

Caeruloplasmin biosynthesis by the human uterus

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The blue copper protein caeruloplasmin is synthesized mainly by hepatocytes. An alternative transcript for caeruloplasmin produced in certain extrahepatic tissues, CP-1, contains an additional 12 nucleotides encoding 4 amino acids not present in the hepatic transcript, CP-2 [Yang, Friedrichs, Cupples, Bonifacio, Sanford, Horton & Bowman (1990) *J. Biol. Chem.* 265, 10780–10785]. We have demonstrated transcription of caeruloplasmin mRNA by a well-differentiated human uterine epithelial adenocarcinoma cell line, Ishikawa, and by human uterine endometrium and purified endometrial glands. Identical CP-2 nucleotide sequences were obtained for partial caeruloplasmin transcripts from human liver and Ishikawa cells, indicating that CP-2 transcripts are produced by uterine epithelial lining cells. The synthesis of caeruloplasmin protein was demonstrated for Ishikawa cells and another uterine adenocarcinoma cell line, ECC1. Peptide-mapping analysis indicated that caeruloplasmin secreted by Ishikawa cells was structurally identical with the protein synthesized by the human hepatoblastoma cell line HepG2. The secretion of a 135000- M_r caeruloplasmin by Ishikawa and ECC1 cells, comparable with that of the human hepatoblastoma cell line, HepG2, indicated similar processing of uterine and hepatic caeruloplasmin. Incorporation of ^{67}Cu into caeruloplasmin was demonstrated for Ishikawa and ECC1 cells, suggesting that the human uterus produces a bioactive form of caeruloplasmin and possesses the necessary metal transporters and intracellular machinery for copper incorporation into this protein.

INTRODUCTION

The human serum glycoprotein caeruloplasmin contains six or seven copper atoms per molecule [1]. These prosthetic copper atoms are necessary for this protein to assist in the mobilization of iron, catalyse oxidation of catecholamines, function in copper transport and delivery, act as an angiogenesis factor (for a review, see [2]) and for its action as an antioxidant [3]. The many protective and metabolically important roles for caeruloplasmin may account for the increased synthesis of this protein as part of the acute-phase response [4].

Caeruloplasmin is present in normal human sera in concentrations of 200–350 mg/litre [5]. The major form of caeruloplasmin peptide isolated from human sera has an M_r of 135000 [6]. Two other minor forms of caeruloplasmin peptide are frequently observed: a 115000- M_r peptide derived from the 135000- M_r form by proteolysis, and another ~200000- M_r form which appears to represent a dimer of the 135000- M_r peptide [7]. These three caeruloplasmin peptides probably contain copper, since they were all present when the copper-containing oxidase-active holo-caeruloplasmin band resolved by non-denaturing SDS/PAGE was further evaluated by second-dimension electrophoresis [7].

The liver appears to be the main site of synthesis and secretion of caeruloplasmin [8]. Recent evidence suggests that the major 135000- M_r form of caeruloplasmin peptide is also synthesized by rat sertoli cells [9,10] and alveolar macrophages [11], rat mammary glands [12], mouse embryonic fibroblasts transformed by the Rous-sarcoma virus [13], human synovial tissue [14] and in human lung (Hs242T) and breast (MCF-7) adenocarcinoma cell lines [12]. Other extrahepatic tissues appear to be able to transcribe caeruloplasmin mRNA [15–20]; however, no evidence has yet been presented for the synthesis of holo-caeruloplasmin.

We now report on the extrahepatic biosynthesis of caeruloplasmin by the human endometrium. In the present study we have demonstrated the transcription of caeruloplasmin mRNA by two well-differentiated human uterine epithelial adeno-

carcinoma cell lines, Ishikawa [21] and ECC1 [22] and by human uterine tissue. Furthermore, we show that the caeruloplasmin which is synthesized and secreted by the uterine cell lines appears to be structurally identical with the hepatic protein. In addition, the ability of Ishikawa and ECC1 cells to incorporate copper biosynthetically into holo-caeruloplasmin suggests that the human uterus produces a bioactive form of this protein.

