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The revelation of expressing region in the processed ceruloplasmin gene in human genome by biocomputational and biochemical methods

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Abstract

Annotation: Translation in all open reading frames (ORF) of human ceruloplasmin (Cp) pseudogene revealed the only translating sequence of 984 nucleotides. The amino acid sequence contains a signal peptide for mitochondrial protein import at N-terminus. The predicted protein without taking the signal peptide into consideration has 92% identity to the corresponding Cp fragment. It contains 20 amino acid substitutions, 8 of them are significant. There is His-X-His motif in the center of a molecule that is typical for copper containing oxidases. Potential copper-binding site appears as a result of the substitution P923H along human Cp sequence. Cp pseudogene transcription product was found in the cultured human cell lines HepG2 and HuTu 80 using RT-PCR strategy. Cp polypeptides with molecular weight of nearly 30 kDa were found in mitochondria of HuTu 80 cells. The possible biological role of mitochondrial Cp is under discussion.

Keywords: Ceruloplasmin; Ceruloplasmin pseudogene expression; Mitochondria

1. Introduction

Ceruloplasmin (Cp, E.C. 1.16.3.1) is a multicopper oxidase of vertebrates, it plays a central role in iron metabolism and copper transport [1,2]. This polyfunctional protein whose functions depend on many parameters (cell type, period development, localization) [3], has several soluble and membrane bound isoforms [4–9]. So far only two Cp-mRNA isoforms are described. They are formed from a common Cp gene transcriptional product in the process of alternative splicing [9]. We used biocomputational strategy for searching for homologous to Cp functional sequences in GenBank database and revealed a potential mitochondrial Cp-like protein encoded by human Cp pseudogene. The experimental data supporting the existence of mitochondrial Cp are also presented.

2. Experimental

Cultured cells of HepG2 line, primarily obtained from human hepatoblastoma, and HuTu 80 line, primarily obtained from human small intestine adenocarcinoma, were used. Cells were cultured in DMEM, containing 2 mM glutamine, 0.1 mg/ml ampicillin, supplemented with 10% fetal calf serum. Subcellular fractions enriched with plasma membranes, mitochondria and intracellular membranes were isolated using differential centrifugation from cellular homogenate in 0.25 M sucrose, 100 mM KCl, 8 mM $MgCl_2$, 10 mM tris–HCl, pH7,4, 5% β-mercaptoethanol, 0.5 μl/ml (in 1:1000 ratio) protease inhibitors cocktail ("Sigma", CIIIA). HepG2 and HuTu 80 RNA was extracted with RNAqueous™ (Ambion, USA) according to the manufacturer's instructions. RT-PCR was performed with 1 μg RNA, oligo d(T)18 primer (1 μM), 500 μM dNTPs ("Promega", USA), 5× RT-buffer and M-Mlv reverse transcriptase (200 U, Amplisens, Russia) at 37 °C for 1 h. 1 μl of RT-sample was added to PCR mix (1.5 mM $MgCl_2$, 200 μM dNTPs, 5× PCR buffer, 1.5 U Taq DNA polymerase) with 0.5 μM of each primer. Western blots were carried out in

