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ISOLATION OF A cDNA CLONE FOR HUMAN CYTOCHROME c_1 FROM A λgt11 EXPRESSION LIBRARY

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Summary: Antiserum directed against a purified preparation of beef heart cytochrome bc_1 complex has been used to screen a human liver cDNA expression library in λgt11. The inserts of two recombinants, which gave strong signals, were found to represent cytochrome c_1 by epitope selection using nitrocellulose filters containing the expressed proteins. The amino acid sequence deduced from the nucleotide sequence of an insert DNA revealed a high degree of homology with the sequence of bovine cytochrome c_1.

Cytochrome c_1 is a key component of cytochrome bc_1 complex as an electron carrier in the mitochondrial electron-transfer chain (1). A recent study on the synthesis of this cytochrome in yeast demonstrated that it is synthesized as a precursor with an amino-terminal extension which is cleaved in two successive steps after its translocation into mitochondria (2). The primary structure of the extension sequence was deduced from the nucleotide sequence of the structural gene for yeast cytochrome c_1 precursor (3).

In the study on biogenesis of this cytochrome in higher eukaryotes, there is an indication that it is also synthesized as a larger precursor which is processed into a mature form in mitochondria (4). Although the amino acid sequence of the mature form of bovine cytochrome c_1 was determined (5), the structure of its presequence is not yet known. As an initial step to study the biogenesis of this cytochrome in mammals, we have attempted to isolate its cDNA clone from a human liver cDNA library.

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Materials and Methods

Materials: A human liver cDNA library in λgt11, which had been constructed by the method of Huynh *et al.* (6), was the product of Clontech Laboratories, Inc., Palo Alto, CA. Restriction endonucleases were obtained from Toyobo Co., Ltd., Osaka and Takara Shuzo Co., Ltd., Kyoto. Cytochrome bc_1 complex (7) and electron-transfer particles (8) from bovine heart mitochondria were prepared as described. Human liver mitochondria were prepared from the liver tissue resected in an operation on bile duct cancer. Antiserum directed against bovine cytochrome bc_1 complex was obtained as described previously (9).

Screening of the Library: The λgt11 human liver cDNA library was screened using antiserum directed against bovine heart cytochrome bc_1 complex essentially as described by Huynh *et al.* (6). The antiserum was previously passed through a column of BioRad Affigel 10 and 15 to which proteins in lysate of *E. coli* strain Y1090 had been bound, and was used at a 1 : 1000 dilution.

Selective Isolation of Antibody with Expressed Protein: Near confluent plaques of cloned bacteriophages were grown on LB agar plates (ϕ, 9 cm), and the fusion protein production was induced with isopropyl-β-D-thiogalactopyranoside-impregnated nitrocellulose filters as was done in the screening of the library. The filters were incubated with anti-cytochrome bc_1 complex antiserum diluted 1000-fold in buffer containing 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.5% skim milk at 20°C overnight. After the filters were washed three times with the above buffer containing 0.5% Triton X-100, the bound antibodies were eluted as described by Weinberger *et al.* (10). The eluted antibodies were used for Western blot analysis.

DNA Sequencing: The inserts in λgt11 recombinants were inserted into pUC19, the resulting pUC19 recombinants were prepared, and the insert DNAs were obtained as described (11). The determination of DNA sequence was performed by the chain-termination method of Sanger *et al.* (12) after subcloning of suitable restriction fragments into a bacteriophage M13 mp18 vector. A M13 sequencing kit (Takara Shuzo Co., Ltd,) was used.

Other Procedures: Protein was assayed by a modification of the Lowry method (13). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described (14) except that 18.25% gel was used. Electroblotting was carried out as described (15) unless otherwise stated, and the blotted filters were stained essentially as described by Glass *et al.* (16).

