Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 19-25

CLONING AND SEQUENCE ANALYSIS OF A cDNA ENCODING THE RIESKE IRON-SULFUR PROTEIN OF RAT MITOCHONDRIAL CYTOCHROME bc_1 COMPLEX

Morimitsu Nishikimi, Yoshitaka Hosokawa, Haruo Toda,
Hiroshi Suzuki, and Takayuki Ozawa

Department of Biomedical Chemistry, Faculty of Medicine,
University of Nagoya, Nagoya 466, Japan

Received January 4, 1989

Summary: We have isolated a cDNA clone for the Rieske iron-sulfur protein of rat cytochrome bc_1 complex, by screening a rat liver cDNA expression library using antiserum directed against the corresponding protein of bovine. The amino acid sequence deduced from the nucleotide sequence of the cDNA indicated that the mature polypeptide of the rat protein consists of 196 amino acid residues with a molecular weight of 21,465, and that it is formed as a precursor with an amino-terminal extension. Northern blot analysis indicated that rat liver possibly contains different sizes of mRNAs for the Rieske iron-sulfur protein, and Southern blot analysis demonstrated that rats and mice possess a single gene for this protein.

Cytochrome bc_1 complex of the mitochondrial respiratory chain catalyzes the transfer of electrons from ubiquinol to cytochrome c coupled to the energy transduction. The complex from bovine heart mitochondria consists of 10-11 subunits (1,2): two core proteins, cytochrome *b*, cytochrome c_1, Rieske Fe-S protein, ubiquinone-binding protein, and four to five low molecular weight subunits. The Fe-S protein possesses one [2Fe-2S] cluster (3), and is essential for the electron-transfer function of cytochrome bc_1 complex, as demonstrated by its isolation and reconstitution experiments (4,5).

The primary structure of the Rieske Fe-S protein of bovine cytochrome bc_1 complex has recently been determined by amino acid sequencing analysis (6). However, no molecular biological study has been done on the structural analysis of the gene for the Fe-S protein of higher eukaryotes, although there is much information regarding the gene structures of the Rieske Fe-S proteins in bacteria (7-10), in lower eukaryotes (11,12), and in chloroplasts (13). In this study, we have isolated a cDNA encoding rat Rieske Fe-S protein and determined its nucleotide sequence. Blot hybridization analyses of mRNA and genomic DNA for this protein have also been carried out.

0006-291X/89 $1.50

Materials and Methods

Materials: Antiserum directed against bovine Rieske Fe-S protein was raised in a rabbit as described previously (14). Total RNA was prepared from freshly-excised rat liver and human fibroblast cells as described by Derman *et al.* (15) and Chirgwin *et al.* (16), respectively. Poly(A)$^+$ RNA was obtained using oligo(dT)-Latex as described (17). High molecular weight DNA was prepared from rat and mouse spleens as described (18). A rat liver cDNA library in λgt11 was purchased from Clontech Laboratories, Inc., nitrocellulose membranes from Bio-Rad; and GeneScreen *Plus* membranes from Du Pont-New England Nuclear. Enzymes and chemicals were obtained from the following sources: restriction enzymes and T4 DNA ligase from Toyobo Co., Ltd., Osaka, Japan; a 7-deaza sequencing kit from Takara Shuzo Co., Ltd., Kyoto, Japan; calf intestinal alkaline phosphatase from Boehringer-Mannheim; and [α-^{32}P]dCTP (3000 Ci/mmol) and a multiprime DNA labeling system from Amersham.

Screening of the Library: The λgt11 rat liver cDNA library was screened using the antiserum directed against bovine Rieske Fe-S protein essentially as described by Huynh *et al.* (19). The antiserum was previously passed through a column of Bio-Rad Affigel 10 and 15 to which proteins in lysate of *Escherichia coli* strain Y1090 had been bound, and was used at a 1:1000 dilution.

