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CONSTRUCTION OF A cDNA CLONE FOR A NUCLEAR-CODED SUBUNIT OF CYTOCHROME c OXIDASE FROM RAT LIVER

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A cDNA library for 6S-9S poly(A)-containing RNA from rat liver was constructed in E. coli. Initial screening of the clones was carried out using single stranded ^{32}P-labeled cDNA prepared against poly(A)-containing RNA isolated from immunoadsorbed polyribosomes enriched for the nuclear-coded subunit messenger RNAs of cytochrome c oxidase. One of the clones, pC089, was found to hybridize with the messenger RNA for subunit VIC. The DNA sequence of the insert in pC089 was carried out and it has got extensive homology with the C-terminal 33 amino acids of subunit VIC from beef heart cytochrome c oxidase. In addition, the insert contained 146 bp, corresponding to a portion of the 3'-non-coding region. Northern blot analysis of rat liver RNA with the nick-translated insert of pC089 revealed that the messenger RNA for subunit VI would contain around 510 bases.

An understanding of the organization of nuclear genes coding for mitochondrial proteins is essential to evaluate the interaction between nuclear and mitochondrial genes involved in the biogenesis of the mitochondrion. Cytochrome c oxidase, an inner membrane protein of the mitochondrion affords a good model system, since this multi-subunit protein is coded for by the mitochondrial and nuclear genes (1). This protein also serves as a

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good system to study the mechanism of translocation of the nuclear-coded subunits into mitochondria. A variety of studies in yeast, Neurospora crassa and rat liver have revealed that the nuclear-coded mitochondrial inner membrane/matrix proteins are synthesized on free polyribosomes, mostly as precursors, released into the cytosol and taken up by mitochondria through specific receptor-mediated mechanisms (2-4). However, evidences are now available in rat liver indicating that atleast in the case of cytochrome c oxidase, endoplasmic reticulum may be involved in the synthesis and translocation of the nuclear-coded subunits into mitochondria (5-6).

Thus, construction of cDNA clones for the nuclear-coded subunits of cytochrome c oxidase would be very useful in identifying genomic clones for these polypeptides as well as to quantify messenger RNA contents in various polyribosome fractions. The present study describes the construction and characterization of a cDNA clone for subunit VIC of rat liver cytochrome c oxidase.

EXPERIMENTAL PROCEDURES

For purposes of cloning, total polysomal, poly(A)-containing RNA from rat liver (7) was fractionated on 5-25% (w/v) sucrose density gradients and each fraction was translated in the reticulocyte cell-free system using ^{35}S-methionine (8). The 6S-9S fraction was found to code for the precursors of the nuclear-coded subunits of cytochrome c oxidase as revealed by the analysis of the immunobound material obtained with holo-cytochrome c oxidase antibody on 20% sodiumdodecylsulfate-polyacrylamide gels (9) followed by fluorography (10). This RNA fraction was converted into double stranded DNA and cloned into E. coli (χ 1776) using Pst-cut pBR 322 as vector and the GC tailing procedure (11). Tetracycline-resistant, ampicillin-sensitive clones were screened with ^{32}P-labelled cDNA prepared against purified messenger RNAs for the cytochrome c oxidase subunits. Purification of the messenger RNAs was achieved by specific immunoadsorption of polyribosomes with the holo-cytochrome c oxidase antibody employing the general protocol described by Kraus and Rosenberg (12). The cDNA insert from the positive clone (pC089) was

characterized on the basis of its size, hybrid selection of a specific messenger RNA coding for a subunit of cytochrome c oxidase, restirction mapping and DNA sequence determination by the procedure of Maxam and Gilbert after strand separation (13). The direct amino acid sequence of subunit VIC of cytochrome c oxidase was also determined (14) and the complete details of this study (Erdweg, Meinecke and Buse) will be published elsewhere. The size of the subunit messenger RNA was ascertained by northern blot analysis of microsomal RNA separated on 6M urea-2.5 % agarose gels (15), transferred to DBM paper and hybridized with nick translated insert of pC089 (11).

RESULTS

The 6S-9S polyribosomal poly(A)-containing RNA fraction from rat liver was cloned into E. coli and 90 tetracycline-resistant, ampicillin- sensitive clones were screened with ^{32}P-cDNA prepared against purified cytochrome c oxidase messenger RNAs as probe. Translation studies indicate that nearly 60-fold purification of subunit messenger RNAs has been achieved by the isolation of cytochrome c oxidase specific polyribosomes using the immunobinding procedure (Fig.1). Hybrid selection studies reveal that plasmid DNA from clone pC089 is able to hybridize with messenger RNA coding for a subunit of cytochrome c oxidase with a molecular weight around 8500 in the reticulocyte cell-free system (Fig.2). The cDNA insert liberated by Pst digestion of the plasmid DNA corresponds to a size around 243 bp (Fig.3).

