocytochrome *c* proteins confirms the cDNA sequence data which indicate that the mutant mRNA codes for an apocytochrome *c* protein containing an extra 19 amino acid residues.

cyc1-1 apocytochrome c, unlike wild-type apocytochrome c, is not imported into mitochondria in vitro

Apocytochrome *c*, the precursor of holocytochrome *c*, binds with high affinity to isolated mitochondria (Hennig *et al.*, 1983). The import of apocytochrome *c* can be prevented when heme attachment is inhibited by the addition of the heme analogue deuteroheme to the import assay (Hennig and Neupert, 1981). Thus it has been demonstrated that the translocation of apocytochrome *c* across the outer mitochondrial membrane is intrinsically coupled with the covalent attachment of heme; a process catalysed by the enzyme cytochrome *c* heme lyase. Holocytochrome *c* formation is also dependent on the presence of NADH and on a cytosolic factor (Nicholson *et al.*, 1987). An assay system has recently been developed to measure directly the process of heme addition to apocytochrome *c*; thus, import of this precursor and its conversion to apocytochrome *c* can now be quantitatively demonstrated (Nicholson *et al.*, 1987). Using this assay system, both the wild-type and mutant apocytochrome *c* species were tested for their ability to bind to wild-type mitochondria and to be converted to holocytochrome *c* on import into mitochondria.

Transcription of both wild-type and mutant cDNA inserts in pDS5 was carried out, followed by translation of the resulting RNAs in reticulocyte lysates in the presence of [^{35}S]cysteine. Isolated mitochondria (75 μg protein) were incubated with reticulocyte lysates either in the presence or absence of NADH, for 10 min at 25°C. Mitochondria and a post-mitochondrial supernatant were resolved and cytochrome *c* was immunoprecipitated using an antiserum which recognized both apo- and holocytochrome *c*. Immunoprecipitates were then subjected to tryptic digestion and resulting peptide mixtures were resolved by reverse-phase h.p.l.c. and [^{35}S]cysteine-containing peptides were collected and radioactivity quantified, as previously described (Nicholson *et al.*, 1987).

In the presence of NADH, a significant amount of wild-type apocytochrome *c* was converted to holocytochrome *c* (Table I). However, in the absence of NADH up to a 50-fold decrease in holocytochrome *c* formation was observed, in agreement with previous results (Nicholson *et al.*, 1987). About 70% of the wild-type holocytochrome *c* formed in the presence of NADH was detected in the post-mitochondrial supernatant. This fraction of

Table I. Mutant cytochrome *c* is not imported into mitochondria *in vitro*

Sample	Total (=100%) apo and holocyt. *c* in assay (pmol)	% Apocyt. *c* associated with mitochondria	% Holocyt. *c* formed
1. Wild-type + NADH	9.6×10^{-4}	10	51.7
2. Wild-type − NADH	6.0×10^{-4}	30	0.9
3. *cyc1-1* + NADH	4.2×10^{-4}	2.3	1.7
4. *cyc1-1* − NADH	2.3×10^{-4}	5.5	0.5

Full-length cDNA inserts for both wild-type and *cyc1-1* cytochrome *c* were transcribed and resulting transcripts were translated in reticulocyte lysates in the presence of [^{35}S]cysteine (see Materials and methods). Isolated mitochondria (75 μg protein) were incubated with the reticulocyte lysates either in the presence or absence of NADH, for 10 min at 25°C. Mitochondria and supernatants were separated by centrifugation and apo- and holocytochrome *c* were co-immunoprecipitated. Immunoprecipitates were eluted with urea, digested with trypsin and resulting peptide mixtures were resolved by reverse-phase h.p.l.c. Fractions containing apo- and holopeptides were collected and radioactivity determinations were carried out by scintillation counting. For each sample, the amount of cytochrome *c* immunoprecipitated from the mitochondrial and supernatant fractions were summed together and set at 100%. The various samples were as follows: (1) wild-type apocytochrome *c* + NADH; (2) wild-type apocytochrome *c* − NADH; (3) *cyc1-1* apocytochrome *c* + NADH; and (4) *cyc1-1* apocytochrome *c* − NADH.

holocytochrome *c* was apparently released from the mitochondria as a result of their disruption due to incubation in reticulocyte lysate or by subsequent centrifugation. Binding of wild-type apocytochrome *c* to mitochondria was not prevented in the absence of NADH.