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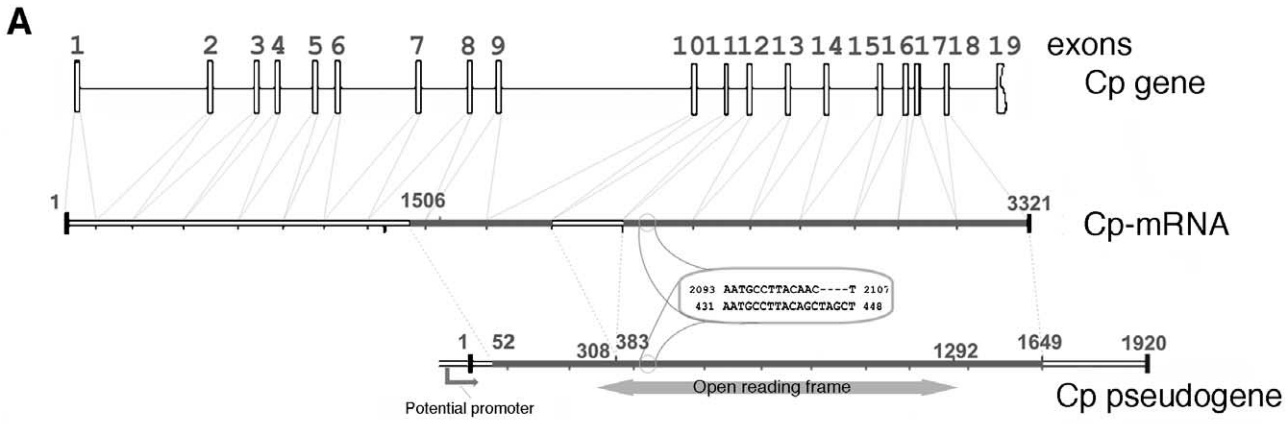

B

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pseudo Cp MRMRVYLLEDNIRMFTTAPDQVDKQMKTFRNLIKCTGLLMLNALQL---------------------------------------------------------- 45
      Cp DENESLLLEDNIRMFTTAPDQVDKEDEDFQESNKMHSMNGFMYGNQPGLTMCKGDSVVWYLFSAGNEADVHGIYFSGNTYLWRGERRDTANLFPQTSLTLHMWPD 690
         ******************    *

pseudo Cp ------------DHYTGGMKQKYTVNQCRWQSEDSTFYLGERTYYIAAVEVEWDYSPQREWEKELHHLQEQNVSNAFLDKGEFYIGSKYKKVVYRQYTDCTFRIP 140
      Cp TEGTFNVECLTTDHYTGGMKQKYTVNQCRRQSEDSTFYLGERTYYIAAVEVEWDYSPQREWEKELHHLQEQNVSNAFLDKGEFYIGSKYKKVVYRQYTDSTFRVP 775
                ***************** ********************************************************************** *** *

pseudo Cp VERKAEEEHLGILALQLHADVGDKVKIIFKNMTTRPYSIHAHGVQTESSTFIPALPGETLTYLWKIPERSGAGTEDSACIPWAYYSTVDQVKDLYSGLIGPLIVC 245
      Cp VERKAEEEHLGILGPQLHADVGDKVKIIFKNMATRPYSIHAHGVQTESSTVTPTLPGETLTYVWKIPERSGAGTEDSACIPWAYYSTVDQVKDLYSGLIGPLIVC 860
         *************  ***************** ********************************************************************

pseudo Cp TESSTFIPALPGETLTYLWKIPERSGAGTEDSACIPWAYYSTVDQVKDLYSGLIGPLIVCRRHYLKVFNPRKKLEFTLLFLVFDENESWYLDDNIKTNSDHPKKV 290
      Cp TESSTVTPTLPGETLTYVWKIPERSGAGTEDSACIPWAYYSTVDQVKDLYSGLIGPLIVCRRPYLKVFNPRRKLEFALLFLVFDENESWYLDDNIKTYSDHPEKV 945
         *****  * ******** ********************************************** ******** **** ******************** **** **

