Vol. 155, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1988 Pages 300-304

MOLECULAR CLONING OF A cDNA FOR α-SUBUNIT OF RAT LIVER ELECTRON TRANSFER FLAVOPROTEIN

Kyoko Shinzawa[1], Taisuke Inagaki[1,2], Nobuko Ohishi[1], Chikako Ichihara[1], Norihiro Tsukagoshi[2], Sigezo Udaka[2], and Kunio Yagi[1,*]

[1]Institute of Applied Biochemistry, Yagi Memorial Park, Mitake, Gifu 505-01, Japan

[2]Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan

Received July 13, 1988

Two cDNA clones for the α-subunit of rat liver electron transfer flavoprotein were isolated and their nucleotide suquences were determined. The longer cDNA contained a protein-coding region of 900 nucleotides and 3'-noncoding region of 335 nucleotides. The identity of the clone was confirmed by matching the amino acid sequence predicted from the cDNA with the sequence of one of the lysyl endopeptidase-digested peptides from the purified α-subunit. The molecular weight of the protein calculated from the protein-coding nucleotides was approx. 3,000 daltons smaller than that of the precursor, suggesting that the cDNA was not of full length. The derived amino acid composition fairly agreed with the chemically determined amino acid composition of the purified α-subunit, indicating that the protein-coding region contains most of the mature α-subunit.

Electron transfer flavoprotein (ETF) located in mitochondria is a specific electron acceptor for mitochondrial flavin enzymes such as branched acyl CoA dehydrogenase, sarcosine dehydrogenase, and dimethylglycine dehydrogenase. ETF consists of two non identical subunits (1,2). The molecular weight of the larger subunit (α-subunit) was estimated to be 31-32 K and that of the smaller one (β-subunit), 26-27 K, for both pig liver ETF (2) and rat liver ETF (3). From studies on in vitro biosynthesis, the α-subunit was shown to be synthesized as a precursor of larger molecular weight (34-35 K) and the β-subunit, as a protein having the same molecular weight as the mature form (3,4). We attempted cDNA cloning for both subunits of rat liver ETF in order to clarify the protein and gene structures. In this paper, we report the isolation of cDNA for the α-subunit.

*To whom correspondence should be addressed.

0006-291X/88 $1.50

MATERIALS AND METHODS

Purification of ETF and its subunits. Rat liver mitochondria were suspended in 20 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 30 mM KCl, to be 100 mg protein/ml, sonicated for 1 min x 4 in an ice bath with a sonicator (Model W-225R, Heat Systems Ultrasonics, Inc., Plainview, NY), and centrifuged for 30 min at 105,000 g and 4°C. The supernatant was fractionated with ammonium sulfate between 50 to 80% saturation. The precipitate was suspended in 10 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 10% glycerol and 0.5 mM EDTA, and dialyzed against the same buffer overnight at 4°C. The solution was applied onto a column (2.5 x 17 cm) of Matrex gel red A (Amicon Corp., Danvers, MA) previously equilibrated with the same buffer, and was eluted with the same buffer containing 60 mM KCl. Fractions containing ETF, which was detected by immunoblotting using anti-pig liver ETF antibody raised in a rabbit (4), were collected and concentrated to be 50 ml. After dialysis against the same buffer, the solution was applied onto a column (1.5 x 8 cm) of Bio-Gel HTP (Bio-Rad Laboratories, Richmond, CA) previously equilibrated with the same buffer. ETF was eluted with a gradient concentration of 10-80 mM potassium phosphate buffer containing 10% glycerol and 0.5 mM EDTA. Fractions containing ETF were collected, concentrated, and subjected to 12% SDS-polyacrylamide gel electrophoresis. The gel was soaked in 4 M ammonium acetate to visualize proteins. Segments containing each subunit were cut out separately and the subunits were eluted by shaking the segments with distilled water overnight at room temperature. The purified α-subunit was used for the following experiments.

Antibody. Antibody against the α-subunit was raised in a rabbit. Immunoblotting analysis showed that the antibody could cross-react with the α-subunit, but not with β-subunit.

Isolation of cDNA clone for rat liver ETF α-subunit. cDNA clones for the α-subunit were isolated by immunoscreening a rat liver cDNA library constructed in λgt11 (Clontech Laboratories, Inc., Palo Alto, CA) according to the method of Young and Davis (5). Screening procedure, analysis of fusion protein, and epitope selection (6) were carried out as described previously (7).