The mutant apocytochrome *c* was not targeted to the mitochondria, as no significant binding of the precursor to mitochondria or conversion to holocytochrome *c* was observed, irrespective of whether NADH was present or not (Table I).

Discussion

The amino acid sequence of wild-type *N. crassa* cytochrome *c* deduced from the cDNA sequence confirmed the previously published protein sequence and the absence of a second AUG codon upstream from the postulated initiation codon demonstrated that the cytochrome *c* precursor is synthesized without a cleavable amino-terminal extension sequence (Zimmermann *et al.*, 1981). Comparison of the *N. crassa* cytochrome *c* mRNA sequence (coding region) with that of iso-1-cytochrome *c* from *S. cerevisiae* (Boss *et al.*, 1981) revealed a high level of homology (65%). *Neurospora crassa*, like chicken (Limbach and Wu, 1983) and *Schizosaccharomyces pombe* (Russell and Hall, 1982), contains only one functional cytochrome *c* gene per haploid genome. It has been previously demonstrated that *Drosophila melanogaster* (Limbach and Wu, 1985b) and *S. cerevisiae* (Montgomery *et al.*, 1980) contain two cytochrome *c* genes per haploid genome. In contrast, mammals seem to contain ~20−30 cytochrome *c*-like sequences (Scarpulla *et al.*, 1982), the majority of which appear to be non-functional pseudogenes (Scarpulla and Wu, 1983).

Sequence analysis of the cDNA from the cytochrome *c*-deficient mutant, *cyc1-1*, revealed the presence of a larger open reading frame, owing to the presence of a 74-bp intervening sequence in the 3′ area of the coding region. The transcript codes for a protein containing an extra 19 amino acids at the carboxy terminus, when compared with the wild-type cytochrome *c* protein. The presence of a GUACAU sequence at the 5′ end of the intervening sequence and a CAG sequence at the 3′ junction led us to suspect that the mutant cytochrome *c* mRNA might contain an unspliced intron. Consistent with this view, a sequence CUAUC was observed, resembling the conserved branch sequence 14 nucleotides upstream from the 3′ junction. To date, it has been demonstrated that only chicken (Limbach and Wu, 1983), mouse (Limbach and Wu, 1985a) and rat (Scarpulla *et al.*, 1981) cytochrome *c* genes contain one intron in their coding regions. These introns would appear to be conserved as they interrupt all three genes at identical positions (between codons 55 and 56 of the coding region) and also appear to be of comparable lengths (Limbach and Wu, 1985a); however, no intron present in the 3′ area of the coding region of a cytochrome *c* gene has been observed to date. Sequence analysis confirmed that the *N. crassa* cytochrome *c* gene contains an intron of 74 bp after codon 102. The 5′ donor splice sequence. GTACGT, the 3′ acceptor splice sequence, CAG, and the branch sequence, CTAAC, conforms to consensus sequences derived from other *N. crassa* introns (Kinnaird and Fincham, 1983; Arends and Sebald, 1984; M.Tropschug and W.Neupert, unpublished results). These conserved sequences in the wild-type intron, however, differ from those found in the mutant intervening sequence. The mutant cytochrome *c* mRNA contains the sequence GUACAU instead of GUACGU at the 5′ splice site and CUAUC instead of CUAAC at the branch site. Splicing of the intron from the mutant mRNA is thus prevented or retarded owing to the presence of these two base exchanges.

In the branch sequence the final A residue, observed in the wild-type genomic sequence, is changed to a U residue. We speculate, therefore, that the formation of the 2′−5′ phosphodiester bond with the first G residue of the 5′ splice sequence is prevented, thus the essential lariat formation does not occur. The occurrence of spliced mRNA in the mutant may indicate unofficial usage of an alternative site.