EXPERIMENTAL

Materials

[^{35}S]Cysteine, ^{125}I , [^{32}P]dCTP and [^{35}S]ATP were obtained from Amersham International, carrier-free ^{67}Cu from Brookhaven National Laboratories, nitrocellulose membranes (0.45 μm pore size) from Schleicher and Schuell, SB 100 and XAR 5 film from Kodak, Protein A and Protein A-Sepharose C14B from Pharmacia, DEAE-20 from James River Corp., En 3 Hance from du Pont, staphylococcal V8 proteinase and Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham from Sigma, minimal essential medium, F-12 Ham nutrient mixture and custom radio-labelling media from Gibco, dishes for cells from Falcon, TA cloning kit from Invitrogen, reagents for DNA sequencing (Sequenase 2.0) from U.S. Biochemicals, Long Ranger polyacrylamide for sequencing gels from AT Biochemicals and random priming kit from Amersham International.

Caeruloplasmin was isolated from human serum by the method of Morell *et al.* [23]. Antisera to human caeruloplasmin was prepared in rabbits as previously described [7]. Samples of human endometrium and placenta were collected [24] and human endometrial glands were prepared as described in [25]. The cell lines Ishikawa [21] and ECC1 [22] were kindly provided by Dr. John White (Department of Obstetrics and Gynecology, Royal Postgraduate Medical School, Hammersmith Hospital, London W.12. U.K.) and Dr. P. G. Satyaswaroop (Department of Obstetrics and Gynecology, Pennsylvania State University, Hershey, PA, U.S.A.). HepG2 cells utilized for these studies were described previously [7].

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum.

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Methods

Cell culture. All cells were maintained at 37 °C under a 5% CO₂ atmosphere. Cells were plated at ~10⁵ cells/dish, then grown to confluence in the appropriate growth medium. HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal-bovine serum (FBS). Ishikawa cells were grown overnight in DMEM nutrient mixture F-12 Ham (DME/F-12) supplemented with 10% FBS and thereafter with phenol-free DMEM/F-12 supplemented with insulin (6.25 µg/ml), transferrin (6.25 µg/ml), sodium selenite (6.25 µg/ml), BSA (1.25 mg/ml) and linoleic acid (5.33 µg/ml). ECC1 cells were grown overnight in F-12 Ham nutrient mixture supplemented with 10% FBS and thereafter in F-12 Ham nutrient mixture supplemented with glucose (0.4 mg/ml), insulin (9.08 µg/ml), transferrin (25 µg/ml), cholera toxin (10 ng/ml) and oestradiol (0.2 ng/ml).

Metabolic radiolabelling. Confluent cells were washed with sterile phosphate-buffered saline (50 mM-NaH₂PO₄/150 mM-NaCl, pH 7.4) and then incubated in cysteine-free MEM with 10% dialysed FBS and [³⁵S]cysteine (100–200 µCi/ml) for up to 3 h.

Metabolic labelling with ⁶⁷Cu was performed using confluent cells grown on 100 mm-diameter dishes. Carrier-free ⁶⁷Cu was added to cells in 5 ml of growth medium (~10⁷ c.p.m./µg of Cu per ml) and then incubated at 37 °C under 5%-CO₂ atmosphere for 14 h.

Protein analysis. Radiolabelled cell lysates or conditioned medium to which phenylmethanesulphonyl fluoride was added to 2 µM, were precleaned by incubation with Protein A–Sepharose at 4 °C for 30 min. The suspension was centrifuged, and 25–50 µl of Protein A–Sepharose prebound with 50–100 µl of rabbit anti-(human caeruloplasmin) antisera was added. The suspension was incubated for 2 h at 4 °C, then centrifuged (15000 g, 3 min). The pellet was saved and washed by resuspension in RIPA buffer (4 °C) [1.0% (w/v) Triton X-100/0.5% (w/v) sodium deoxycholate/50 mM-Tris/150 mM-NaCl, pH 7.8] and again centrifuged. This was repeated three times with RIPA buffer and then once with 0.15 M-NaCl/10 mM-Tris, pH 7.4 (4 °C). The bound antibody–antigen complex was released from the Protein A–Sepharose and separated by heating in 50 µl of SDS/PAGE sample buffer at 90 °C for 10 min. The heated mixture was centrifuged and the supernatant recovered.