DNA sequence analysis. cDNA inserts were inserted into EcoRI site of pUC19 and restriction fragments were subcloned into M13mp18 phage vector. DNA was sequenced by the M13 chain termination method of Sanger et al. (8).

Amino acid sequence and amino acid composition analysis. Rat liver ETF α-subunit prepared as described above was digested with lysyl endopeptidase (Wako Pure Chemicals Ind., Ltd., Osaka). The digested peptides were separated by reverse-phase HPLC and amino acid sequence of one of the peptides was determined in a gas-phase sequenator (Model 470A, Applied Biosystems, Foster City, CA). In order to determine the amino acid composition of the α-subunit, the protein was hydrolyzed with 6N HCl containing 4% thioglycolic acid at 110°C for 22 h, and amino acid analysis was performed with an amino acid analyzer (Model 835, Hitachi, Tokyo).

RESULTS AND DISCUSSION

When a rat liver λgt11 cDNA library was screened with anti-pig liver ETF antibody, five candidate phages were obtained among 5 x 10^5 recombinant phages and one of them, designated Eα-2, was identified by epitope selection as a clone containing cDNA of the α-subunit. The size of the insert was estimated to be 700 bp by analysis on agarose gel electrophoresis.

We therefore attempted to obtain a clone having a much longer cDNA insert, and a rat liver λgt11 cDNA library was screened with anti-rat liver ETF α-subunit antibody. Ten candidate phages were obtained among 5 x 10^5 recombinant phages. One of them, designated Eα-8, was identified by epitope selection as a clone containing cDNA of the α-subunit, and the insert was estimated to be 1,250 bp.

Restriction maps and sequencing strategy for Eα-2 and 8 are shown in Fig. 1, indicating that clone Eα-8 has a longer nucleotide sequence overlapping the sequence of Eα-2. The nucleotide and deduced amino acid sequences are shown in Fig. 2. The open reading frame of 900 nucleotides ends with TGA terminal codon. The amino acid sequence of a peptide (10 amino acids) from rat liver ETF α-subunit perfectly matched that deduced from the DNA sequence (Fig. 2, underlined amino acids). This confirmed the identity of the cDNA clone. However, we assume that the cDNA is not of full length, since the molecular weight (31 K) of the reading frame was almost the same as that of the mature α-subunit and smaller than that of the precursor (34-35 K). The amino acid composition obtained by chemical analysis of the α-subunit of rat liver or pig liver ETF (2) fairly agreed