DNA Sequence Analysis: The cDNA inserts in λgt11 recombinants were ligated into the *Eco*RI site of pUC19, and the recombinant plasmids were prepared. Their inserts were digested into smaller fragments with appropriate restriction enzymes for subcloning into M13 mp18. The determination of DNA sequence was carried out by a modification of the dideoxy chain termination method (20), in which 2'-deoxy-7-deazaguanosine triphosphate was used in place of dGTP (21). Analysis of sequence data was performed using the DNASIS program (Hitachi SK, Yokohama, Japan).

Northern Blot Analysis: Poly(A)$^+$ RNA (2 μg) was electrophoresed in a 1% agarose/formaldehyde gel and was transferred onto a GeneScreen *Plus* membrane as described (22). Hybridization was carried out at 60°C for 15 h in 10% dextran sulfate/1% sodium dodecyl sulfate/1 M NaCl/denatured salmon sperm DNA (100 μg/ml)/^{32}P-labeled probe DNA (5 x 10^5 dpm/ml). The membrane was successively washed twice each time with 2 x SSC at room temperature for 5 min, with 2 x SSC/1% sodium dodecyl sulfate at 65°C for 30 min, and with 0.1 x SSC at room temperature for 30 min, and were subjected to autoradiography using two intensifying screens at - 80°C for 30 h. The insert cDNA for Rieske Fe-S protein was labeled with [α-^{32}P]dCTP using a multiprime labeling system according to the manufacturer's manual (Amersham) and used as the probe.

Southern Blot Analysis: DNA (5 μg) was digested with *Bam*HI (30 units) at 37°C for 5 h, separated in a 0.6% agarose gel, and transferred onto a GeneScreen *Plus* membrane. Hybridization and washing of the membrane were carried out under the same conditions as specified in Northern blot analysis except that the temperature for hybridization was 65°C and that the exposure time in autoradiography was 48 h.

Results and Discussion

Molecular Cloning: Rabbit antiserum directed against bovine Rieske Fe-S protein was found to cross-react with the corresponding protein of rat liver mitochondria by Western blot analysis (data not shown). We used this antiserum to screen a rat liver cDNA expression library in λgt11 and isolated nine positive clones. The insert DNAs of these clones were approximately 900 base pairs, and one of them extending longest toward the 5'-side was analyzed in detail.

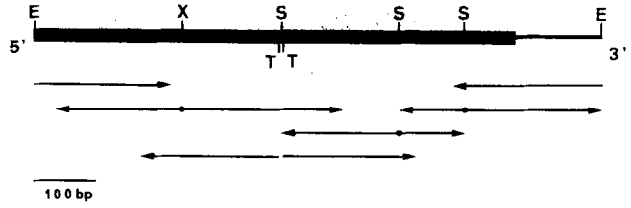

Fig. 1. Restriction map and sequencing strategy for the cDNA encoding rat Rieske Fe-S protein. The strategy for sequencing is indicated with the arrows representing the direction and length of the sequencing runs. The protein coding region is indicated by the thick line. bp, base pairs. E, *Eco*RI; S, *Sau*3AI; T, *Taq*I; X, *Xba*I.

Sequence Analysis: Figure 1 shows a restriction map and sequencing strategy for the cloned cDNA, and the nucleotide sequence of this cDNA and its deduced amino acid sequence are shown in Fig. 2. Schägger *et al.* (6) reported the primary structure of bovine Rieske Fe-S protein determined by amino acid sequencing analysis. Based on this sequence, it is predicted that rat Rieske Fe-S protein consists of 196 amino acid residues with a molecular weight of 21,465, and that it is formed as a precursor with an N-terminal extension. Since there is no methionine upward from the N-terminus of the mature protein, the presequence of the rat Rieske Fe-S protein precursor is presumed to comprise more than 60 amino acid residues. The molecular weight of the predicted part of the presequence is 6241, which is in the range of the value (6000-7000) estimated for the presequence of the rat Rieske Fe-S protein precursor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23). This indicates that the part of the presequence determined in this study misses only a small number of N-terminal residues of the presequence. Although the determination of the sequence of the presequence was incomplete, it became clear that the rat presequence has several interesting features when it was compared with the presequences of Rieske Fe-S proteins from other species (Fig. 3A). It is much longer than the presequences of the mitochondrial Rieske Fe-S proteins of *Neurospora* (11) and yeast (12). It is rich in basic amino acids and hydroxyl group-containing amino acids, as is the case with presequences of other mitochondrial proteins (24); however, it unusually contains three or more acidic residues. The occurrence of acidic residues is reported for the presequence of the Rieske Fe-S protein of spinach chloroplasts (13), but the presequences of the lower eukaryotes contain no such residues. It is also noted that there is a consecutive stretch of 14 uncharged amino acids near the C-terminus of the presequence. This feature is similar to the case of the presequences of the cytochrome c_1 precursors (25,26). In addition to the coding sequence, the cDNA has a 144-nucleotide 3'-noncoding region, but it contains neither a polyadenylation signal nor a poly(A) tail.

