Isolation and Partial Characterization of cDNA Clone of Human Ceruloplasmin Receptor

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An individual clone, presumably carrying a 3 bp fragment of ceruloplasmin receptor cDNA was isolated from the expression library of human placenta cDNA using polyclonal specific antibodies to ceruloplasmin receptors. *EcoR1*-hydrolysate of isolated DNA was cloned in a *pTZ19* bacterial vector and sequenced in the forward and reverse direction. The comparison of the revealed sequence with known sequences of human genome revealed its high similarity to ceruloplasmin cDNA.

Key Words: *ceruloplasmin receptor; cloning; cDNA sequencing; copper metabolism*

Ceruloplasmin receptor (CPR), a part of the metabolic system providing copper homeostasis, was identified in 1984 [4] and electrophoretically purified. CPR consists of a single polypeptide chain with a molecular weight of approximately 130 kD, which is hydrolyzed after binding with ceruloplasmin (CP) [1,2] yielding several peptides with molecular weights ~60, 40, and 20 kD. Triptic hydrolysates of radioiodinated electrophoretically pure CP and CPR contain a group of identical peptides presumably responsible for partial immunological similarity of these proteins [2]. The function of CPR is not definitely determined, but it undoubtedly plays an important role in copper metabolism [6]. The most valuable scientific information seems to be provided by data on the cDNA structure of this protein. The present work was centered around the cloning and determination of the partial structure of CPR cDNA.

MATERIALS AND METHODS

The following materials were used: expression library of human placenta in λgt11 vector, *E. coli* (Y1090 and DH5-α strains), pTZ19 plasmid vector, EcoR1 and HindIII restriction endonucleases, T4 phage DNA-ligase, Klenov's fragment of DNA polymerase 1, Multiprime DNA-labeling system (Amersham), α-^{32}P-dATP, α-^{3}3P-dATP, and ^{125}I-Na (Isotop, St. Petersburg), type V agarose for nucleic acid electrophoresis, low-melting agarose, agar, microbial culture media components, and electrophoresis reagents (Sigma, ISN Flow, and Serva). A Gibco kit was used for polymerase chain reaction (PCR), primary DNA structure was analyzed with an f-mol DNA Sequencing System (Promega). Fuji X-ray film was used for autoradiography.

The cDNA library was screened using specific antibodies to CPR isolated from human erythrocyte plasma membranes. Immune complexes were detected with donkey ^{125}I-antibodies to rabbit immunoglobulins. Separation of immunological clones, isolation, restriction, and electrophoretic analysis of phage and plasmid DNA, isolation and cloning of recombinant DNA, DNA/DNA and RNA/DNA hybridization, analysis of PCR products, isolation of polyribosomal RNA, and preparation of ^{32}P-DNA probe were performed as described elsewhere [8].

RESULTS

Clones containing DNA sequences encoding immunologically active polypeptides were isolated from the expression library of human placenta cDNA in λgt11