Results and Discussion

Specificity of Antiserum: To examine whether the antiserum directed against bovine cytochrome bc_1 complex cross-reacts with the corresponding human complex, bovine electron-transfer particles and a membrane preparation of human liver mitochondria were analyzed by Western blot analysis. The antiserum reacted with core proteins I and II, cytochrome c_1, iron-sulfur protein, and ubiquinone-binding protein in bovine cytochrome bc_1 complex (Fig. 1). The corresponding subunits of human cytochrome bc_1 complex were also recognized by the antiserum, although only one band was observed at the position of the core proteins. The band of ubiquinone-binding protein was not obviously seen in the photograph when electroblotting was carried out with a

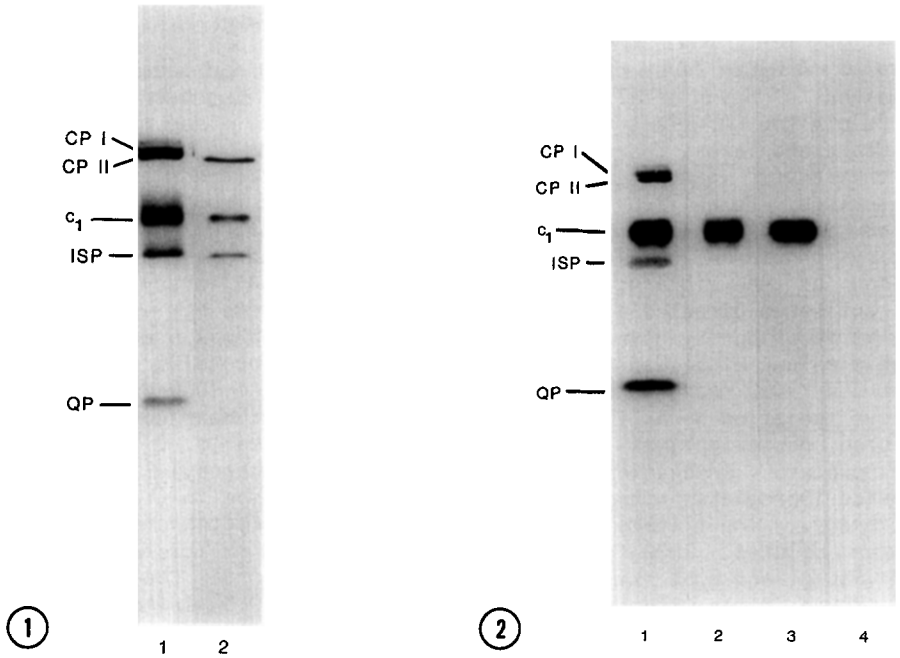

Fig. 1. Characterization of antiserum directed to bovine cytochrome bc_1 complex by Western blotting. Beef heart electron-transfer particles (6.8 µg of protein, lane 1) and a membrane preparation of human liver mitochondria (31 µg of protein, lane 2) were subjected to SDS gel electrophoresis and transferred onto a nitrocellulose filter in the presence of 0.1 % SDS. The blotted filter was stained by the peroxidase-anti-peroxidase method. CP1, core protein 1; CP2, core protein 2; c_1, cytochrome c_1; ISP, iron-sulfur protein; and QP, ubiquinone-binding protein.
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Fig. 2. Identification of cytochrome c_1 cDNA clones by epitope selection. Two recombinants, which gave strong signals, and λgt11 were used to select antibody as specified under Materials and Methods. Bovine cytochrome bc_1 complex (0.5 µg of protein each) was electrophoresed on a SDS gel and transferred to a nitrocellulose filter in buffer containing no SDS. The blotted filter was cut into each lane and stained using anti-cytochrome bc_1 complex antiserum (diluted 1:1000, lane 1) and antibodies selected with the expressed proteins of λIII17 (lane 2), λIII60 (lane 3), and λgt11 (lane 4). CP1, core protein 1; CP2, core protein 2; c_1, cytochrome c_1; ISP, iron-sulfur protein; and QP, ubiquinone-binding protein.

blotting buffer containing SDS, but it was clearly visualized when blotting buffer contained no SDS (data not shown).