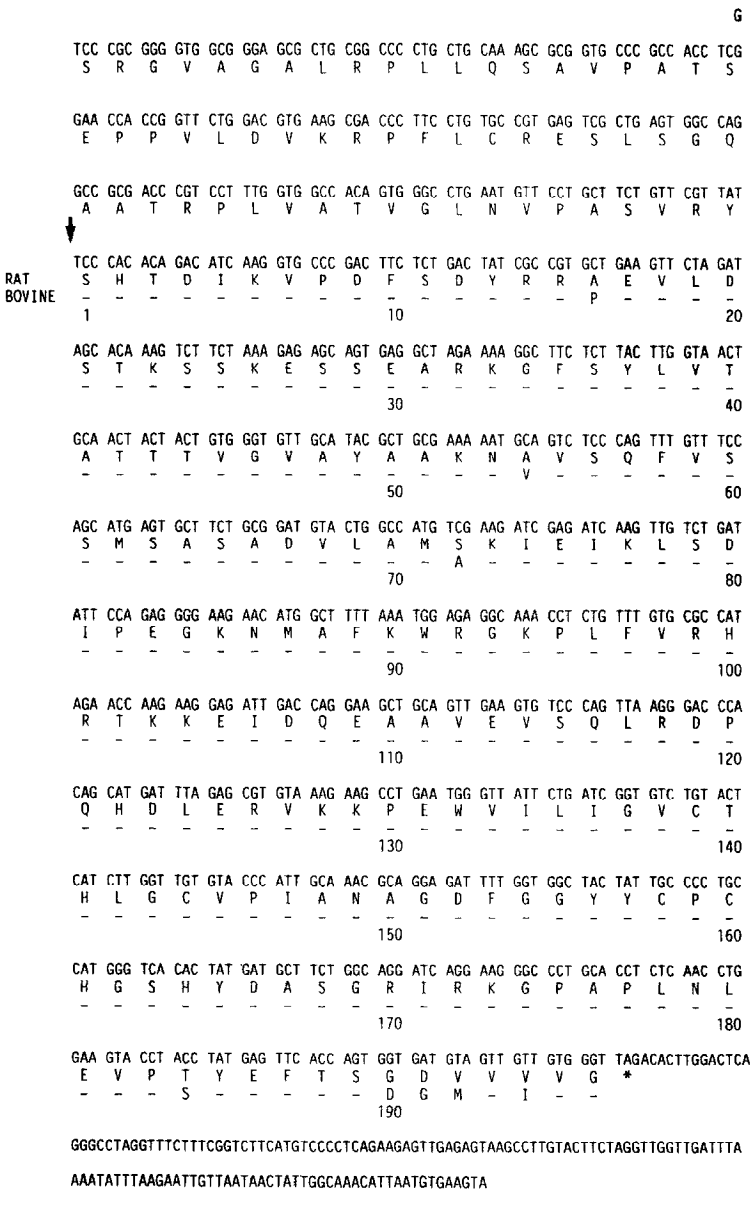

Fig. 2. Nucleotide sequence of the cDNA encoding rat Rieske Fe-S protein and its deduced amino acid sequence. The amino acid sequence of bovine Rieske Fe-S protein (6) is included for comparison. The dashes represent identical amino acids. The arrow indicates the start of the mature form of the protein as inferred from the bovine sequence obtained by amino acid sequencing analysis; the N-terminal serine of the presumed mature protein is numbered 1.