pseudo Cp NKDDEEFIESNKMHAVNGRMFGNPQGLTMHMGDEANGR------------------------------------ 328
      Cp NKDDEEFIESNKMHAINGRMFGNLQGLTMHVGDEVNWYLMGMGNEIDLHTVHFHGHSFQYKHRGVYSSDVFDIFP 1020
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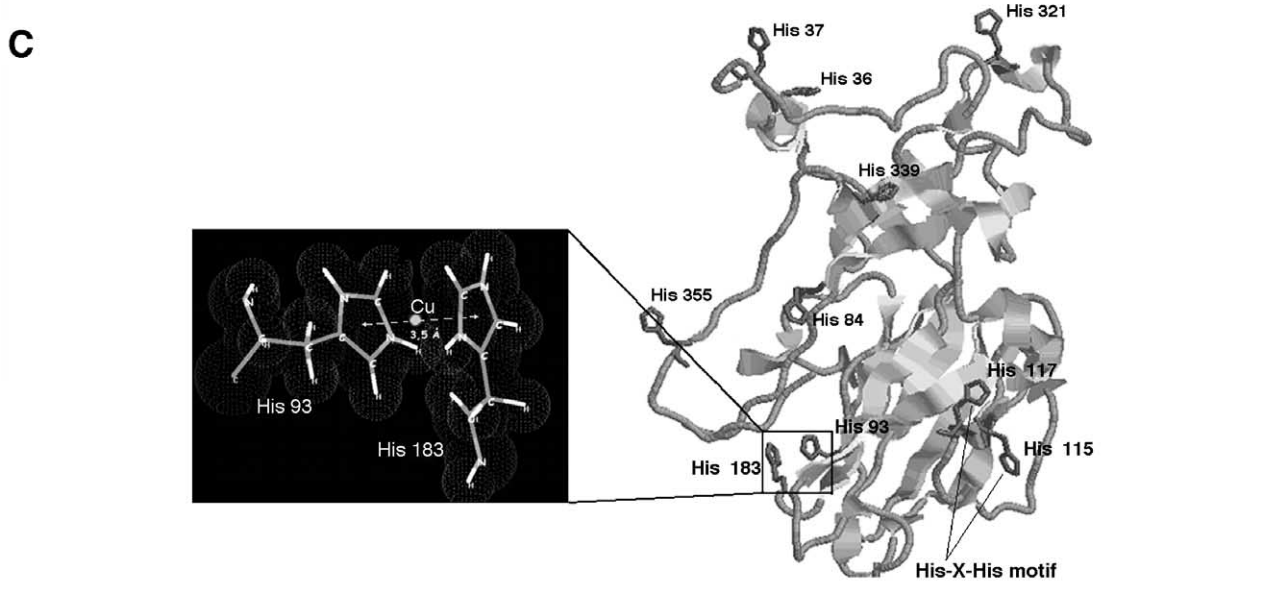

Fig. 1. Comparison of nucleotide and amino acid sequences of Cp and Cp pseudogene. (A) Above: exon/intron structure of human Cp gene. Vertical rectangles are exons. Below: mRNAs of Cp and *pseudo*Cp. Homologous regions are overshadowed; 4-nucleotide insertion is shown in a frame. (B) SPMPI is marked out with light font, His-X-His motif and histidines forming three dimensional sandwich are shown in a frame. (C) A model of the predicted mature expression product of Cp pseudogene. The model is built on the basis of corresponding region of human Cp molecule, obtained by the method of X-ray analysis [10], by replacement of non-corresponding amino acids and subsequent energy minimization using Polak-Ribiere algorithm (HyperChem 7.0.1. program). Graphical representation of a molecule was made using RasMol 2.7.1. program. Incut: sandwich with the bilateral symmetry of imidazole rings of His93 and His183, distance between the rings is 3.5Å.

7.5% SDS-PAGE and transferred to nitrocellulose membrane ECL Hybond ("Amersham", England) using semi-dry method. Rabbit antibodies to human Cp were obtained as described early [8]; anti-rabbit goat antibodies ("Amersham", England) were used as secondary antibodies. Western blots were developed using chemiluminescence. The programs ClustalX 1.83, MitoProt II 1.0a4, PSORT II, HyperChem 7.01, RasMol 2.7.1, program packages Gene Runner 3.0, PAML 3.0E and GenBank database were used for computer analysis.

