Alignment of the amino terminal amino acid sequence of human cytochrome c oxidase subunits I and II with the sequence of their putative mRNAs

Anne Chomyn, Michael W. Hunkapiller and Giuseppe Attardi

Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA

Received 1 December 1980

ABSTRACT

Thirteen of the first fifteen amino acids from the NH$_2$-terminus of the primary sequence of human cytochrome c oxidase subunit I and eleven of the first twelve amino acids of subunit II have been identified by microsequencing procedures. These sequences have been compared with the recently determined 5'-end proximal sequences of the HeLa cell mitochondrial mRNAs and unambiguously aligned with two of them. This alignment has allowed the identification of the putative mRNA for subunit I, and has shown that the initiator codon for this subunit is only three nucleotides away from the 5'-end of its mRNA; furthermore, the results have substantiated the idea that the translation of human cytochrome c oxidase subunit II starts directly at the 5'-end of its putative mRNA, as had been previously inferred on the basis of the sequence homology of human mitochondrial DNA with the primary sequence of the bovine subunit.

INTRODUCTION

Human cytochrome c oxidase has been recently isolated from placental material and its subunit composition, characterized.$^1$ Furthermore, it has been shown that, in HeLa cells, as in yeast and Neurospora crassa,$^2$ the three largest among the seven polypeptide components of the enzyme are synthesized in mitochondria, and are in fact among the major mitochondrial translation products in these cells.$^1$ Previous evidence indicating that all the mRNAs utilized for mitochondrial translation in HeLa cells are endogenous$^3,4$ had given support to the idea that the three mitochondrially synthesized subunits of the human enzyme are encoded in mitochondrial DNA (mtDNA), as is true for the homologous subunits of the yeast enzyme.$^5,6$ The recent progress in the sequence analysis of human mtDNA and mitochondrial RNA has made it possible to verify this idea and to investigate in detail the mtDNA sequences coding for the three subunits. Thus, the gene for cytochrome c oxidase subunit II (COII) has been mapped in human mtDNA on the basis of the homology existing between the amino acid sequence of subunit II of the bovine enzyme and an amino acid sequence encoded in a stretch of human mtDNA.$^7$ A striking result of this analysis has been that the reading frame for human COII appears to be joined end-to-end, on its 5'-side, to the tRNA$^{\text{Asp}}$ gene. More recently, a sequencing analysis of the mitochondrial poly(A)-containing RNA mapping in
the region of the COII gene (RNA 16, in the classification of Amalric et al.\textsuperscript{8} (Fig. 1))\textsuperscript{9} has revealed that this RNA starts directly with the presumptive initiator codon of COII.\textsuperscript{10} This close proximity of the initiator codon to the 5'-end of the mRNA may be a common feature of human mitochondrial mRNAs. A recent sequence analysis has shown that nine of the ten identified mitochondrial heavy (H)-strand coded mRNAs from HeLa cells have a triplet AUG or AUA (AUA is also a methionine codon in human mitochondria)\textsuperscript{11} at or very close to their 5'-end;\textsuperscript{12,13} in the case of the tenth mRNA, which starts with an AUU, there is suggestive evidence that this triplet as well functions as an initiator codon for the corresponding polypeptide.\textsuperscript{12} In the present work, we have provided direct evidence that the AUG following the tRNA\textsuperscript{Asp} gene is indeed the initiator codon for the amino acid sequence of human cytochrome c oxidase subunit II. Furthermore, we have identified the mRNA for cytochrome c oxidase subunit I (COI) and shown that, in this RNA (RNA 9), the initiator codon (AUG) is located three nucleotides from the 5'-end. While this work was in progress, we learned from personal communication that F. Sanger and collaborators have identified in human mtDNA the reading frame of COI by comparison with the amino acid sequence of the bovine subunit.\textsuperscript{14}