The amino acid sequences of the Rieske Fe-S proteins from eight phylogenetically diverse species were determined: three from mitochondria (bovine (6), *Saccharomyces cerevisiae* (12), and *Neurospora crassa* (10)), three from bacteria (*Paracoccus denitrificans* (9), and two strains of *Rhodobacter capsulatus* (7,8)), one from chloroplasts (spinach (13)), and one from cyanobacteria (*Nostoc* PCC 7906 (11)). Comparison of these sequences and the

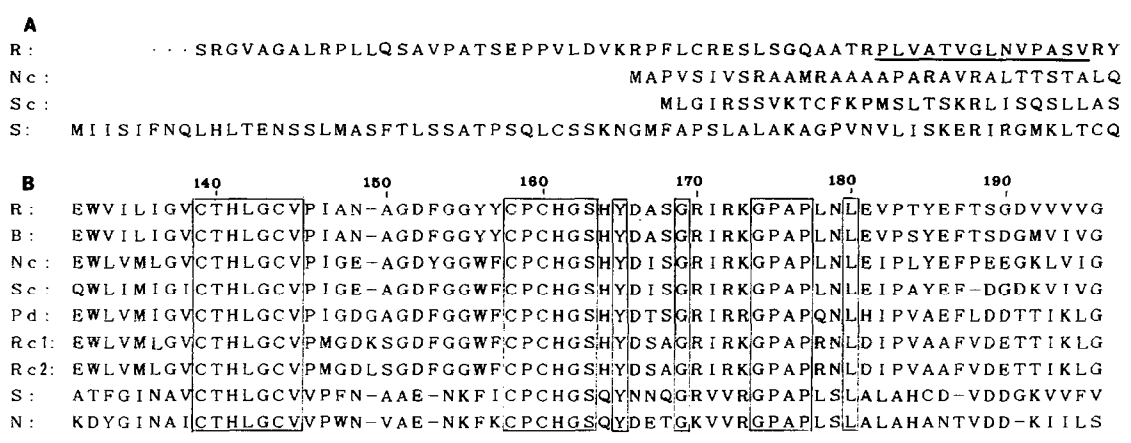

Fig. 3. Comparison of amino acid sequences of the presequence (A) and of the C-terminal part (B) of Rieske Fe-S proteins from various species. R, rat (this study); B, bovine (6); Nc, *Neurospora crassa* (10); Sc, *Saccharomyces cerevisiae* (12); Pd, *Paracoccus denitrificans* (9); Rc1 and Rc2, *Rhodobacter capuslatus* (7,8); S, spinach chloroplasts (13); and N, *Nostoc* PCC 7906 (11). The stretch of consecutive uncharged residues in the presequence of the rat protein is underlined. Amino acids conserved among all the sequences in B are boxed. The figures above the sequences in B are the amino acid residue numbers for the rat sequence.

rat sequence demonstrates that the C-terminal part is strongly conserved (Fig. 3B). In particular, there are a large number of amino acid residues that are conserved among all the nine sequences: the heptapeptide CTHLGCV (positions 139-145), the hexapeptide CPCHGS (158-163), Y165, G169, the tetrapeptide GPAP (164-167), and L180. This C-terminal part is expected to coordinate the [2Fe-2S] cluster. Several lines of evidence from the studies of *Thermus thermophilus* Rieske-type Fe-S protein (27) and yeast Rieske Fe-S protein (28) indicate that the Fe-S cluster is coordinated by one or more nitrogen ligands in addition to cysteines. H141 and H161 are the most probable candidates as such ligands, since they are conserved among all the sequences.

Figure 4 shows the hydropathy profile of rat Rieske Fe-S protein displayed by the method of Kyte and Doolittle (29). The mean hydropathic index for the mature protein is - 0.29, indicating that this protein is hydrophilic as a whole. The relatively hydrophobic region at positions 34-72 possibly binds the protein to the mitochondrial inner membrane, as suggested previously for other Rieske Fe-S proteins (6,11). The conserved heptapeptide at positions 139-145 is located in a hydrophobic stretch, and there is another short hydrophobic region at the C-terminus.