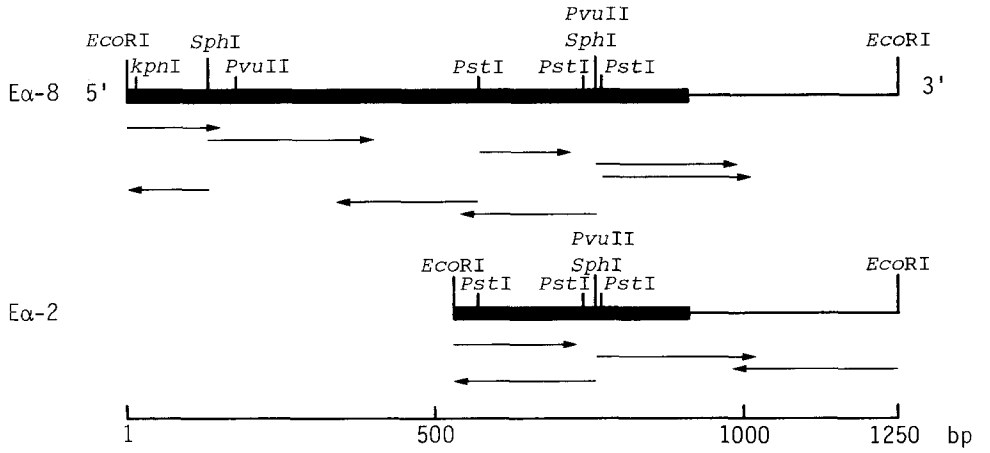

Fig. 1. Restriction maps and sequence strategy for rat liver ETF α-subunit cDNA clone (Eα-2 and 8). The arrows represent the direction and length of the sequence runs. The boxed region of the cDNA indicates protein-coding regions.

```
GAATTCCGGGAGGGTACCGCTGGGCTGACTAGAACAGAGACAATGTTTCGAGCAGCGGCGCCTGGGCAGCTCCGGCGGGCGGCCTCGTTG   90
        GluGlyThrAlaGlyLeuThrArgThrGluThrMETPheArgAlaAlaAlaProGlyGlnLeuArgArgAlaAlaSerLeu

CTCCGTTTTCAGAGCACCTTGGTAATAGCTGAGCATGCGAATGATTCTCTAGCACCTATTACTCTAAACACTATCACTGCAGCTGGACGT  180
LeuArgPheGlnSerThrLeuValIleAlaGluHisAlaAsnAspSerLeuAlaProIleThrLeuAsnThrIleThrAlaAlaGlyArg

CTTGGAGGTGAAGTGTCCTGCTTAGTAGCTGGAACAAAATGTGACAAGGTGGTACAGGATCTCTGTAAAGTAGCAGGCGTGGCAAAGGTT  270
LeuGlyGlyGluValSerCysLeuValAlaGlyThrLysCysAspLysValValGlnAspLeuCysLysValAlaGlyValAlaLysVal

CTGGTGGCTCAGCATGATGCGTACAAAGGCCTTCTTCCAGAGGAACTCACACCATTGATTTTGGAAACTCAGAAGCAGTTCAGTTACACA  360
LeuValAlaGlnHisAspAlaTyrLysGlyLeuLeuProGluGluLeuThrProLeuIleLeuGluThrGlnLysGlnPheSerTyrThr

CACATCGTTGCTGGAGCATCTGCTTTTGGAAAGAACCTTCTGCCCAGAGTAGCAGCCAAACTTAATGTTGCCCCAGTTTCTGACATCATT  450
HisIleValAlaGlyAlaSerAlaPheGlyLysAsnLeuLeuProArgValAlaAlaLysLeuAsnValAlaProValSerAspIleIle

GAGATCAAGTCACCTGACACATTTGTGAGAACTATCTATGCAGCAAATGCATTGTGTACAGTGAAATGTGATGAGAAAGTGAAGGTGTTT  540
GluIleLysSerProAspThrPheValArgThrIleTyrAlaAlaAsnAlaLeuCysThrValLysCysAspGluLysValLysValPhe

TCTGTTCGAGGAACATCTTTTGAGGCTGCAGCAGCAAGTGGAGGTAGTGCCAGTTCAGAAAAGGCACCAAGTTCTTCATCAGCAGGAATA  630
SerValArgGlyThrSerPheGluAlaAlaAlaAlaSerGlyGlySerAlaSerSerGluLysAlaProSerSerSerSerAlaGlyIle

TCAGAGTGGCTTGACCAGAAATTGACAAAAAGTGACCGACCAGAGCTAACTGGTGCCAAAGTGGTGGTATCTGGTGGTCGGGGCTTGAAG  720
SerGluTrpLeuAspGlnLysLeuThrLysSerAspArgProGluLeuThrGlyAlaLysValVlaValSerGlyGlyArgGlyLeuLys

AGTGGAGAGAACTTTAAACTGCAGTATGACTTGGCAGATCAGCTGCATGCTGCAGTTGGTGCTTCCCGTGCTGCTGTTGATGCTGGCTTT  810
SerGlyGluAsnPheLysLeuGlnTyrAspLeuAlaAspGlnLeuHisAlaAlaValGlyAlaSerArgAlaAlaValAspAlaGlyPhe

GTTCCCAATGACATGCAAGTTGGTCAGACAGGAAAAATAGTAGCTCCAGAACTTTATATTGCTGTTGGAATATCTGGAGCCATCCAGCAT  900
ValProAsnAspMETGlnValGlyGlnThrGlyLysIleValAlaProGluLeuTyrIleAlaValGlyIleSerGlyAlaIleGlnHis

TTAGCTGGATGAAGGACAGCAAGACAATTGTGGCTATTAACAAAGACCCAGAAGCTCCAATTTTCCAAGTGGCAGATTATGGAATAGTTG  990
LeuAlaGly***

CAGATTTATTTAAGGTGGTTCCTGAAATGACAGAAATACTGAAGAAAAAATAAGTCAAAATCATATCTTAAGGAAACTGTCAAAAGTATT 1080

CAGATTGAAATCATAGCAACTTCATAATCCAAAGAAAGAAAATATGCTAACAGCAGCTACAATGCCTTTTGATGAATCAATTATTATATT 1170

CCTTTTTAATTTTTTGTGATTCCAAACAATTATTTTTACATTTTCTAATTCTATAATAAAAAATATAATAAAGCGAATTC
```