As a consequence of this defective mRNA splicing, *cyc1-1* synthesizes an apocytochrome *c* with an altered carboxy terminus. The polypeptide sequence is changed from amino acid residue 102 onwards, resulting in an apocytochrome *c* which is 19 amino acids longer than the corresponding wild-type protein, thus the final 27 amino acids are of an unrelated sequence. The mutant apocytochrome *c* is not imported into mitochondria either *in vitro* or *in vivo*; in fact, *in vivo* the mutant apocytochrome *c* accumulates in the cytosol (B.Hennig and W.Neupert, unpublished results). The reduction in levels of holocytochrome *c* present in *cyc1-1* mitochondria is wholly due to this defect in the apocytochrome *c* molecule itself, which renders it incompetent for binding to the mitochondrial surface, rather than due to a defect in the mitochondrial import machinery. Number and affinity of the apocytochrome *c* binding sites have been found to be unaffected in isolated *cyc1-1* mitochondria (results not shown). It is thus concluded that the interaction of the apoprotein with either the outer mitochondrial membrane and/or receptor is disturbed. A very small amount of mutant holocytochrome *c*, however, is formed *in vivo* and peptide analysis confirms the sequence derived from the cDNA (B.Hennig and W.Neupert, unpublished results).

The altered polypeptide sequence is rather hydrophilic and contains one negative and three positive charges. It is highly remarkable that this relatively minor alteration at the carboxy terminus of cytochrome *c* confers inability for import, when one considers that diverse passenger proteins (non-mitochondrial hydrophilic proteins) can be efficiently imported into mitochondria if a mitochondrial targeting sequence is linked to the amino terminus. This is independent confirmation for the view that the import pathway of cytochrome *c* is unique with respect to all other mitochondrial

proteins studied to date. Matsuura *et al.* (1981) postulated that an addressing signal is contained in a cytochrome *c* fragment extending from amino acid residue 66 to the carboxy end of the protein, which serves to target the apocytochrome to mitochondria. It cannot be concluded from our findings that a 'targeting signal' is located at the carboxy terminus of apocytochrome *c*. The addition of extra amino acids at the carboxy terminus may simply serve to alter the overall folding of the apo-protein which could prevent binding. A specific overall conformation of the apo-protein, rather than a targeting signal, could as well be required for mitochondrial targeting. Thus, results presented here reinforce the importance of the carboxy terminus in the targeting of apocytochrome *c* to the mitochondria. Apocytochrome *c*, in traversing only the outer membrane, apparently interacts in a delicate manner with the outer membrane and the apocytochrome *c* binding protein. This interaction is obviously affected in the case of the mutant apocytochrome *c*.

Materials and methods

Strains of N. crassa and growth of cultures

Strains of *N. crassa* used in this study were the wild-type strain 74A and a derivative strain, *cyc1-1*, obtained from H.Bertrand, Regina, University of Saskatchewan, Canada. Cultures were grown for 14 h at 25°C with vigorous aeration in Vogel's minimal medium supplemented with 2% (w/v) sucrose. The inoculum used was 1×10^6 conidia/l medium and the cultures were grown under bright illumination.

Isolation of mitochondria and in vitro protein import

Mitochondria used in import experiments were isolated using Percoll gradients (Nicholson *et al.*, 1987). The conditions used for *in vitro* import of apocytochrome *c* into mitochondria were essentially as described (Nicholson *et al.*, 1987).

Preparation of RNA, synthesis and cloning of cDNA

Total RNA and poly(A)$^+$ RNA were isolated according to the methods of Michel *et al.* (1979) and Sheldon *et al.* (1972). About 10 μg of both wild-type and mutant poly(A)$^+$ mRNA were used for cDNA synthesis according to the method of Gubler and Hoffman (1983). Approximately 40 ng of the resulting cDNA were dC-tailed and annealed with 1.3 μg of *Pst*I-cut dG-tailed pBR322 (Villa-Komaroff *et al.*, 1978). Recombinant plasmids were transformed into *E. coli* 5K (Hubacek and Glover, 1970) by the method of Hanahan (1983).