SDS/PAGE was performed as described by Laemmli [26], using 3% stacking gels and 7.5% resolving gels. Gels were prepared for autoradiography by fixation in methanol/acetic acid/water (10:1:9, by vol.) then incubation in fluorescent enhancer (En³Hance), followed by methanol/acetic acid/water (5:1:14, by vol.) before vacuum drying. Dried gels were exposed to SB-100 film at –70 °C.

Peptide mapping of caeruloplasmin was performed using the SDS/PAGE-resolved 135 kDa band of radiolabelled protein recovered by immunoprecipitation. The band was treated with staphylococcal V8 proteinase [27], the peptides resolved by SDS/PAGE, and the gel prepared for autoradiography as described above.

Transblot of proteins resolved by SDS/PAGE to nitrocellulose membranes was performed with a buffer of 25 mM-Tris/5.5% (w/v) glycine/30% (v/v) methanol using a semi-dry blotting apparatus to which 100 mA was applied for 3 h. Western blotting was performed as described by Towbin *et al.* [28] using a blocking solution of 2% non-fat dry milk in 10 mM-Tris, pH 7.4, 0.15 M-NaCl, a wash solution containing 0.5% Tween-20 in 10 mM-Tris, pH 7.4, 0.15 M-NaCl, rabbit anti-(human caeruloplasmin) antisera diluted 1:1000 in blocking solution and ¹²⁵I-Protein A for detection of bound antibody. Radiolabelled Protein

A was detected by exposure of the dried membranes to XAR-5 film at –70 °C.

Secreted proteins from HepG2, Ishikawa and ECC1 cells were analysed for copper incorporation into caeruloplasmin after their incubation with growth medium containing ⁶⁷Cu, as described above. Medium containing radiolabelled protein was partially purified by anion-exchange chromatography using DEAE-20 [23] and concentrated by precipitation with 4 vol. of ethanol at 4 °C. Caeruloplasmin was then resolved by non-denaturing SDS/PAGE, transferred to nitrocellulose membranes, and ⁶⁷Cu-labelled protein identified by autoradiography [7]. For each individual ⁶⁷Cu labelling experiment, holo-caeruloplasmin and copper-free apo-caeruloplasmin standards were applied to neighbouring SDS/PAGE lanes and were subjected to Western-blot analysis with rabbit anti-(human caeruloplasmin) antisera, followed by ¹²⁵I-Protein A and autoradiography after the decay of the ⁶⁷Cu (*t*_{1/2} ~ 60 h).

Nucleic acid techniques. Total RNA from normal human liver, uterus, uterine epithelial glands, placenta, Ishikawa and ECC1 cell lines were prepared as described in [24]. Total RNA were reverse-transcribed in the presence of 0.2 mM random hexanucleotides as primers using Maloney-murine-leukaemia-virus reverse transcriptase (20 units/µg of RNA) in a buffer containing 12 mM-Tris/HCl, pH 8.3, 75 mM-KCl, 35 mM-MgCl₂, 15 mM-dithiothreitol, acylated BSA (40 µg/ml) and 0.7 mM each of the four NTPs for 90 min at 37 °C, followed by 5 min at 90 °C for enzyme inactivation. cDNA amplification was performed using 1–2 µl aliquots of reverse-transcribed products in the presence of 1.0 µM each of caeruloplasmin-specific primers (sense 5'-ACGGC-CATAGCTTCCAATACAA; antisense 5'-AGTTGTATGCT-TCCAGTCTTCT [20]) and 5 units of *Taq* polymerase in a buffer containing 10 mM-Tris/HCl, pH 8.3, 50 mM-KCl, 4.0 mM-MgCl₂ and 0.2 mM each of the four dNTPs. The reaction mixture was subjected to 40 cycles of an amplification sequence as follows: denaturation at 94 °C (1 min), annealing at 60 °C (2 min), extension at 72 °C (2.5 min). PCR products were resolved by electrophoresis on 2%-(w/v)-agarose gels and revealed by ethidium bromide fluorescence.