Fig. 2. Sequence of rat liver ETF α-subunit cDNA and deduced amino acid sequence. The amino acid residues that were confirmed by amino acid sequencing of the purified α-subunit are underlined. Polyadenylation signal is indicated by broken lines. The predicted N-glycosylation site is boxed. ***, termination codon.

with that calculated from the deduced amino acid sequence (Table 1), indicating that the reading frame contains most of the sequence of the mature α-subunit. A preliminary experiment showed the N-terminus to be blocked. The reading frame contains one potential N-glycosylation site (Asn-X-Ser or Thr), but there is no evidence that the protein is a glycoprotein. The cDNA insert contains a 3'-noncoding region of 335 nucleotides, which contains two polyadenylation signals (AATAAA) (Fig. 2, nucleotides with broken lines).

In the last decade, evidence obtained by immunoblotting techniques has indicated a genetic deficiency of ETF to be one of the causes of metabolic disorders such as glutaric aciduria type II and ethylmalonic adipic aciduria (9-11). Further, Ikeda et al. (3) have reported that in a patient of glutaric aciduria type II, the synthesis of the α-subunit, but not that of

Table 1. Amino acid composition of rat liver ETF α-subunit

Amino acid	Deduced from DNA sequence (nucleotide No. 10-909)	Protein analysis	
		Rat liver	Pig liver[a]
Ala	44	57.6	37.4
Asp/Asn	20	22.6	28.6
Arg	12	5.1	7.2
Cys	5	4.5	N.D.
Glu/Gln	27	20.8	25.4
Gly	27	52.6	27.9
His	5	3.7	5.1
Ile	14	14.8	15.8
Leu	29	25.2	27.8
Lys	19	20.4	23.7
Met	2	2.4	2.7
Phe	9	6.2	8.4
Pro	11	13.7	17.4
Ser	24	22.0	19.0
Thr	19	15.8	16.8
Trp	1	0.6	0.2
Tyr	5	4.0	6.0
Val	27	27.3	26.4
Mr	31,176	31,000	31,100

The values are numbers of residues of ETF α-subunit. [a]Data of McKean et al. (2). N.D., not detemined.

the β-subunit, was defective. Accordingly, the cDNA encoding the α-subunit we isolated in the present study should be useful for investigating ETF deficient disorders.

REFERENCES

1. Husain, M., and Steenkamp, D. J. (1983) Biochem. J. 209, 541-545.
2. McKean, M. C., Beckmann, J. D., and Frerman, F. E. (1983) J. Biol. Chem. 258, 1866-1870.
3. Ikeda, Y., Keese, S. M., and Tanaka, K. (1986) J. Clin. Invest. 78, 997-1002.
4. Yagi, K., Inagaki, T., Ichihara, C., Koshizaka, T., Ohishi, N., Ghisla, S., Tsukagoshi, N., Udaka, S., Nishikimi, M., and Ozawa, T. (1988) in Flavins and Flavoproteins 1987 (Edmondson, D. E., and McCormick, D. B. ed), pp. 491-500.
5. Young, R. A., and Davis, R. W. (1983) Proc. Natl. Acad. Sci. USA 80, 1194-1198.
6. Weinberger, C., Hollenberg, S. M., Ong, E. S., Harmon, J. M., Brower, S. T., Cidlowski, J., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985) Science 228, 740-742.
7. Inagaki, T., Ohishi, N., Rasched, I., Frank, R. W., Ghisla, S., Tsukagoshi, N., Udaka, S., and Yagi, K. (1987) Biochem. Int. 15, 1051-1056.
8. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467.
9. Goodman, S. I., McCabe, E. R. B., Fennessey, P. V., and Mace, J. W. (1980) Pediatr. Res. 14, 12-17.
10. Rhead, W. J., Mantagos, S., and Tanaka, K. (1980) Pediatr. Res. 14, 1339-1342.
11. Mantagos, S., Genel, M., and Tanaka, K. (1979) J. Clin. Invest., 64, 1580-1589.