Figure 1. Portion of the HeLa cell mtDNA genetic and transcription map illustrating the COI and COII gene region. The diagram shows the precise positions of the H-strand transcripts corresponding to the COI and COII genes as well as the positions of several H-strand (◼) and L-strand coded (●) tRNA genes (modified from ref. 9). The positions and identities of the tRNA genes were derived from the mtDNA sequence (ref. 7, 14; F. Sanger and B. Barrell, personal communication). The hatched bars represent the H-strand transcripts numbered according to the classification of Amalric et al. The scale in the upper portion of the diagram measures the distance in kilobases from the origin of replication in the direction of H-strand transcription (indicated by the rightward arrow in the lower part of the Figure). H: heavy strand; L: light strand.
MATERIALS AND METHODS

Materials. Sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide, N,N,N',N'‐tetramethylethylenediamine (TEMED), ammonium persulfate, and SM2 beads were purchased from Biorad; "Ultra‐pure" urea, from Schwarz‐Mann; and all the other reagents, from Sigma.

Isolation of Subunits. Cytochrome c oxidase was isolated according to the procedure of Hare et al.1 from human placenta and from human heart tissue derived from autopsy material. Fractionation on SDS/urea polyacrylamide gel of the subunits of the purified enzyme was carried out as described, with the following modification. The enzyme solution, after the last purification step and prior to gel electrophoresis, was shaken with SM2 beads: this step had the purpose of reducing, by adsorption to the beads, the concentration of Triton X‐100,15 which limits considerably the amount of enzyme that can be loaded on a preparative SDS/urea gel.

For the isolation of the individual subunits for sequence analysis, two procedures were used: in one procedure, the entire gel was fixed, stained and destained,16 and gel slices corresponding to the COI and COII bands were excised, neutralized with phosphate buffered saline and stored frozen at −20°C until use; in the other procedure, a longitudinal slice cut out of each lane containing the human enzyme was stained, destained, and used as a guide in cutting bands of the frozen, unstained gel corresponding to COI and COII. Protein was eluted from the stained gel fragments by shaking in 8 M urea, 6% SDS, 0.1 M phosphoric acid, adjusted to pH 8 with Tris, for two days at 25°C, and from unstained gel slices according to the method of Anderson et al.17 The protein was dialyzed first against 0.15 M NaCl, 0.1% SDS for 50 h; then against 0.1% SDS for 19 h; and finally against 0.02% SDS for 44 h. The dialyzing solutions also contained 0.01 M NaHCO₃ and 0.001 M dithiothreitol or 0.001 M β‐mercaptoethanol or 0.01 M thioglycolate. The protein solution was finally lyophilized.

Sequence Analysis. Since the bovine COI and COII are blocked at their NH₂‐termini by formyl groups,18,19 we anticipated the presence of blocked NH₂‐termini in the human subunits as well. Therefore, human COI and COII were exposed to deblocking conditions, i.e., 0.5 M HCl in methanol,20 for two or three days at 25°C, and then lyophilized; in order to hydrolyze glutamyl and aspartyl methyl esters which may have formed during the deblocking treatment,19 the samples were dissolved in 0.5 M NH₄OH and incubated overnight at room temperature. Automated Edman degradation was performed on 5 to 25 μg of protein at a time in a spinning cup sequenator designed for sequencing picomole to nanomole quantities of protein.21 Phenylthiohydantoin (Pth) amino acids were identified by high pressure liquid chromatography (HPLC) on a DuPont Zorbax CN column.
RESULTS

Isolation of cytochrome c oxidase subunits I and II. Figure 2 shows the electrophoretic pattern in an SDS-urea polyacrylamide gel of human cytochrome c oxidase purified from human placenta according to the method of Hare et al.\textsuperscript{1} up to the DEAE-cellulose chromatography step (lane b), and, for comparison, the pattern of the bovine enzyme purified by the Capaldi and Hayashi procedure\textsuperscript{22} (lane a). The bovine enzyme exhibits the typical seven subunits, with a small amount of high molecular weight contaminants and, in addition, three contaminants reproducibly observed in this type of preparation,\textsuperscript{1,22} one migrating between subunits IV and V and two others between subunits V and VI (they presumably correspond to the impurities denoted 'a', 'b' and 'c',

