The mitochondrially made subunit 2 of *Neurospora crassa* cytochrome aa_3 is synthesized as a precursor protein

Paul van den Boogaart, Simon van Dijk and Etienne Agsteribbe

Laboratory of Physiological Chemistry, State University, Bloemsingel 10, 9712 KZ Groningen, The Netherlands

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1. INTRODUCTION

Biogenesis of mitochondria requires the coordinate activity of two genetic systems: most of the mitochondrial proteins are synthesized in the cytoplasm on mRNAs transcribed from nuclear genes, whereas only a limited number of polypeptides is coded for by the mitochondrial genome and synthesized inside the organelle [1,2]. Most of the mitochondrially synthesized proteins are located in the mitochondrial inner membrane. Little is known about the way in which these very hydrophobic proteins are integrated into the membrane and assembled into a functional enzyme complex.

In yeast the mitochondrially made subunit 2 and in *Neurospora* subunit 1 of cytochrome aa_3 are translated as larger precursor proteins, most likely with an NH_2-terminal extension [3,4]. This indicates that in yeast and *Neurospora* mitochondria a transient NH_2-terminal 'signal sequence' might play a role in the initial targeting of integral polypeptides to the membrane [5]. Here, we present evidence that in *Neurospora crassa* also subunit 2 of cytochrome aa_3 is translated as a precursor protein with an NH_2-terminal extension. Its 'signal sequence' is compared with that of the corresponding pre-protein of yeast.

2. EXPERIMENTAL

Mitochondrial DNA from *Neurospora crassa* strain ANT-1 was isolated as in [6,7]. After restriction with *Eco*RI, according to the directions of the manufacturer (Boehringer, Mannheim), the fragments were separated by electrophoresis on a 0.7% agarose gel in 40 mM Tris–HCl (pH 7.8), 20 mM sodium acetate and 2 mM EDTA. *Eco*RI fragment 4 was recombined with pBR322 and cloned in *E. coli* C600 according to [8–10]. Restriction enzyme mapping followed the procedure outlined in [11]. The gene for subunit 2 of *Neurospora crassa* cytochrome oxidase was localized on the restriction map by hybridizing a nick-labeled [12], yeast mitochondrial DNA probe containing the subunit 2 gene [13] to restriction enzyme digests of *Eco*RI fragment 4 of *Neurospora* mitochondrial DNA that had been electrophoretically separated and fixed to a nitrocellulose filter [14]. The yeast probe, clone pMT36, was a generous gift from Dr T.D. Fox (Biocenter, Basel). Hybridizations were carried out in 0.1% sodium dodecyl sulfate, 0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll and 3×SSC at 55°C as in [6]. Nucleotide sequence determination of appropriate restriction fragments followed the protocol detailed in [15].

3. RESULTS AND DISCUSSION

We have shown that *Eco*RI fragment 4 of *Neurospora crassa* mitochondrial DNA contains the gene for subunit 2 of cytochrome aa_3 [6]. Fig.1A shows a detailed physical map of *Eco*RI fragment 4 with the positions of the ATPase subunit 9 gene [16] and the gene for subunit 2 of cytochrome aa_3 indicated. Part of the nucleotide sequence of the latter gene is given in fig.1B. The coding region has been written in triplets and translated according to the genetic code for *Neurospora* mitochondria [17]. The predicted amino acid sequence of subunit 2 is