Hybrid selection

A 280-bp apocytochrome *c* cDNA insert (A12/20) was identified by hybridization selection of mRNA (Parnes *et al.*, 1981; Viebrock *et al.*, 1982), subsequent translation in a reticulocyte lysate (Pelham and Jackson, 1977) and immunoprecipitation of the ^{35}S-labelled product with a cytochrome *c* specific antibody (Zimmermann *et al.*, 1979). Insert A12/20 was used to identify full-length cDNA inserts by means of colony hybridization (Grunstein and Hogness, 1975).

Construction of N. crassa genomic DNA library

A *N. crassa* (74A) genomic library was constructed in the following manner. Genomic DNA was partially digested with *Sau*3A and 4–6 kb DNA fragments were cloned into *Bam*HI cleaved pBR322. The recombinant plasmids were transformed into *E. coli* 5K (Hanahan, 1983).

Screening of the cDNA and genomic DNA libraries

Neurospora crassa wild-type cDNA library was screened with cDNA insert A12/20 (see above). A full-length cytochrome *c* cDNA (A5) was identified and was used as a hybridization probe to screen the *cyc1-1* mutant cDNA library and wild-type genomic DNA library by colony hybridization (Grunstein and Hogness, 1975).

Sequencing of the cytochrome c cDNAs and genomic DNA

Both the wild-type and mutant cytochrome *c* cDNAs were sequenced in the following manner. Plasmids containing hybridization-positive cDNA fragments were isolated (Birnboim and Doly, 1979) and digested with *Pst*I. The resulting inserts were separated from the linearized plasmids by agarose gel electrophoresis and purified by electroelution. The purified inserts were further digested with various restriction enzymes (see Figure 1) and ligated into vector pUC19. The sequences of all fragments of the wild-type cDNA were determined on both strands, except for the two end fragments which were not determined from the tailed ends. Sequence analysis was carried out with denatured plasmids (Chen and Seeburg, 1985) according to the dideoxy termination method (Sanger *et al.*, 1977) using ^{35}S-labelled dATP (Amersham; 600 Ci/mmol) (Biggin *et al.*, 1983).

The 5′ and 3′ ends of both cytochrome *c* cDNAs were also sequenced using cytochrome *c* specific synthetic primers. The primers 5′GGCACCCTTCTTGGAATC3′ and 5′GGCCTTCGGTGGTCTCAAGAAG3′ hybridized specifically to nucleotide positions corresponding to codons 6–12 (anti-sense strand) and codons 85–91 (sense strand) respectively, of the wild-type cytochrome *c* cDNA (see Figure 2).

Sequencing of cytochrome c gene

The cytochrome *c* specific primer, complementary to codons 85–91 of the wild-type cytochrome *c* cDNA was used to sequence the 3′ end of the coding region and part of the noncoding region of the cytochrome *c* gene.

Analysis of RNA by Northern blotting

Poly(A)$^+$ mRNA from wild-type (74A) and mutant (*cyc1-1*) *N. crassa* was fractionated on 2.5% agarose gels containing formaldehyde (Maniatis *et al.*, 1982) and transferred overnight to Biodyne A membranes. The probe used in the Northern blot analysis was a nick-translated (Rigby *et al.*, 1977) A5 cytochrome *c* cDNA insert.

Analysis of genomic DNA by Southern blotting

Southern blot analysis of genomic DNA isolated from both the wild-type and mutant *cyc1-1 N. crassa* was carried out as previously described (Southern, 1975; Maniatis *et al.*, 1982). The hybridization probe used was the A5 cDNA insert containing the complete coding region for the wild-type cytochrome *c*. Hybridization to the filter-bound DNA was carried out at 65°C in 6 × SSC, 0.5% SDS, 5 × Denhardt's solution (Denhardt, 1966), 100 μg/ml denatured salmon sperm DNA and the ^{32}P-labelled probe for 12–30 h. Filters were washed twice for 10 min in 2 × SSC, 0.5% SDS and twice in 2 × SSC, 0.1% SDS at 25°C and finally twice for 10 min in 0.1 × SSC, 0.5% SDS at 65°C.