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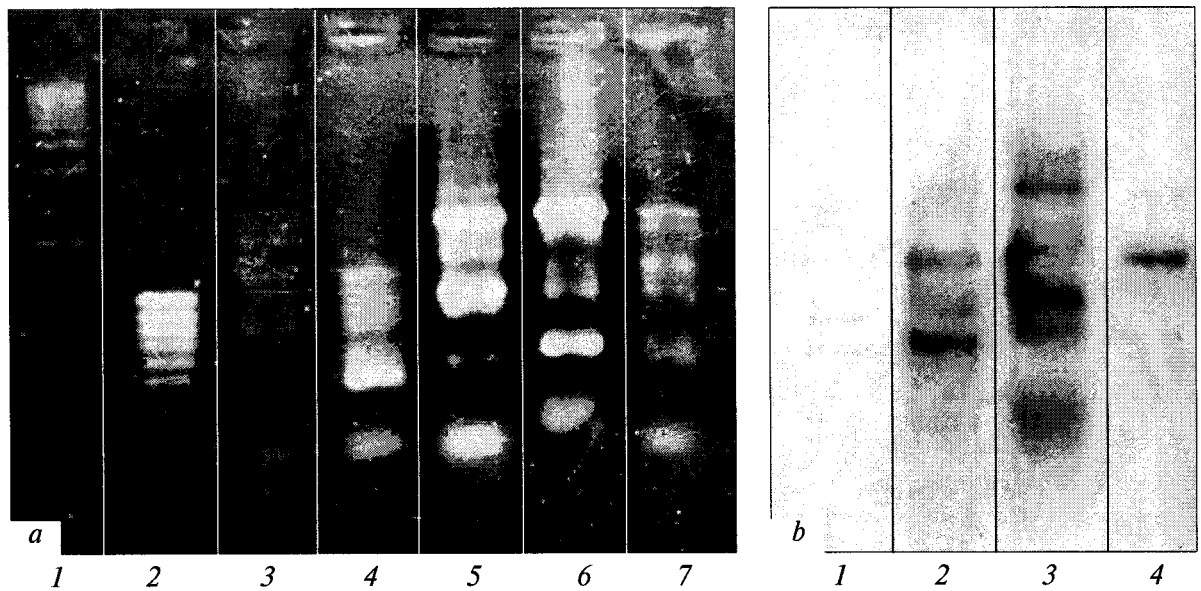

Fig. 1. Analysis of DNA from isolated clone presumably carrying a fragment of ceruloplasmin receptor cDNA. *a*: electrophoresis of PCR products of cloned phage DNA: *1*) standard set of DNA fragments (6100 to 1000 bp) differing by approximately 1000 bp; *2*) standard set of DNA fragments (1000 to 500 bp) differing by 100 bp; *3*) phage λgt11 DNA without insertion; *4*) highly purified DNA of cloned λgt11 phage; *5-7*) freshly isolated DNA of cloned λgt11 phage. Ethidium bromide staining (0.5 μg/ml); *b*: blot-hybridization of PCR products of cloned phage DNA with ^{32}P-DNA probe: *1*) highly purified DNA of cloned λgt11 phage isolated by immunoscreening; *2-4*) phage λgt11 DNA from immunopositive lysates.

vector using antibodies to CPR. A phage culture with clear immunoreactive plaques throughout the entire plate area was obtained after 4 passages in a selective medium.

Phage DNA was isolated from a single plaque. Intact DNA purified in a CsCl- ethidium bromide gradient and wild-type λ-phage DNA were used as PCR matrices. Primers complementary to λgt11 DNA flanking sequences were used. The test clone DNA had an insertion copied as a set of fragments with a maximal length of 1.55 kb (Fig. 1, *a*, rows 3 and 4). Then PCR was performed with DNA freshly isolated from the lysates of 3 randomly selected plaques on a Petri dish with bacteria infected with the selected phage clone. This approach provided the synthesis of DNA fragments with similar size and maximum length of about 3 kb (Fig. 1, *a*, rows 5-7). To characterize cDNA insertions in the selected clone, the longest fragment of PCR-amplified DNA (Fig. 1, *a*, row 6) was eluted after electrophoresis from low-melting agarose and used as a matrix for ^{32}P-DNA probe. Hybridization of this probe with PCR products of DNA insertions from all selected phages showed that all PCR-amplified DNA fragments revealed by electrophoresis had sequences homologous to the probe fragment (Fig. 1, *b*). This suggests the identity of all clones used in PCR. Thus, an individual clone with a 3 kb insertion was isolated from the cDNA library.

Blot-hybridization of this probe with total polyribosomal RNA from human placenta and cultured hepatoma cells (HepG2) revealed the presence of specific 8.2 kb transcripts only in placental polyribosomal RNA (Fig. 2). These results agree with previous data showing no CPR gene expression in hepatocytes [6]. The length of mRNA sequence (8.2 kb) is sufficient for encoding CPR polypeptide (130-150 kD). These data suggest that the test cDNA insertion in λgt11 is cDNA of human CPR.