Products of the PCR reaction using caeruloplasmin-specific primers for human liver cDNA and Ishikawa-cell cDNA were ligated with the TA Cloning Vector and used to transform competent *Escherichia coli* JM109. Colonies containing transformants were selected, and overnight cultures prepared in Luria broth with 25 µg of kanamycin/ml for plasmid isolation [29]. The purified plasmid DNA was utilized for DNA sequencing by the enzymic dideoxy termination method utilizing ³⁵S-ATP for detection of sequence reaction products, and plasmid-specific primers, as previously described [25]. Products of the sequencing reaction were resolved on 6%-(w/v)-urea/PAGE, and the gel vacuum-dried before autoradiographic exposure. Once the sequence of the cloned amplicon from human liver was determined to be caeruloplasmin (see Fig. 4 below), the insert from this clone obtained by restriction digestion with *Eco*R1 and *Hind*III was purified and utilized as a probe for Southern blotting.

PCR products resolved by 2%-(w/v)-agarose-gel electrophoresis were denatured and transferred to nylon membranes by vacuum blotting, then utilized for Southern blotting as previously described [25]. Hybridization of the [³²P]dCTP radiolabelled cDNA probe for human caeruloplasmin was detected by autoradiography.

RESULTS

Synthesis of caeruloplasmin by uterine cells

Metabolic radiolabelling and specific immunoprecipitation studies of the human adenocarcinoma cell lines Ishikawa and

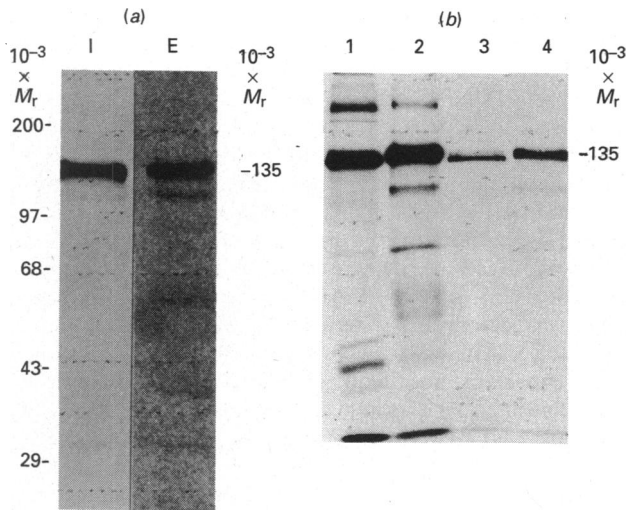


Fig. 1. Synthesis and secretion of caeruloplasmin protein by Ishikawa (I) and ECC1 (E) cells

(a) Autoradiogram demonstrating synthesis and secretion of a 135000- M_r protein recovered by immunoprecipitation with antisera specific for human caeruloplasmin from the media of Ishikawa and ECC1 cells incubated with [35 S]cysteine for 3 h. Immunoprecipitates were resolved by SDS/PAGE using a 3% stacking gel and 7.5% resolving gel, and prepared for autoradiography as described in the Experimental Section. (b) Autoradiogram of caeruloplasmin immunoprecipitates from HepG2-cell lysate (lane 1) and medium (lane 2) and Ishikawa-cell lysate (lane 3) and medium (lane 4) after a 3 h incubation of cells with [35 S]cysteine. A slightly decreased M_r for the intracellular caeruloplasmin immunoprecipitates was noted in both HepG2 and Ishikawa cells. In addition, the previously reported higher- M_r form of caeruloplasmin [7] was seen only in HepG2 (lanes 1 and 2).