Northern and Southern Blot Analyses: In Northern blot analysis using the cloned cDNA as a probe (Fig. 5), rat liver poly(A)$^+$ RNA gave a somewhat broad band as compared to a band observed with human fibroblast poly(A)$^+$ RNA, indicating possible occurrence of multiple species of mRNAs for the Rieske

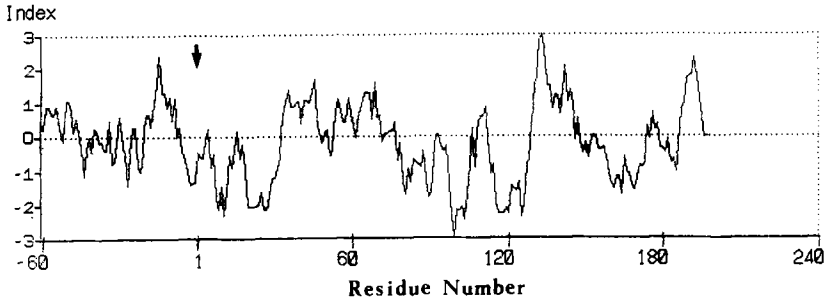

Fig. 4. Hydropathy plot of the amino acid sequence of rat Rieske Fe-S protein. The relative hydropathy of each residue was calculated by the method of Kyte and Doolittle (29) using a windowing average of 7 residues. Positive values indicate hydrophobic regions whereas negative values indicate hydrophilic regions. The arrow indicates the start of the mature protein. The numbering of residues is the same as in Fig. 2.

Fe-S protein in rat liver. The sizes of the rat and human mRNAs were estimated to be 1200-1400 and 1200 nucleotides, respectively. Southern blot analysis of rat genomic DNA (Fig. 6) showed that the cDNA probe hybridized to a single fragment produced by digestion with *Bam*HI, indicating that the gene for the Rieske Fe-S protein is a single copy gene. The mouse genome was also found to contain a single gene for this protein.

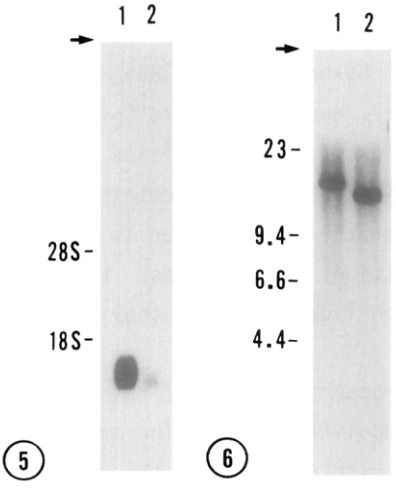

Fig. 5. Northern blot analysis of poly(A)$^+$ RNA from rat liver and human fibroblast cells. Poly(A)$^+$ RNAs (2 μg) from rat liver (lane 1) and human fibroblast cells (lane 2) were electrophoresed in a 1% agarose/formaldehyde gel and blotted onto a nylon membrane. The membrane was hybridized with the ^{32}P-labeled cDNA encoding rat Rieske Fe-S protein. The arrow indicates the origin. Size markers are 18S (1869 nucleotides) and 28S (4721 nucleotides) ribosomal RNAs.

Fig. 6. Southern blot analysis of rat and mouse genomic DNA using the cDNA encoding rat Rieske Fe-S protein as a probe. Rat (lane 1) and mouse (lane 2) DNAs (5μg each) were digested with *Bam*HI, electrophoresed in a 1% agarose/formaldehyd gel, and transferred onto a nylon membrane. The membrane was hybridized with the ^{32}P-labeled cDNA encoding rat Rieske Fe-S protein. The arrow indicates the origin. The size markers are in kilobase pairs.

Acknowledgments

We thank Dr. Yasuhiro Furuichi for kindly donating oligo(dT)-Latex. This work was supported in part by a grant-in-aid for Scientific Research on Priority Areas (62617002).