![Figure 2. Purification of human cytochrome c oxidase subunits I and II. (a) and (b): electrophoretic patterns in SDS-urea polyacrylamide gels of beef heart cytochrome c oxidase purified according to Capaldi and Hayashi\textsuperscript{22} (a), and of human enzyme purified according to Hare et al.\textsuperscript{1} up to the stage of DEAE-cellulose chromatography (b); the numbers near the human subunits represent their previously estimated molecular weights in kilodaltons;\textsuperscript{1} (c) and (f): electrophoretic behavior of human subunits I (c) and II (f), eluted from preparative gels as described in Materials and Methods, and rerun on an SDS-urea-polyacrylamide gel in parallel with a beef heart enzyme marker [(d) and (e)].]
respectively, in Capaldi et al.). The human enzyme pattern shows also seven subunits, which migrate identically or similarly to the beef heart enzyme subunits; as previously reported, human subunit II has an electrophoretic mobility distinctly higher than the bovine COII, while human subunit I migrates somewhat slower and subunit IV somewhat faster than the homologous beef heart components. Indicated in lane b are the apparent molecular weights of the seven human subunits, as previously estimated. There is a contaminant, reproducibly observed, migrating slightly slower than subunit III, another contaminant migrating slightly faster than subunit IV and small amounts of contaminants with mobilities similar to those of the impurities, mentioned above, present in the beef heart enzyme. Although most of these contaminants can be removed by running the DEAE-cellulose purified enzyme through a sucrose gradient, in the present work, this further step of purification was not performed, in order to avoid losses, since subunits I and II appeared to be well resolved from any contaminant. The bands corresponding to these subunits were excised from stained or unstained gels, and the subunits, eluted and concentrated. In order to check the purity of the subunits and to obtain a rough estimate of the amount recovered in the elution, a portion of each subunit preparation was rerun on a gel in parallel with a known amount of beef heart enzyme marker. Figure 2 shows the electrophoretic behavior of subunit I eluted from an unstained gel (lane c) and subunit II eluted from a stained gel (lane f). No contamination of either of the two subunits by other subunits or extraneous proteins is observed; furthermore, the two subunits migrated with the same mobilities they have in the whole enzyme sample, as can be judged from comparison with the behavior of the beef heart enzyme subunits (lanes d and e). The yield of protein extracted from unfixed and unstained gel (40 to 50%) was greater than the yield from fixed and stained gels (~20%).

Protein Sequencing Analysis. The limited amounts of purified subunits available demanded the use of microsequencing procedures. In order to remove the formyl group which was expected to block the NH2-termini of human COI and COII, as is the case for the homologous bovine subunits, the two human subunits eluted from the preparative gels were treated, prior to sequencing, with methanolic hydrochloric acid for two or three days. That human COI and COII indeed have their NH2-termini blocked is indicated by the observation that a shorter deblocking treatment (2 h) gave, for an equivalent amount of protein, a considerably lower yield (<5% versus 25-30%) of Pth-amino acid at each cycle of the automated Edman degradation. In view of the limited efficiency of the deblocking treatment, the purity of the protein analyzed was essential, since any contaminating protein without a blocked NH2-terminus would form the first Pth-derivative more readily and would therefore be favored in its detection also in the following sequencing cycles. Figure 3a shows the amino terminal amino acid sequence of human COI derived after 15 cycles. All the first 15 amino acids, except the
Correlation of Protein and RNA Sequences. A comparison of the amino terminal amino acid sequences of human COI and COII determined here with the 5'-end proximal sequences of the ten presumptive mitochondrial H-strand coded mRNAs identified in HeLa cells has allowed the unambiguous alignment of the two protein sequences with two RNA sequences. In fact, a screening of all RNA sequences reveals that only the 5'-end proximal sequence of RNA 9 (Fig. 3a) could fit the COI amino terminal amino acid sequence. RNA 5, for which only a partial sequence of a very short 5'-end proximal region had been determined (AY---Y--G, where Y stands for pyrimidine), due to the extremely minute amounts of material available, could also be aligned with the COI amino terminal amino acid sequence on the basis of these limited data; however, the corresponding DNA sequence, identified first from transcription mapping data and then by alignment with the human mtDNA sequence (F. Sanger and B. Barrell, personal communication) is ATAACCATATG, which would not fit the observed amino acid sequence. The 5'-end proximal sequence of all other putative mitochondrial mRNAs showed varying degrees of incompatibility with the COI sequence, with 2 out of 4 to 11
out of 13 inconsistencies in different cases (computed using the human mitochondrial genetic code\textsuperscript{11} and the experimentally determined RNA sequences, with the pyrimidine undetermined).\textsuperscript{10,13} In Figure 3a it should be noticed that the sixth amino acid from the amino terminal of COI, i.e., tryptophan, corresponds in the RNA sequence to UGA, an observation which confirms the previously reached conclusion about the use of the 'opal' termination codon as tryptophan codon in human mitochondria.\textsuperscript{7} From the RNA 9 sequence it appears that the ninth and tenth codons are a serine and threonine codon, respectively. The failure to identify the ninth and tenth amino acid in the microsequencing analysis is presumably explained by the fact that the Pth derivatives of these amino acids are obtained in much lower yields than those of the other amino acids, and are therefore more difficult to detect when they are present in low concentrations.