ECC1 demonstrate the synthesis and secretion of caeruloplasmin (Fig. 1a). Caeruloplasmin recovered for both cell lysates and medium of Ishikawa cells had an M_r identical with that of caeruloplasmin synthesized by HepG2 cells (Fig. 1b), although the amount of caeruloplasmin synthesized by Ishikawa cells appears to be less than that of HepG2, since comparable numbers of cells were utilized for this experiment. Synthesis of a mature 135000- M_r form of caeruloplasmin by Ishikawa and ECC1 cells (Fig. 1a), comparable with that of HepG2 cells (Fig. 1b), suggests similar processing of this protein by these cell lines.

Peptide mapping was performed on proteinase-treated Ishikawa-cell-derived caeruloplasmin for comparison with the corresponding protein synthesized by HepG2 (Fig. 2). The Ishikawa- and HepG2-cell caeruloplasmins were found to give peptide patterns identical with those of the proteins originally isolated from human sera [7]. This indicates that the primary structures of the uterine-derived and human serum proteins are identical.

Transcription of caeruloplasmin mRNA by uterine tissue and Ishikawa cells

Active transcription of caeruloplasmin mRNA by Ishikawa cells, uterine endometrium, purified endometrial-gland cells, placenta and liver was demonstrated by the presence of PCR products obtained using caeruloplasmin specific primers and cDNA from these sources (Fig. 3a). The identity of these PCR products was confirmed by Southern-blot analysis, demonstrating hybridization with a cDNA probe specific for human caeruloplasmin (Fig. 3b).

DNA sequencing of the cloned caeruloplasmin PCR products obtained from Ishikawa cells and human liver (Fig. 4) indicates

that both contain DNA sequences identical with that reported for hepatic caeruloplasmin [30], including the absence of the additional 12 bp insert found in CP-1 transcripts reported by Yang *et al.* [20,30]. This suggests that similar processing of caerulo-

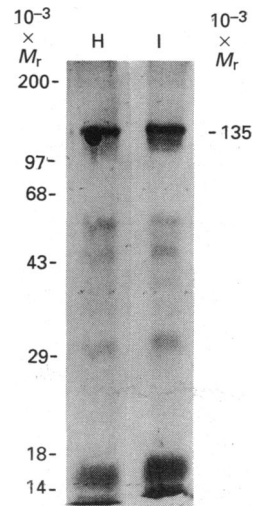


Fig. 2. Peptide mapping of newly synthesized caeruloplasmin from HepG2 (H) and Ishikawa (I) cells

Shown are autoradiograms demonstrating the caeruloplasmin peptides generated by staphylococcal-V8-proteinase treatment of the 135000- M_r band recovered by immunoprecipitation with antisera specific for human caeruloplasmin from the media of Ishikawa and HepG2 cells incubated with [35 S]cysteine for 3 h. Peptides were resolved by SDS/PAGE using a 3% stacking gel and 10% resolving gel, and prepared for autoradiography as described in the Experimental section. Vacuum-dried gels were exposed to SB-100 film at -70°C for 1 week to reveal HepG2 caeruloplasmin peptides and for 3 weeks for Ishikawa caeruloplasmin peptides.

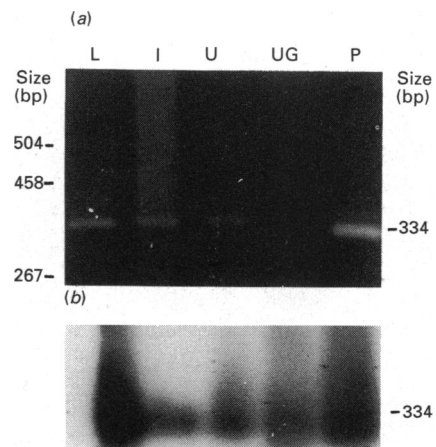


Fig. 3. Presence of caeruloplasmin mRNA in human tissue samples and in Ishikawa cells

(a) Ethidium bromide detection of the products of PCR reactions using caeruloplasmin-specific primers and cDNA made from RNA extracted from human endometrium (U), purified endometrial glands (UG), placenta (P), Ishikawa cells (I) and liver (L). DNA standards are shown for comparison. (b) Autoradiogram of a Southern blot of the same samples as in (a), demonstrating hybridization of the PCR products with a ^{32}P -labelled probe for human caeruloplasmin.