Molecular Cloning of Human Cytochrome c_1 cDNA: In an attempt to obtain cDNA clones for different subunits of cytochrome bc_1 complex at one time, the antiserum containing antibodies directed against the several subunits was used to screen a λgt11 human liver cDNA library. From 5×10^5 recombinants, 9 clones, which gave strong signals, were selected. The insert DNAs prepared from all the clones were approximately 950 bp and showed apparently the same pattern of restriction fragments when digested with *Taq*I, indicating that all

the selected clones eventually represent the same protein. Two clones of these, designated λIII17 and λIII60, were analyzed in detail.

In order to determine which subunit of cytochrome bc_1 complex these clones represent, antibodies which selectively bind to the fusion proteins produced by these two clones were used to stain nitrocellulose filters onto which the subunits of bovine cytochrome bc_1 complex were electroblotted after SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2, both of the selected antibodies recognized specifically cytochrome c_1.

Nucleotide Sequence Analysis: The inserts from λIII17 and λIII60 were inserted into the cloning site of pUC19, and clones containing the respective inserts (pIII17 and pIII60) were obtained. These two inserts were found to be identical with each other by sequencing both termini of each insert: the amino acid sequence deduced from the nucleotide sequence of 5'-terminal portion were highly homologous with the sequence near the amino-terminus of bovine cytochrome c_1. The four fragments generated by *Taq*I digestion of the insert of pIII17 were sequenced, and their deduced amino acid sequences matched, with close homology, the bovine amino acid sequence determined by Wakabayashi *et al.* (5) as shown in Fig. 3. The characteristic sequence for the covalent binding of heme corresponding to Cys-37, Cys-40, and His-41 of bovine cytochrome c_1 is conserved in the human cytochrome. Met-160 of the bovine sequence, which is suggested as the sixth ligand of the heme in comparison with the yeast sequence (3), is also conserved in the human sequence. The suggestion of Met-208 of bovine cytochrome c_1 as the ligand (5) may not be true, since this methionine is not conserved in the yeast cytochrome. Like bovine and yeast cytochrome c_1, the human cytochrome contains a stretch of 15 uncharged amino acids near the carboxyl terminus. This uncharged region may play an role to anchor the protein to the mitochondrial inner membrane, as suggested by Wakabayashi *et al.* (5). Further study is in progress to clone a recombinant which possesses an insert containing the nucleotide sequence corresponding to the presequence.

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        CGT GGC CTC CTC TCT TCC TTG GAC CAC ACC AGC ATC CGG AGG GGT TTC CAG GTA TAT AAG
Human   Arg Gly Leu Leu Ser Ser Leu Asp His Thr Ser Ile Arg Arg Gly Phe Gln Val Tyr Lys
Bovine   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
         15                  20                  25                  30

        CAG GTG TGC GCC TCC TGC CAC AGC ATG GAC TTC GTG GCC TAC CGC CAC CTG GTG GGC GTG
        Gln Val Cys Ala Ser Cys His Ser Met Asp Phe Val Ala Tyr Arg His Leu Val Gly Val
         -   -   -  Ser  -   -   -   -   -   -  Tyr  -   -   -   -   -   -   -   -   -
         35                  40                  45                  50

        TGC TAC ACG GAG GAT GAA GCT AAG GAG CTG GCT GCG GAG GTG GAG GTT CAA GAC GGC CCC
        Cys Tyr Thr Glu Asp Glu Ala Lys Glu Leu Ala Ala Glu Val Glu Val Gln Asp Gly Pro
         -   -   -   -   -   -   -   -  Ala  -   -  Glu  -   -   -   -   -   -   -   -
         55                  60                  65                  70