References

1. Shimomura, Y. and Ozawa, T. (1982) Biochem. Int. 5, 1-6.
2. Schägger, H., Link, T.A., Engel, W.D., and von Jagow, G. (1986) Methods Enzymol. 126, 224-237.
3. Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195-247.
4. Trumpower, B.L., and Edwards, C.A. (1979) J. Biol. Chem. 254, 8697-8706.
5. Shimomura, Y., Nishikimi, M., and Ozawa, T. (1984) J. Biol. Chem. 259, 14059-14063.
6. Schägger, H., Borchart, U., Machleidt, W., Link, T.A., and von Jagow, G. (1987) FEBS Lett. 219, 161-168.
7. Gabellini, N., and Sebald, W. (1986) Eur. J. Biochem. 154, 569-579.
8. Davidson, E., and Daldal, F. (1987) J. Mol. Biol. 195, 13-24.
9. Kurowski, B., and Ludwig, B. (1987) J. Biol. Chem. 262, 13805-13811.
10. Kallas, T., Spiller, S., and Malkin, R. (1988) Proc. Natl. Acad. Sci. USA 85, 5794-5798.
11. Harnisch, U., Weiss, H., and Sebald, W. (1985) Eur. J. Biochem. 149, 95-99.
12. Beckmann, J.D., Ljungdahl, P.O., Lopez, J.L., and Trumpower, B.L. (1987) J. Biol. Chem. 262, 8901-8909.
13. Steppuhn, J., Rother, C., Hermans, J., Jansen, T., Salnikow, J., Hauska, G., and Herrmann, R.G. (1987) Mol. Gen. Genet. 210, 171-177.
14. Nishikimi, M., Shimomura, Y., and Ozawa, T. (1983) Biochem. Int. 7, 793-798.
15. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, Jr., J.E. (1981) Cell 23, 731-739.
16. Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. (1979) Biochemistry 18, 5294-5299.
17. Kuribayashi, K., Hikata, M., Hiraoka, O., Miyamoto, C., and Furuichi, Y. (1988) In Nucleic Acids Research Symposium Series No. 19 (Hayatsu, H. Ed.), pp.61-64 IRL Press, Oxford.
18. Arrand, J.E. (1985) In Nucleic Acid Hybridization: A Practical Approach (B.D. Hames and S.J. Higgins, Eds.) pp.17-45. IRL Press, Oxford.
19. Huynh, T.V., Young, R.A., and Davis, R.W. (1985) In DNA cloning: A Practical Approach (D.M. Glover, Ed.) Vol.I, pp.49-78. IRL Press, Oxford.
20. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
21. Mizusawa, S., Nishimura, S., and Seela, F. (1986) Nucleic Acids Res. 14, 1319-1324.
22. Selden, R.F. (1987) In Current Protocols in Molecular Biology (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, Eds.) pp.4.9.1-4.9.8. John-Wiley & Sons, New York.
23. Kuzela, S., Joste, V., and Nelson, B.D. (1986) Eur. J. Biochem. 154, 553-557.
24. Reid, G.A. (1985) Curr. Top. Membr. Transp. 24, 295-336.
25. Sadler, I., Suda, K., Schatz, G., Kaudewitz, F., and Haid, A. (1984) EMBO J. 3, 2137-2143.
26. Nishikimi, M., Ohta, S., Suzuki, H., Tanaka, T., Kikkawa, F., Tanaka, M., Kagawa, Y., and Ozawa, T. (1988) Nucleic Acids Res. 16, 3577.
27. Fee, J.A., Findling, K.L., Yoshida, T., Hille, R., Tarr, G.E., Hearshen, D.O., Dunham, W.R., Day, E.P., Kent, T.A., and Münck, E. (1984) J. Biol. Chem. 259, 124-133.
28. Telser, J., Hoffman, B.M., LoBrutto, R., Ohnishi, T., Tsai A.-L., Simpkin, D., and Palmer, G. (1987) FEBS Lett. 214, 117-121.
29. Kyte, J., and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105-132.