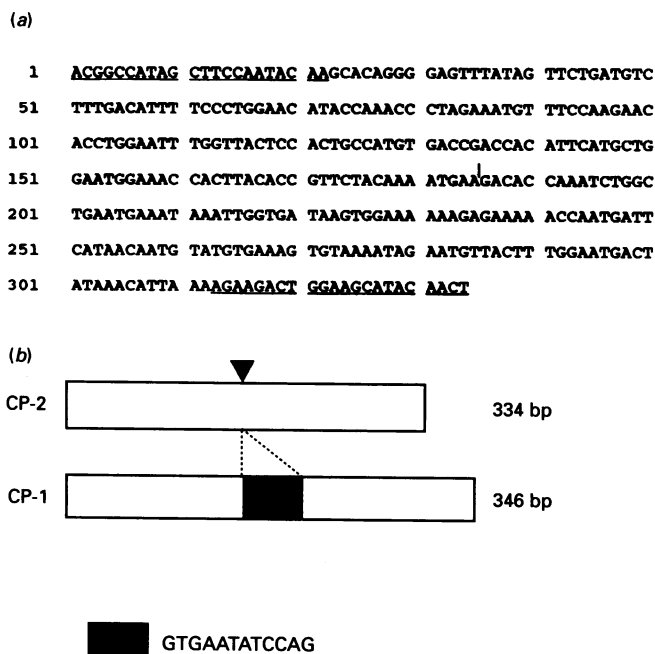


Fig. 4. Analysis of caeruloplasmin transcripts in Ishikawa cells and normal human liver

Shown are the identical DNA sequences of the cloned PCR amplification products obtained using caeruloplasmin-specific primers (underlined) and Ishikawa-cell or human liver cDNA. This sequence was identical with the previously reported sequence for hepatic caeruloplasmin, CP-2 [30]. The site where the 12 additional nucleotides are located in the alternatively spliced extrahepatic transcript of caeruloplasmin, CP-1 [20,30], indicated by the vertical line (above) and inverted arrow (below). A model of the CP-2 transcript present in human liver and Ishikawa cells is shown for comparison with CP-1.

plasmín transcripts occurs in liver and uterine endometrial tissue.

Metabolic incorporation of copper into caeruloplasmin by uterine cell lines

The biosynthetic incorporation of copper into caeruloplasmin was demonstrated for Ishikawa, ECC and HepG2 cells by metabolic radiolabelling of the protein with ^{67}Cu (Fig. 5). The radiolabelled protein, detected by autoradiography of the transblot, was identified as the copper-containing protein holo-caeruloplasmin by its characteristic migration on non-denaturing SDS/PAGE (Fig. 5; [7]). The differential migration of the purified human serum apo- and holo-caeruloplasmin standards, present on adjacent lanes of the same transblot as the ^{67}Cu caeruloplasmin, were identified by Western-blot analysis using ^{125}I -Protein A for detection of bound antibody once the ^{67}Cu had decayed (^{67}Cu $t_{1/2}$ = 60 h) (Fig. 5).

DISCUSSION

We now provide the first evidence for the active transcription, translation and secretion of caeruloplasmin protein by two uterine epithelial cell lines and for its transcription by human uterine endometrial tissue and placenta. Caeruloplasmin synthesized by the uterine cell lines (Figs. 1a and 1b) was observed to be of the same M_r , as that normally present for these proteins within the circulation, and the same as that synthesized by HepG2 cells (Fig. 1b). Peptide mapping of caeruloplasmin synthesized by Ishikawa cells showed identity with the corresponding HepG2 protein (Fig. 2). Furthermore, DNA sequence

analysis corresponding to partial transcripts of caeruloplasmin from Ishikawa cells and human liver (Fig. 4) indicates identity with previously reported sequences for this protein [20,30]. Therefore it is likely that the primary structure and intracellular processing of these proteins is similar for uterine epithelial cells and hepatocytes.