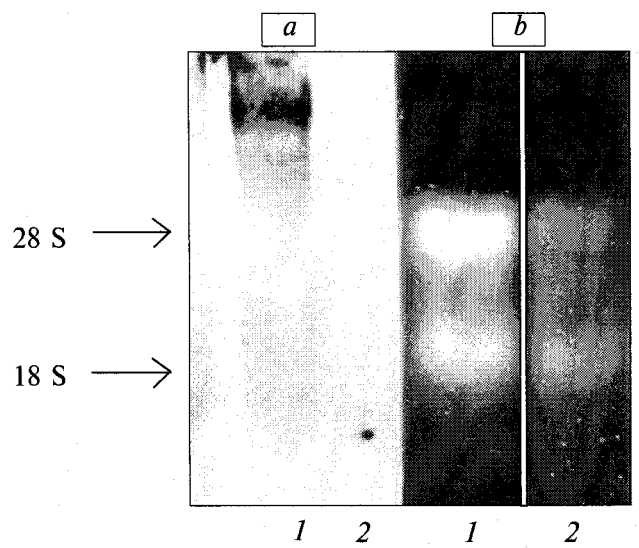

Fig. 2. Blot-hybridization of total polyribosomal RNA from human placenta (*1*) and HepG2 cells (*2*) with ^{32}P-DNA probe. *a*: blotting autoradiogram. Each row contains 70 μg RNA; *b*: staining with ethidium bromide after gel electrophoresis; row *1* contains 30 μg RNA, row 2 – 15 μg RNA.

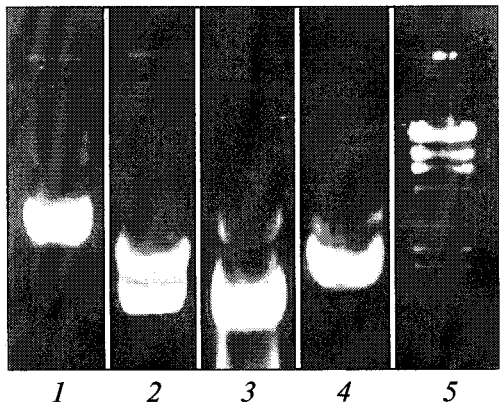

Fig. 3. Electrophoresis of pCpR1 DNA containing a fragment of ceruloplasmin receptor cDNA. *1*) 2 μg pCpR1 DNA; *2*) after treatment with EcoR1 restriction endonuclease; *3*) 2 μg intact supertwisted pTZ19 DNA; *4*) after treatment with EcoR1 restriction endonuclease, linearized DNA; *5*) 1 μg wild-type λ phage DNA after treatment with Hind III restriction endonuclease.

EcoR1-hydrolysate of highly purified phage DNA of a selected clone was -recloned in a pTZ19 plasmid vector to obtain a recombinant DNA clone, pCpR1, with a 2.3 kb fragment (presumably, a fragment of CPR cDNA) inserted into pTZ19 plasmid vector (Fig. 3). This recombinant DNA was used to determine the partial nucleotide sequence.

The pCpR1 sequence determined at forward transcription (257 bp, number accession AF211153) contained a 194 bp segment (Fig. 4) homologous by 76% to exon 16 of CP gene encoding a part of domain 6 in mature CP [5]. This segment was bordered with a 10 bp segment homologous by 81% to exon 15. Other nucleotide sequences were not found in the database. Translation of this sequence using DNASIS software revealed a single amino acid sequence showing no homology with CP region encoded by cDNA fragment homologous to CPR cDNA or with GenBank sequences. According to the Hoop and Woods hydrophilicity scale, this new amino acid sequence had a hydrophobic region containing 27 residues (Fig. 4, *1*). As predicted by a PC/GENE program, this hydrophobic region contains a transmembrane domain consisting of 16 residues (^{53}H-Y^{68}).