Restriction enzyme analysis of the fragment indicates sites for Eco RI, Mbo II and Hinf I and the orientation of the fragment is as indicated (Fig.4a). The DNA sequence analysis of the insert reveals the presence of AATAAA sequence, which is a polyadenylation signal occurring near the 3' end of many eukaryotic messenger RNAs (16). In this particular reading frame, the cDNA-derived aminoacid sequence corresponds very closely to the sequence of 33

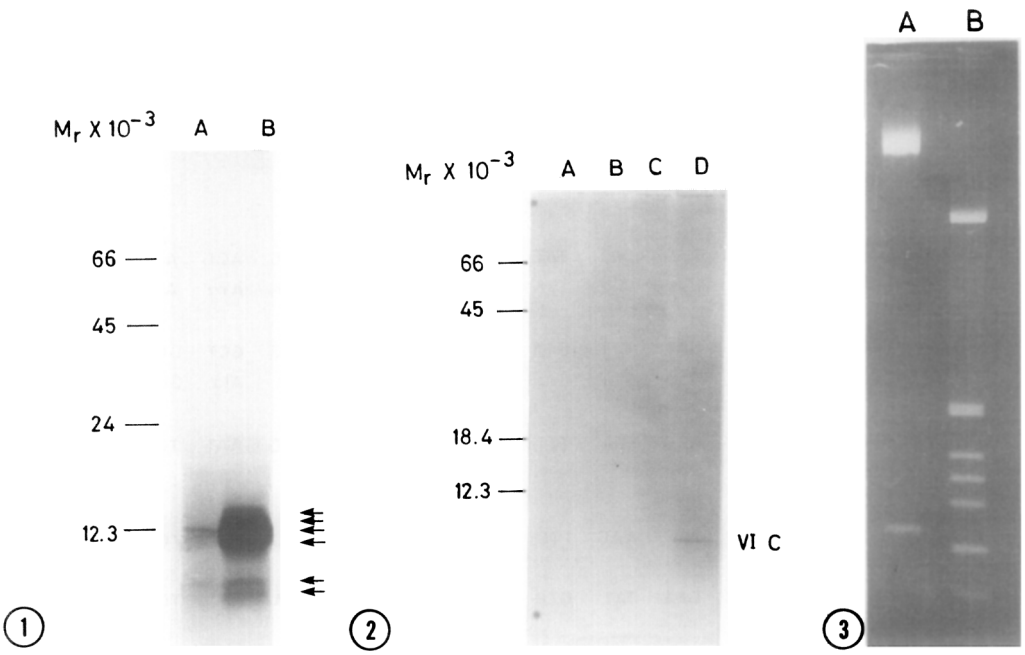
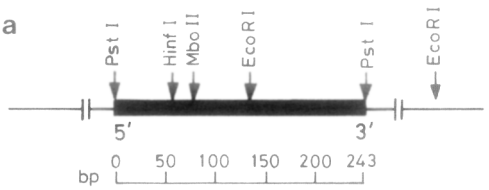


Fig.1. Translation of poly(A)-containing RNA isolated from specific polysomes isolated by immunobinding to holo-cytochrome c oxidase antibody. The experimental details are given in text. Lane A refers to the nuclear-coded subunits of cytochrome c oxidase synthesized in the reticulocyte cell-free system programmed with total polysomal, poly(A)-containing RNA (2 µg RNA used and 1.6 x 10^6 cpm processed for immunobinding). Lane B refers to similar data obtained with purified messenger RNA for the nuclear-coded subunits (330 ng RNA used for translation and 6.95 x 10^5 cpm processed for immunobinding). The gel system used was 15% acrylamide, 0.2% bisacrylamide, essentially as described by Merle and Kadenbach (21). Arrows indicate the positions of the putative precursors.

Fig.2. Translation of hybrid selected RNA with cloned pCO89. Plasmid DNAs (50 µg) from tetracycline-resistant, ampicillin - sensitive clones were loaded on to DBM paper and the hybrid-selected RNAs were analysed for their ability to code for the nuclear-coded subunits of cytochrome c oxidase in the reticulocyte cell-free system. Lane A: Endogenous cell-free products of the lysate system immunoprecipitated with holo-cytochrome c oxidase antibody. Lane B: Pattern obtained with pBR DNA. Lane C: Pattern with pCO89 DNA but using anti-BSA antibody. Lane D: Pattern obtained with pCO89 DNA using holo-cytochrome c oxidase antibody.

Fig.3. Size of the cDNA insert in pCO89. The plasmid DNA (3 µg) was cut with Pst, analysed on 2% (w/v) agarose gels and stained with ethidium bromide. Lane A: Pst-cut pCO89 DNA. Lane B: pBR DNA size markers obtained with Hinf I.



b

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5'- GAA  CCA  AGA  AAG  AAG  GCT  TAT  GCA  GAT  TTC  TAC  AGG  AAT  TAT
    Glu  Pro  Arg  Lys  Lys  Ala  Tyr  Ala  Asp  Phe  Tyr  Arg  Asn  Tyr

    GAC  TCC  ATG  AAA  GAT  TTT  GAA  GAG  ATG  AGG  CAG  GCT  GGT  GTC
    Asp  Ser  Met  Lys  Asp  Phe  Glu  Glu  Met  Arg  Gln  Ala  Gly  Val