Miscellaneous

Plasmid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979), followed by centrifugation in caesium chloride–ethidium bromide gradients. Mini-preparations of plasmid DNA were performed according to the method of Birnboim and Doly (1979), which was modified for direct sequencing to include an RNase treatment and a polyethylene glycol precipitation of the plasmid DNA.

Preparation of the rabbit reticulocyte lysates and cell-free protein synthesis, programmed by *N. crassa* poly(A)$^+$ mRNA, was performed essentially as described before (Pelham and Jackson, 1977), except where stated [^{35}S]cysteine (sp. act. 1100–1400 Ci/mmol, Amersham) instead of [^{35}S]methionine (1000 Ci/mmol, Amersham) was used in an amino acid mixture which lacked unlabelled cysteine.

In vitro transcription of the cytochrome *c* cDNA inserts, cloned into the *Pst*I sites of the pDS5 plasmid was carried out as described (Stüber *et al.*, 1984). After an ethanol precipitation step, capped transcripts were translated in a rabbit reticulocyte lysate system (Pelham and Jackson, 1977). Following translation a post-ribosomal supernatant was prepared by centrifugation for 30 min at 226 000 *g* (Beckman, Ti50 rotor) which was then made iso-osmotic (for mitochondria) by the addition of sucrose to a final concentration of 0.25 M. Analysis of translation products was carried out by SDS–PAGE (Laemmli, 1970) and fluorography (Amplify, Amersham).

Transformation of *E. coli* strains 5K and HB101 was performed according to the method of Hanahan (1983).

Acknowledgements

The authors would like to thank Dr H.Bertrand, Regina, for the generous gift of the *cyc1-1* mutant and Dr W.Sebald, Würzburg, for assistance given in the initial cDNA cloning procedures. We are also grateful to Dr R.Mertz, Genzentrum München, for synthesizing the oligonucleotides used in the sequence analysis. We thank Dr H.Domdey, Genzentrum München, and Dr D.W.Nicholson for critically reading the manuscript. We also appreciate very much the assistance provided by Dr D.W.Nicholson with the heme lyase assay. The authors would also like to acknowledge the work of Axel Laminet. This work was supported by the Genzentrum München, and a fellowship to R.A.S. from the Deutscher Akademischer Austauschdienst.

References

Arends,H. and Sebald,W. (1984) *EMBO J.*, **3**, 377–382.
Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
Boss,J.M., Gillam,S., Zitomer,R.S. and Smith,M. (1981) *J. Biol. Chem.*, **256**, 12958–12961.
Chen,E.J. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Denhardt,D.T. (1966) *Biochem. Biophys. Res. Commun.*, **23**, 641–646.
Douglas,M.G., Geller,B.L. and Emr,S.D. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3983–3987.