The reverse sequencing of the same DNA revealed a 631 bp segment (number accession AF211154), homologous by 81% to the exon 4-exon 7 segment of CP cDNA (Fig. 4, b, 2). According to the DNASIS model, this sequence is translated within a single transcription framework producing an amino acid sequence homologous by 83% to the ^{216}E-E^{427} region of CP involving domains 2 and 3. Substitutions in 63 codons were synonymous and induced no changes in amino acid sequence. The sequence contained 37 substituted amino acid residues, of them 22 substitutions were significant. The homologous region of CP includes 2 glycosylation sites, 358N and 397N, the latter is also present in CPR molecule. Amino acid residues (295H, 338C, 343H, and 348L) forming a type 1 mononuclear Cu-binding center in domain 2 [10] and responsible for ferroxidase properties of CP are conserved in CPR. It can be suggested that the N-terminal sequence of CPR includes a long segment homologous to CP. CPR seems to be a glycoprotein with ferroxidase properties containing a C-terminal transmembrane domain. It can be assumed that CPR participates in iron metabolism.

Three membrane proteins structurally homologous to that of soluble CP are now identified. The first one is FET3 protein encoded by a Fet3 gene and participating in Fe transport in the lower eukaryotes [3]. Another member of this family is hephestin encoded by mouse Heph 1 gene [9]. Its structure is homologous to CP by more than 50%. Heph 1 gene is expressed in intestinal cells and control the transport of Fe ions. Membrane-bound CP with a glycosylphosphatidylinositol-anchoring signal was identified in astrocyte plasma membranes [7]. These proteins contain more than 1 Cu atom and possess oxidase activity. Our findings suggest that CPR belongs to the family of CP-like Cu^{2+}-containing membrane oxidases.

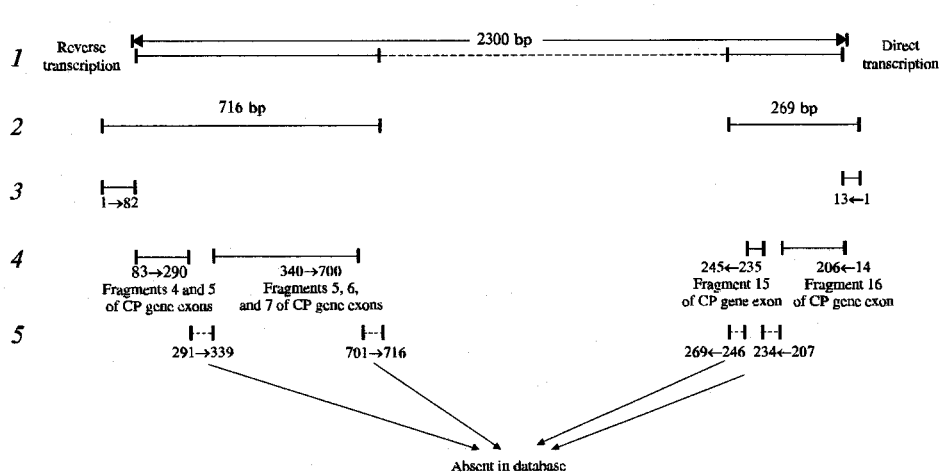

Fig. 4. Nucleotide sequence of ceruloplasmin receptor (CPR) cDNA fragment inserted into pCpR1. Sequencing of pCpR1 insertion: *1*) CPR cDNA fragment; *2*) position of sequenced segment; *3*) plasmid vector DNA; *4*) CPR cDNA segments (solid line–segments homologous to ceruloplasmin (CP) DNA; *5*) CPR cDNA segments without homologous sequences.