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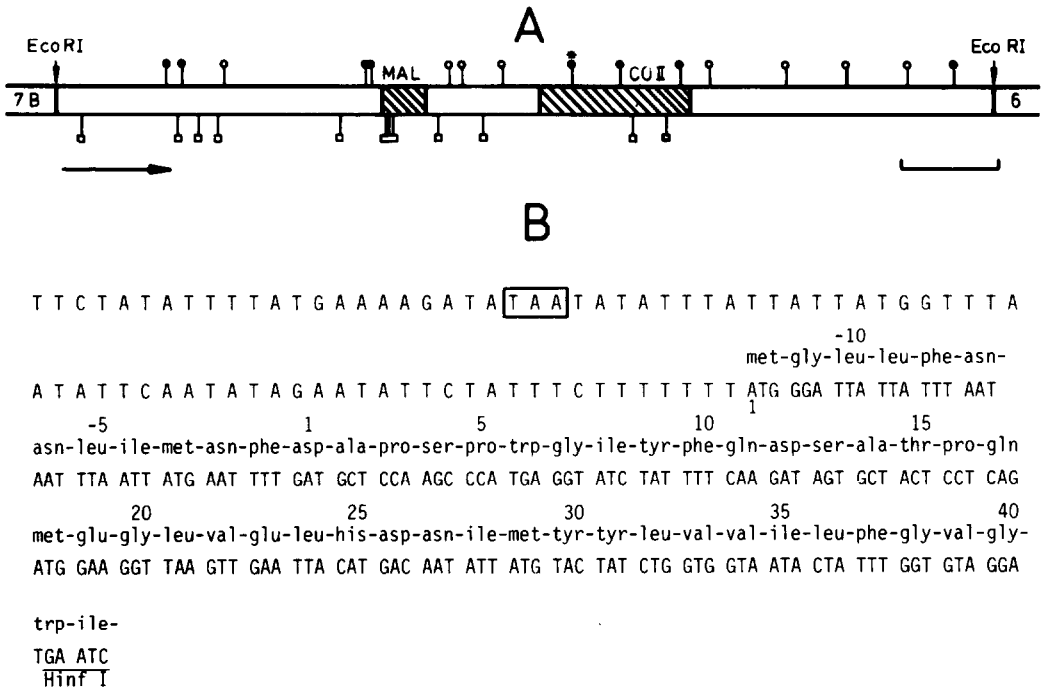

Fig.1. Sequence analysis of the *Neurospora* mitochondrial gene for cytochrome aa_3 subunit 2. (A) Physical map of *Eco*RI fragment 4 of *Neurospora* mitochondrial DNA with the positions of the mitochondrial ATPase proteolipid-like gene (MAL) [16] and the gene for subunit 2 of cytochrome aa_3 (COII). The following restriction sites are indicated: (•) *Hin*fI, (○) *Hae*III and (□) *Alu*I (not complete). The arrow indicates the direction of transcription. The scale line corresponds to a length of 500 basepairs. (B) Base sequence of part of the COII gene. The *Hin*fI site, underlined in the sequence, corresponds to the *Hin*fI site indicated by an asterisk on the physical map. The sequence of the non-coding strand is numbered starting with A of the first possible initiation codon. The ochre termination codon upstream from and in frame with this initiation codon has been boxed. The predicted amino acid sequence has been numbered starting with the NH_2-terminal aspartic acid of the mature subunit [18].

identical to that published for the mature subunit 2 of cytochrome aa_3 [18] with the exceptions:

(i) We find tyrosine at position 30 of the mature protein instead of aspartic acid as in [18];

(ii) The predicted amino acid sequence has an NH_2-terminal extension.

The existence of a larger M_r precursor for subunit 2 of cytochrome aa_3 in *Neurospora crassa* has been suggested in [18], since its amino acid sequence starts with an aspartic acid and not a formylmethionine at the N-terminus. The data in fig.1 definitively prove the existence of a reading frame for an NH_2-terminal elongated precursor for subunit 2 in *Neurospora crassa*. The length of the pre-sequence however remains unclear since the determined sequence preceding the codon specifying the first amino acid of the mature protein contains 2 in-frame initiation codons before an in-frame stop codon is encountered. The length of the pre-sequence might therefore be either 3 or 12 amino acid residues long (the remote possibility that the initiation codon is even further upstream and is spliced to the reading frame of fig.1B is not further considered here).

In yeast, subunit 2 of cytochrome aa_3 is synthesized as a precursor protein which is 1500 M_r larger than mature subunit 2 and most likely is a form of subunit 2 with an NH_2-terminal extension [3]. Moreover, if one compares the amino acid sequence of subunit 2 from beef heart cytochrome aa_3 [19] with that predicted from the corresponding gene from yeast [20] it appears that the first 16 amino acids of the yeast protein are absent in the beef heart subunit 2. It is quite likely therefore that