    TTT  CAG  AGT  GCG  AAG  TGA*  TTT  CAG  AAT  GCA  AAG  AAT  TCT  TTG
    Phe  Gln  Ser  Ala  Lys

    GAT  TGT  GCT  CCA  TAG*  AAG  TTC  ACT  GCT  GAC  CTG  TGT  TCC  TGA*

    ACT  ATG  AAA  CGT  GAA  TAT  GTG  GGC  TAA  GGA  GTA  GTT  TCT  CTC

    AAT  AAA  CAT- 3'
```

c

Ser - Thr - Ala - Leu - Ala - Lys - Pro - Gln - Met - Arg - Gly - Leu - Leu - Ala - Arg - Arg - Leu - Arg - Phe - His

Ile - Val - Gly - Ala - Phe - Met - Val - Ser - Leu - Gly - Phe - Ala - Thr - Phe - Tyr - Lys - Phe - Ala - Val - Ala

Glu - [Lys] - Arg - Lys - Lys - Ala - Tyr - Ala - Asp - Phe - Tyr - Arg - Asn - Tyr - Asp - Ser - Met - Lys - Asp - Phe

Glu - Glu - Met - Arg - [Lys] - Ala - Gly - [Ile] - Phe - Gln - Ser - Ala - Lys

Fig.4. Restriction map and DNA sequence of clone pC089 and aminoacid sequence of subunit VIC of beef heart cytochrome c oxidase. (a) Thick line represents the 243 bp insert of pC089. Thin line represents the pBR DNA sequence not drawn to scale. (b) DNA sequence and the derived aminoacid sequence. *Indicates termination codons. The polyadenylation signal sequence is underlined. The tailed G residues are not shown in the sequence. (c) Direct aminoacid sequence of subunit VIC of beef heart cytochrome c oxidase. The boxed aminoacids indicate the differences with the cDNA-derived amino acid sequence. The aminoacid sequence covered by the cloned DNA is underlined.

aminoacids near the C-terminal of subunit VIC of beef heart cytochrome c oxidase (Fig.4b). The beef heart subunit VIC has 73 aminoacids and its calculated molecular weight of 8480 corresponds closely to the molecular weight of the rat liver product synthesized in the cell-free system

programmed with the hybrid-selected messenger RNA (Fig.2). At this stage, it is not clear whether the messenger for subunit VIC codes for the mature protein or a short precursor. The rat liver cDNA-derived aminoacid sequence shows three differences with the aminoacid sequence obtained directly for subunit VIC of beef heart cytochrome c oxidase (Fig.4c). The differences are probably due to species and/or tissue specificity. The cDNA sequence in addition to coding for the C-terminal 33 aminoacids of subunit VIC, contains a sequence of 146 bp representing a portion of the 3'-non-coding region. A northern blot analysis, to determine the size of the subunit VIC messenger RNA using nick-translated probe, reveals its size to be around 510 bases (Fig.5).

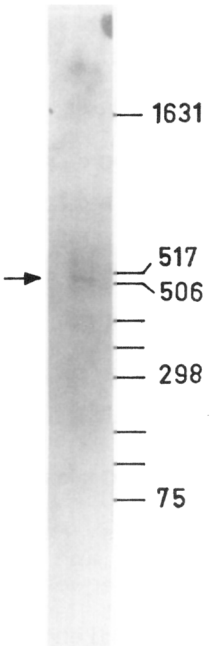

Fig.5. Northern blot analysis of rat liver microsomal poly(A)-containing RNA with nick-translated pC089. Poly(A)-containing RNA (10 µg) was analysed on 2.5 % (w/v) agarose-urea gels, blotted onto DBM paper and hybridized as described in text. Size of the subunit VIC messenger RNA was calculated using pBR 322 DNA size markers obtained with Hinf I.

DISCUSSION

It is generally held that cytochrome c oxidase consists of 7 or 8 subunits as exemplified by studies in yeast and Neurospora (17,18). Recently convincing chemical and immunochemical evidences have been provided to indicate that cytochrome c oxidase from mammalian sources contains a total of 12-13 subunits which includes the 3 subunits of mitochondrial origin (14,19-21). The exact number of nuclear-coded subunits forming an integral part of the enzyme complex is, however, being debated (21).

This is the first report where a nuclear-coded subunit messenger RNA for cytochrome c oxidase has been cloned and it corresponds to subunit VIC in the nomenclature of Kadenbach as well as Buse (19). The close similarity of the cDNA-derived amino acid sequence from rat liver with the direct aminoacid sequence of the subunit from beef heart cytochrome c oxidase reveals that atleast this subunit is conserved in cytochrome c oxidase preparations from different mammalian and tissue sources. As already indicated, this cDNA clone would be of use in identifying genomic clones and quantifying the subunit messenger RNA contents in the different polysome preparations. Preliminary studies indicate that the membrane bound polyribosome is enriched for the subunit VIC messenger RNA content.

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