3. Results

Translation of human Cp pseudogene (acc. no. NG_001106 in GenBank database) revealed the sequence of 984 nucleotides (from 308 to 1292 nucleotide) translated with ORF to amino acid sequence of 328 aa (Fig. 1). Comparison of the translating region of Cp pseudogene and the corresponding Cp cDNA region showed that the region, corresponding to exon 11 of Cp gene, is entirely deleted in Cp pseudogene, but it does not break ORF in Cp pseudogene. At the N-terminus of the estimated polypeptide the signal peptide for mitochondrial protein import (SPMPI), predicted using MitoProt II 1.0a4 program (with probability 0.9505), is localized. Search for the potential promoter in Cp pseudogene was made by the way of revelation of sequences close to TATA-box, capping signal and CAAT-box consensuses. Simultaneously all the three consensuses at the close to the consensus distances were found only in one region of DNA sequence of Cp pseudogene (Fig. 1). The first

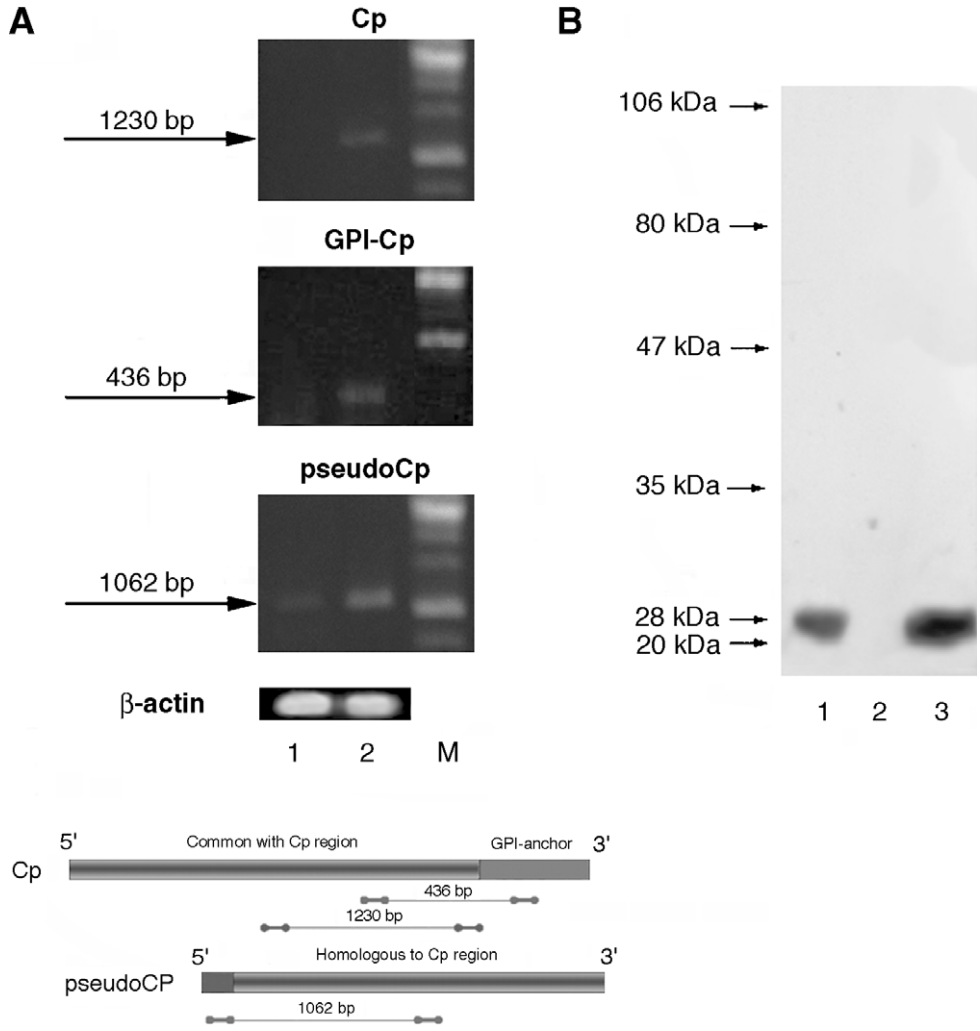

Fig. 2. Expression of Cp pseudogene in HuTu 80 and HepG2 cell lines. (A) *RT-PCR analysis*. The sizes of PCR products are shown with arrows. Lanes: 1—HuTu 80 cells; 2—HepG2 cells; M—markers. The amplification protocols were as follows: 94 °C for 2 min, 30 cycles of 94 °C for 1 min, annealing temperature for 45 s, 72 °C for 1 min 30 s, and a final extension time of 7 min at 72 °C. RT-PCR for β-actin was carried out at an annealing temperature of 60 °C for 30 cycles. RT-(−) control reaction were also performed to ensure that the amplified fragments originated from RNA rather than from genomic DNA. Below: Schematic presentation of used primers. The following primers were used to amplify secreted Cp region 5′ -gggatatactttcaggaaatacct and 5′ -tcgtattccacttatcaccaattta; GPI-Cp region 5′ -agtaaacaaagatgatgaggaattc and 5′ -ctccttggtagatatttggaataaa; *pseudo*Cp region 5′ -cttcacctctctaatgtgacctttc and 5'-agtttccttctgggattgaatactt. (B) *Western blot analysis* of subcellular fractions isolated from 10^7 cells of HuTu 80 line. Lanes: 1—intracellular membranes, 2—plasma membranes, 3—mitochondria. The arrows show the positions of markers (BioRad, USA).

transcribed nucleotide has +21 position along Cp pseudogene sequence. 5′ -UTR long of the putative mRNA is 282 nucleotides.

Juxtaposition of the aligned amino acid sequences of Cp pseudogene and Cp showed that there are 46 amino acid substitutions between these two sequences, moreover half of them corresponds to N-terminus (Fig. 1). *Pseudo*Cp has 20 amino acid substitutions in the homologous to Cp region, 8 substitutions are significant. The potential amino acid sequence of *pseudo*Cp does not have any glycosylation sites and copper binding sites of Cp. However