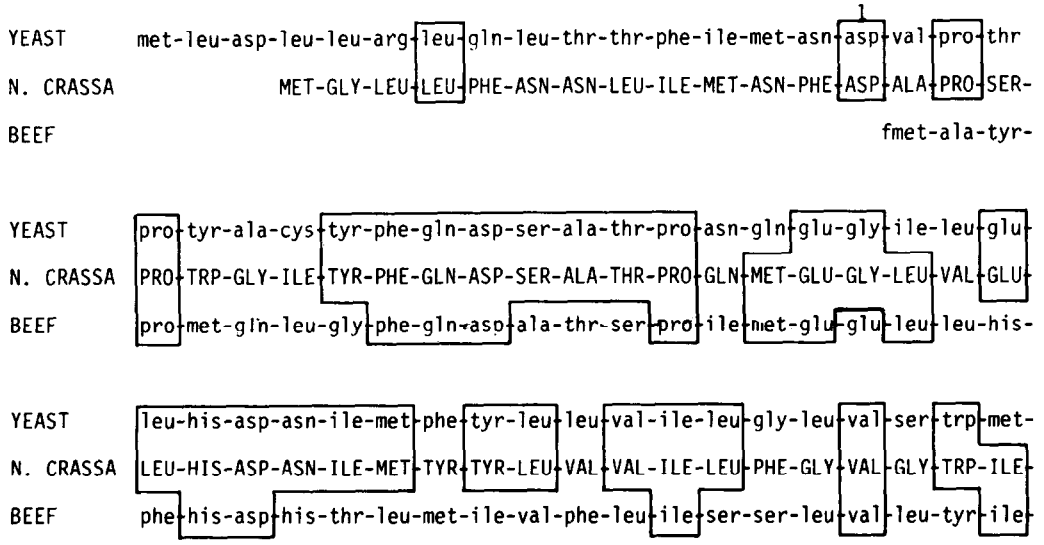

Fig.2. Comparison of the predicted amino acid sequence of *Neurospora* cytochrome oxidase subunit 2 with the sequence of the corresponding proteins from yeast and beef heart. The predicted *Neurospora* sequence is written in block type; the amino acid sequence of the yeast protein [20] is given above and for the beef protein [19] below the *Neurospora* sequence. The first amino acid of the mature *Neurospora* protein has been numbered 1. Amino acid sequence identities between *Neurospora* and yeast and between *Neurospora* and beef heart have been boxed.

the pre-sequence of the yeast protein is located entirely at the NH_2-terminus and would thus be ~15 amino acid residues long. This number comes close to that of 12 amino acid residues which the pre-sequence of the *Neurospora* protein probably contains.

Taken together these considerations have led to the homology comparison between subunit 2 from beef heart, yeast and *Neurospora* cytochrome aa_3 as indicated in fig.2. As expected the homology between the two fungal proteins is much higher than the homology between the beef heart protein and either one of its fungal counterparts. However, it is interesting to note that whereas the homology between mature subunit 2 from *Neurospora* and the corresponding part of the yeast protein is almost 65%, a homology between both pre-sequences is almost absent. The only remarkable identical sequence is Ile–Met–Asn, which in yeast is contiguous to the first amino acid of the mature protein. In *Neurospora* this sequence is separated by one amino acid residue from the N-terminal amino acid. The absence of homology in primary structures indicates that the possible similar function of the pre-sequence in both organisms is not directly related to primary structure as is the case with the signal sequence found on a large number of secretory proteins [5]. The occurrence of co-translational processing would of course strongly support the idea that the pre-sequences of subunit 2 from both organisms are functionally similar to such a signal sequence. It has been shown that processing occurs very rapidly [18]; however, direct evidence for cotranslational processing is still lacking. Alternatively, the lack of homology between both mitochondrial pre-sequences might point to a different function for each pre-sequence. Both organisms need not necessarily follow the same process for assembly of cytochrome aa_3. For instance, in *Neurospora* subunit 1 of cytochrome aa_3 is translated as a larger precursor protein [4]. In man no precursor for this subunit exists [21]. Since the predicted amino acid sequence of subunit 1 from yeast cytochrome aa_3 shows a homology of $>50\%$ with the human protein immediately from the NH_2-terminus [22], presumably no precursor for this subunit in yeast exists. Therefore a difference may exist between the integration process of this subunit in the inner mitochondrial membrane and/or assembly with the other subunits of

cytochrome aa_3 in *Neurospora* and yeast. Detailed analysis of such differences might shed more light on the specific as well as the general features of the biosynthesis of the enzyme complexes of the mitochondrial inner membrane.

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