As concerns the COII amino terminal amino acid sequence, the only 5'-end proximal RNA sequence with which it could be aligned was that of RNA 16; this RNA had been previously identified as the putative COII mRNA on the basis of mapping data and of an alignment of the RNA sequences with the DNA sequence of the COII gene.\textsuperscript{7,9,10} All other RNA sequences exhibited from 8 out of 11 to 5 out of 5 inconsistencies with the COII sequence.

**DISCUSSION**

The unusual features of the 5'-end terminal sequences of human mitochondrial mRNAs, which all appear to start at the initiator codon or to have only a few nucleotides preceding this codon,\textsuperscript{12,13} have made it possible, in the present work, to align the amino terminal amino acid sequence of human COI and COII with the corresponding RNA sequence. For subunit I, this alignment has led to the identification of RNA 9 as its putative mRNA. This RNA is one of the three, among the mRNAs identified and mapped in HeLa cell mtDNA, which is long enough to accommodate the COI coding sequence,\textsuperscript{9} and the only mRNA whose 5'-end proximal sequence could be aligned with the amino terminal amino acid sequence determined here. The apparent lack of intervening sequences in animal cell mitochondrial genes\textsuperscript{9,24} strongly suggests that the whole COI sequence is encoded in RNA 9. The sequences coding for this RNA are located in the HeLa cell mtDNA map between coordinates 34/100 and 44/100 (relative to the origin taken as 0/100), and are flanked, on the 5'-side, by a group of four L-strand tRNA genes and, on the 3'-side, by the tRNA\textsuperscript{Ser} gene of the H-strand\textsuperscript{9} (Fig. 1). From the human mtDNA transcription map\textsuperscript{9} it appears that the sequences coding for poly(A)-containing RNA 6 overlap the RNA 9 coding stretch, extending beyond this on the 5'-side, so as to include the sequences complementary to the four L-strand tRNA genes, and coinciding with the RNA 9 coding sequence at the 3'-end.
(Fig. 1). However, there are good reasons for believing that RNA 6 is not utilized in vivo as COI mRNA. Rather, this RNA, because of its presence in only marginal amounts in partially purified polysomal structures and of its relatively short half-life, is very probably a precursor of RNA 9, which would derive from RNA 6 by removal of the 5'-end proximal stretch containing the sequences complementary to the four L-strand coded tRNA species. The synthesis of this RNA segment on the 5'-side of RNA 9, which extends as far as to reach the 3'-end of the tRNA\textsuperscript{Trp} (Fig. 1), is presumably a result of the mode of H-strand transcription: this transcription in fact, appears to occur in the form of a single molecule, which is processed by precise endonucleolytic cleavages before and after each tRNA sequence to yield the mature products or, in some cases, processing intermediates, like the putative precursors of the rRNAs (RNA 4) and of RNA 9 (RNA 6).