there is His-X-His motif in the sequence that is a part of a trinuclear center of Cp [10]. Such motif is typical for copper containing oxidases. Furthermore three dimensional sandwich with the bilateral symmetry of imidazole rings of His 93 and His 183 is formed in the putative pseudogene product because of the substitution of Pro at position 923 along human Cp sequence to His (Fig. 1C). The estimated distance between imidazole rings is about 3.5 Å. Imidazole rings in histidyl-histidine dipeptide and in His–Cu–His complex, coordinating bivalent copper ion, are located at the same distance according to the data of estimations made using HyperChem7.0 program. Estimations showed that the energy of retention of Cu^{2+} is higher than for Cu^{1+}.

The existence Cp pseudogene transcription product was detected using RT-PCR. RNA isolated from cell lines HuTu 80 and HepG2 were exposed to RT-PCR analysis with selective primers for secreted Cp, glycosylphosphatidylinositol (GPI) anchored Cp and *pseudo*Cp. The transcripts of secreted Cp and GPI-Cp are not presented in HuTu 80 cells, whereas primers for *pseudo*Cp detect PCR-products in RNA of both cell lines (Fig. 2A). The search of *pseudo*Cp polypeptides using Western blot was carried out in HuTu 80 cells where neither Cp-mRNA nor GPI-Cp mRNA had been detected, and therefore their protein products could not interfere the detection of *pseudo*Cp-mRNA translation products. The data showed that, HuTu 80 cells contain immunoreactive Cp polypeptides with molecular mass of about 30 kDa that are localized in mitochondrial fractions and in fraction of intracellular membranes (Fig. 2B).

4. Discussion

Computer analysis of human Cp pseudogene sequence showed that it could be translated in ORF to the sequence of 328 amino acids, 66 of which form N-terminal SPMPI. Specific for oxidases copper-binding His-X-His motif is presented in the center of a molecule. Three dimensional site that hypothetically holds one copper atom appears on the surface of *pseudo*Cp globule as a result of Pro923His substitution along blood Cp sequence. Estimated molecular mass of mature *pseudo*Cp is about 30 kDa. Here we demonstrate that in human cultured cells Cp pseudogene is transcribed and its polypeptides react with antibodies to human Cp. These polypeptides are localized in mitochondria, and their molecular weight corresponds to the size of putative mature *pseudo*Cp. At the same time there is no strong confidence that Cp pseudogene expression takes place in vivo. It is possible that it occurs only in tumorigenic cell line. To answer the question, whether Cp is required in mitochondria, further research is necessary.

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