        AAT GAA GAT GGG GAG ATG TTC ATG CGG CCA GGG AAG CTG TTC GAC TAT TTC CCA AAA CCA
        Asn Glu Asp Gly Glu Met Phe Met Arg Pro Gly Lys Leu Phe Asp Tyr Phe Pro Lys Pro
         -   -   -   -   -   -   -   -   -   -   -   -   -  Ser  -   -   -   -   -   -
         75                  80                  85                  90

        TAC CCC AAC AGT GAG GCT GCT CGA GCT GCC AAC AAC GGA GCA TTG CCC CCT GAC CTC AGC
        Tyr Pro Asn Ser Glu Ala Ala Arg Ala Ala Asn Asn Gly Ala Leu Pro Pro Asp Leu Ser
         -   -   -  Pro  -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
         95                  100                 105                 110

        TAC ATC GTG CGA GCT AGG CAT GGT GGT GAG GAC TAC GTC TTC TCC CTG CTC ACG GGC TAC
        Tyr Ile Val Arg Ala Arg His Gly Gly Glu Asp Tyr Val Phe Ser Leu Leu Thr Gly Tyr
         -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
        115                 120                 125                 130

        TGC GAG CCA CCC ACC GGG GTG TCA CTG CGG GAA GGT CTC TAC TTC AAC CCC TAC TTT CCT
        Cys Glu Pro Pro Thr Gly Val Ser Leu Arg Glu Gly Leu Tyr Phe Asn Pro Tyr Phe Pro
         -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -   -
        135                 140                 145                 150

        GGC CAG GCC ATT GCC ATG GCC CCT CCC ATC TAC ACA GAT GTC TTA GAG TTT GAC GAT GGC
        Gly Gln Ala Ile Ala Met Ala Pro Pro Ile Tyr Thr Asp Val Leu Glu Phe Asp Asp Gly
         -   -   -   -  Gly  -   -   -   -   -   -  Asn Glu  -   -   -   -   -   -   -
        155                 160                 165                 170

        ACC CCA GCT ACC ATG TCC CAG ATA GCC AAG GAT GTG TGC ACC TTC CTG CGC TGG GCA TCT
        Thr Pro Ala Thr Met Ser Gln Ile Ala Lys Asp Val Cys Thr Phe Leu Arg Trp Ala Ser
         -   -   -   -   -   -   -  Val  -   -   -   -   -   -   -   -   -   -   -  Ala
        175                 180                 185                 190

        GAG CCA GAG CAC GAC CAT CGA AAA CGC ATG GGG CTC AAG ATG TTG ATG ATG ATG GCT CTG
        Glu Pro Glu His Asp His Arg Lys Arg Met Gly Leu Lys Met Leu Met Met Met Ala Leu
         -   -   -   -   -   -   -   -   -   -   -   -   -   -   -  Leu  -   -  Gly  -
        195                 200                 205                 210

        CTG GTG CCC CTG GTC TAC ACC ATA AAG CGG CAC AAG TGG TCA GTC CTG AAG AGT CGG AAG
        Leu Val Pro Leu Val Tyr Thr Ile Lys Arg His Lys Trp Ser Val Leu Lys Ser Arg Lys
         -  Leu  -   -   -   -  Ala Met  -   -   -   -   -   -   -   -   -   -   -   -
        215                 220                 225                 230

        CTG GCA TAT CGG CCG CCC AAG TGA CCC TGT CCA GTG TCT GCT TGC CAT CCT GCC AGA ACA
        Leu Ala Tyr Arg Pro Pro Lys ***
         -   -   -   -   -   -
        235                 240

        GGC CCT AAG CCA AGA GCA •••••
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Fig. 3. Nucleotide sequence of the cDNA insert that codes for human cytochrome c_1 and its deduced amino acid sequence. The amino acid sequence of bovine cytochrome c_1 (5) is included for comparison. The dashes represent homologous amino acids. Numbering for the amino acid sequence is based on that for the sequence of mature bovine cytochrome c_1. The typical amino acid sequence for covalent binding of heme is placed in the boxes. The underlined nucleotides represent the *Taq*I sites.

Acknowledgement

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