An interesting result of the alignment of the amino terminal amino acid sequence of human COI with RNA 9 is that the initiator codon for this polypeptide is situated at three nucleotides from the 5'-end. Similarly, the alignment of the amino terminal sequence of human COII with RNA 16 has provided direct evidence that the 5'-terminal AUG of this RNA is indeed the initiator codon for the polypeptide, as had been previously inferred on the basis of the sequence homology of human mtDNA with the primary sequence of the bovine subunit. Other observations, based on a comparison with the sequences of the yeast cytochrome b and cytochrome c oxidase subunit III (COIII)\textsuperscript{6} genes, have recently led to the assignment of the cytochrome b and COIII genes to two reading frames in mtDNA and to the identification of the corresponding poly(A)-containing RNAs, RNA 11 and RNA 15, as cytochrome b and COIII mRNA, respectively: in these RNAs as well, the AUG found immediately at the 5'-end appears to be the initiator codon for the corresponding polypeptide. Thus, it seems reasonable to extrapolate from these results to interpret the terminal or subterminal AUG, and probably also the 5'-proximal AUU or AUU, of the other H-strand coded mRNAs likewise as initiator codons.

The observation that most, if not all human mitochondrial mRNAs either start directly at the initiator codon or have only a few nucleotides preceding this codon indicates that in these mRNAs, in contrast to the known prokaryotic and eukaryotic mRNAs, ribosome attachment can occur with adequate efficiency in the absence of a 5'-noncoding stretch. It is conceivable that special features of human and, in general, mammalian mitochondrial ribosomes or some initiation factor make them suitable for recognizing and binding directly to a terminal or subterminal initiator codon. It is also conceivable that the secondary structure of these mRNAs is such that it would exclude all internal sites containing AUG, AUU or AUU codons, exposing only the terminal or subterminal initiator codons.
The present results also have relevance to the question of the possible processing of the cytochrome c oxidase subunits. The putative reading frame for subunit one in human mtDNA is 1539 nt long (F. Sanger and B. Barrell, personal communication), i.e., about 40% longer than required to account for the molecular weight of this subunit, as estimated from its electrophoretic mobility in SDS-urea polyacrylamide gels (43,600 daltons). Similarly, the reading frame for subunit II is 681 nt long, i.e., about 35% longer than expected for a polypeptide with an estimated molecular weight of 20,100 daltons. While this difference is probably due to a great extent to the inaccuracy of molecular weight determinations of hydrophobic proteins in SDS-urea polyacrylamide gels, the question has to be posed whether the above mentioned reading frames code for precursors destined to be processed to mature products by removal of amino- or carboxyl-terminal segments. In yeast, there is suggestive evidence from in vitro synthesis experiments and from DNA sequence data that the COII polypeptide may be synthesized in form of a precursor with a transient leader sequence. In Neurospora crassa, there is likewise evidence for the occurrence of amino terminally extended precursor polypeptides for COI and COII. The results presented here, indicating that in human cytochrome c oxidase the amino terminal methionine of the mature COI is blocked, presumably by a formyl group, and furthermore that this methionine corresponds to the second codon of the putative COI mRNA, tend to exclude the possibility that any processing of the newly synthesized COI polypeptide occurs at the NH2-terminus. However, any definitive conclusion about the possible occurrence of longer precursors of human COI must await the determination of the sequence at the COOH-terminus of the mature subunit. In the case of the COII of the beef heart enzyme, a perfect correspondence has been found between the length of the polypeptide and that of the homologous human mtDNA sequence, a finding which argues strongly against the existence of longer precursors of the bovine subunit. The present results extend the above conclusion to the human COII as concerns the NH2-terminus; if further work will prove that the same applies to the COOH-terminus, a factor other than a difference in the length of the polypeptide chain must be responsible for the higher electrophoretic mobility consistently observed for the human COII as compared to the bovine subunit.

ACKNOWLEDGEMENTS

This work was supported by research grants from the USPHS (GM-11726) and the Weingart Foundation and was done under the tenure of a Research Fellowship of the American Heart Association, Greater Los Angeles Affiliate, to A. C. We are very grateful to Drs. F. Sanger and B. Barrell for communicating to us mitochondrial DNA sequence data and sending us a manuscript prior to publication and to Dr. E. Ching for a
gift of bovine cytochrome c oxidase.

REFERENCES