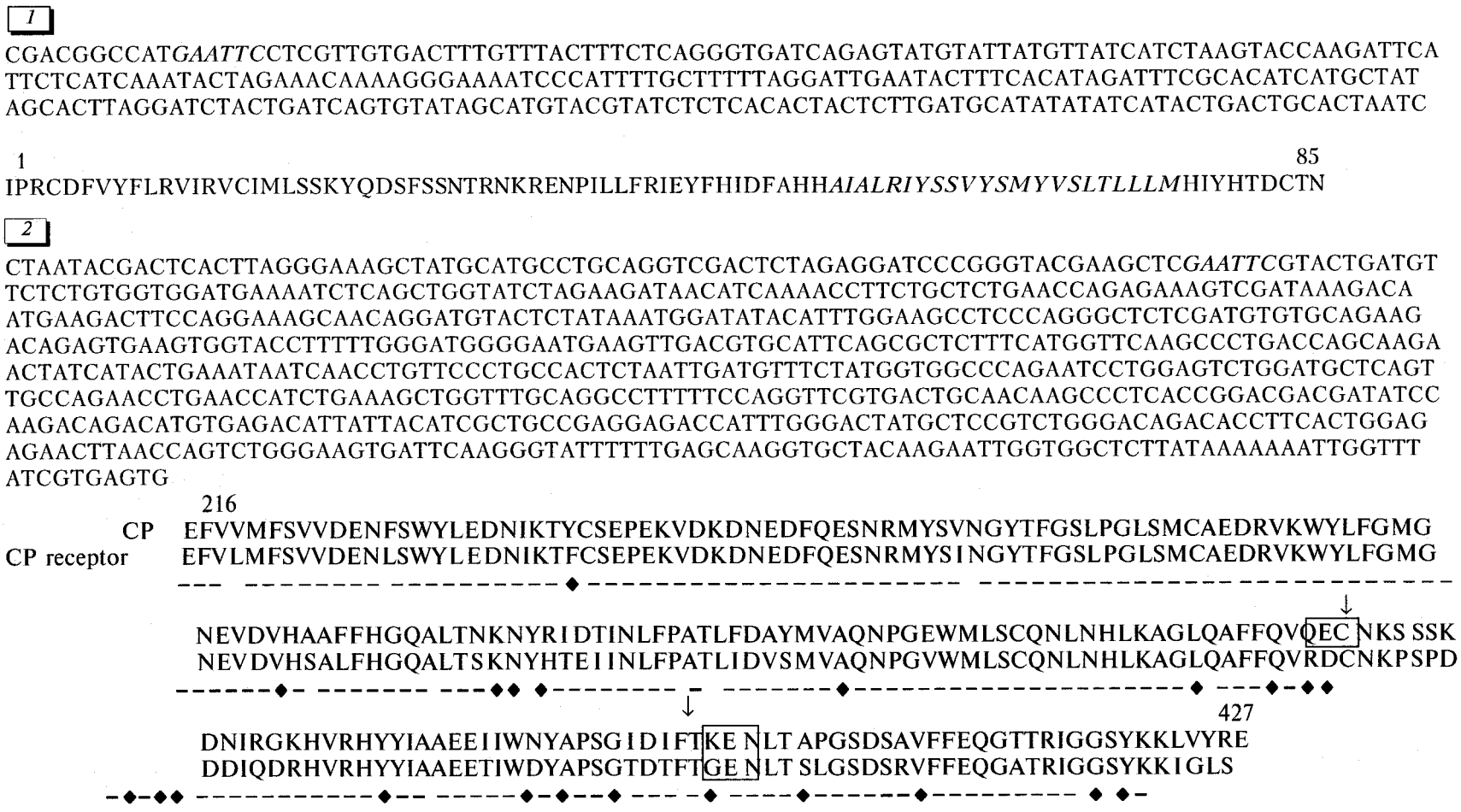

Fig. 5. Nucleotide sequence of pCpR1 insertion in direct (*1*) and reverse (*2*) sequencing primers and corresponding amino acid sequence (DNASIS software). EcoR1 sites (nucleotide sequences and hydrophobic domain) are underlined, glycosylation sites are framed and marked by arrows. Fragment 2: stright letters: identical amino acid residues; underlined letters: Cu^{2+}-ligands; fine italic letters: homologous substitutions; bold italic: significant substitutions. Sequencing was performed by G.Buchlow on a LI-COR automatic infrared sequencer at the Institute of Biochemistry, Berlin Free University.

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