Grunstein,M. and Hogness,D. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 3961–3965.
Gubler,U. and Hoffman,B.J. (1983) *Gene*, **27**, 263–269.
Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580.
Harmey,M.A. and Neupert,W. (1985) In Martonosi,A. (ed.), *The Enzymes of Biological Membranes*. Plenum, New York, Vol. 4, pp. 431–464.
Hase,J., Müller,U., Riezman,H. and Schatz,G. (1984) *EMBO J.*, **3**, 3157–3164.
Heller,J. and Smith,E.L. (1966) *J. Biol. Chem.*, **241**, 3165–3180.
Hennig,B. and Neupert,W. (1981) *Eur. J. Biochem.*, **81**, 533–544.
Hennig,B., Köhler,H. and Neupert,W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4963–4967.
Hubacek,J. and Glover,S.W. (1970) *J. Mol. Biol.*, **50**, 111–127.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149–3156.
Hurt,E.C., Pesold-Hurt,B., Suda,K., Oppliger,W. and Schatz,G. (1985) *EMBO J.*, **4**, 2061–2068.
Kinnaird,J.H. and Fincham,J.R.S. (1983) *Gene*, **26**, 253–260.
Kruse,B. and Sebald,W. (1985) In Papa,S., Altendorf,K., Ernster,L. and Packer,L. (eds), *H^+-ATPase (ATP Synthase): Structure, Function, Biogenesis: The F_0F_1 Complex of Coupling Membranes*. ICSU Press, Italy.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Lederer,F. and Simon,A.M. (1974) *Biochem. Biophys. Res. Commun.*, **56**, 317–328.
Limbach,K.J. and Wu,R. (1983) *Nucleic Acids Res.*, **11**, 8931–8950.
Limbach,K.J. and Wu,R. (1985a) *Nucleic Acids Res.*, **13**, 617–630.
Limbach,K.J. and Wu,R. (1985b) *Nucleic Acids Res.*, **13**, 631–644.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.
Matsuura,S., Arpin,M., Hannum,C., Margoliash,E., Sabatini,D.D. and Morimoto,T. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4368–4372.
Michel,R., Wachter,E. and Sebald,W. (1979) *FEBS Lett.*, **101**, 373–376.
Mihara,K. and Sato,R. (1985) *EMBO J.*, **4**, 769–774.
Montgomery,D.L., Leung,D.W., Smith,M., Shalt,P., Faye,G. and Hall,B.D. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 541–545.
Nicholson,D.W. and Neupert,W. (1987) In Das,R.C. and Robbins,P.W. (eds), *Protein Transfer and Organelle Biogenesis*. Academic Press, New York, in press.
Nicholson,D.W., Köhler,H. and Neupert,W. (1987) *Eur. J. Biochem.*, **164**, 147–157.
Parnes,J.R., Velan,B., Felsenfeld,A., Ramanathan,L., Ferrini,U., Appela,E. and Seidman,J.G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2253–2257.
Pelham,H.R.B. and Jackson,R.J. (1976) *Eur. J. Biochem.*, **67**, 247–256.
Pfanner,N. and Neupert,W. (1985) *EMBO J.*, **4**, 2819–2825.
Proudfoot,N.J. and Brownlee,G.G. (1976) *Nature*, **263**, 211–214.
Rietveld,A., Ponjee,G.A.E., Schiffers,P., Jordi,W., van de Coolwijk,P.J.F.M., Demel,R.A., Marsch,D. and de Kruijff,B. (1985) *Biochim. Biophys. Acta*, **818**, 398–409.
Riezman,H., Hase,T., van Loon,A.P.G.M., Grivell,L.A., Suda,K. and Schatz,G. (1983) *EMBO J.*, **2**, 2161–2168.
Rigby,P.W.J., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.*, **113**, 237–251.
Russell,P.R. and Hall,B.D. (1982) *Mol. Cell. Biol.*, **2**, 106–116.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Scarpulla,R.C. and Wu,R. (1983) *Cell*, **32**, 473–482.
Scarpulla,R.C., Agne,K.M. and Wu,R. (1981) *J. Biol. Chem.*, **256**, 6480–6486.
Scarpulla,R.C., Agne,K.M. and Wu,R. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 739–743.
Sheldon,R., Jurale,C. and Kates,J. (1972) *Proc. Natl. Acad. Sci. USA*, **69**, 419–421.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Stüber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143–3148.
Taniuchi,H., Basile,G., Taniuchi,M. and Veloso,D. (1983) *J. Biol. Chem.*, **258**, 10963–10966.
Viebrock,A., Perz,A. and Sebald,W. (1982) *EMBO J.*, **1**, 565–571.
Villa-Komaroff,L., Efstratiadis,A., Broome,S., Lomedico,P., Tizard,R., Naker, S.P., Chick,W.L. and Gilbert,W. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 3727–3731.
Zimmermann,R. (1986) In Strauss,A.W., Boime,I. and Kreil,G. (eds), *Protein Compartmentalization*. Springer Verlag, New York, pp. 119–136.
Zimmermann,R., Paluch,U. and Neupert,W. (1979) *FEBS Lett.*, **108**, 141–146.
Zimmermann,R., Hennig,B. and Neupert,W. (1981) *Eur. J. Biochem.*, **116**, 455–460.

Received on March 31, 1987; revised on April 22, 1987