Copper-containing holo-caeruloplasmin isolated from human serum can be identified by its characteristic migration and preserved oxidase activity on non-denaturing SDS/PAGE and transblot [7]. We have demonstrated that the two uterine cell lines studied are capable of synthesizing ^{67}Cu caeruloplasmin (Fig. 5), indicating that uterine cells possess the necessary intracellular machinery to incorporate copper into this protein. That this process is not unique to hepatocytes has never before been demonstrated for caeruloplasmin synthesized by non-hepatic cells [9–20]. That the ^{67}Cu is fortuitously bound by the caeruloplasmin synthesized by these cells is unlikely, since the exchange of copper into caeruloplasmin requires very specific non-physiological conditions [31]. The process of copper incorporation into caeruloplasmin must involve copper transport across cellular and intracellular membranes and insertion of the metal into binding sites either co-translationally or immediately post-translationally. At present we can only assume that the stoichiometry of the copper binding of uterine caeruloplasmin is the full six or seven copper molecules per caeruloplasmin molecule [1] and that these copper atoms contain the same multiplicity of oxidation states as caeruloplasmin synthesized by hepatocytes.

Two distinct human caeruloplasmin transcripts have been reported [20,30]. The transcript CP-2 is the main transcript present in liver tissue and encodes the amino acid sequence for caeruloplasmin isolated from human sera. We have found this to be the transcript produced by our sample of human liver and by Ishikawa cells (Fig. 4). Therefore the alternative splicing which was postulated to have produced the caeruloplasmin transcript CP-1 in certain extrahepatic tissues [30] probably does not occur in uterine tissue.

The transcription of caeruloplasmin mRNA by human uterine endometrial tissue and its reported presence in rat uterus [17] strongly suggest that the synthesis of this protein by Ishikawa

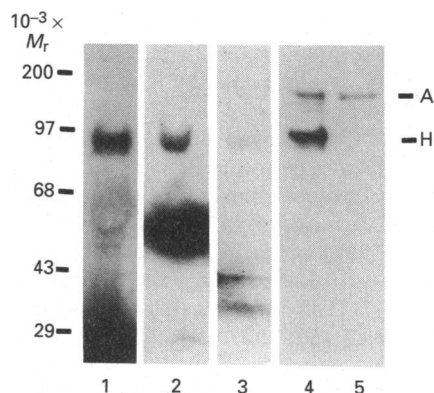


Fig. 5. Biosynthetic incorporation of copper into caeruloplasmin

Autoradiograms shown are of ^{67}Cu -containing proteins from the media of HepG2 (lane 1), Ishikawa (lane 2) and ECC1 (lane 3)-cell lines resolved by non-denaturing SDS/PAGE and transferred to nitrocellulose membranes after partial purification by anion-exchange chromatography and ethanol precipitation. Lanes 5 and 6 are representative autoradiograms of Western blots of holo-caeruloplasmin (H) and apo-caeruloplasmin (A) standards probed with rabbit anti-(human caeruloplasmin) antisera followed by ^{125}I -Protein A. These standards were subjected to SDS/PAGE in lanes adjacent to the ^{67}Cu -labelled proteins for each experiment. Western blots for caeruloplasmin were performed after the ^{67}Cu had decayed ($t_{1/2}$ ~ 60 h).

and ECC1 cells was not due to the transformation of the uterine lining cells which were the progenitors of the cell lines utilized in the present study. The presence of caeruloplasmin transcripts in purified human endometrial-gland epithelium as well as in these two human uterine epithelial adenocarcinoma cell lines indicates that the uterine epithelial lining cells synthesize this protein, but does not exclude its synthesis by other uterine cells. The ability of Ishikawa and ECC1 cells to incorporate copper into caeruloplasmin suggests that the human uterus produces a functional protein.

The uterine endometrial lining cells have been shown to contribute actively towards the creation of a unique micro-environment within the uterus in preparation for embryonic development, synthesizing growth factors, prostaglandins and even interferons [32,33]. Their synthesis of caeruloplasmin may be important for cell growth, for cellular iron utilization and for its antioxidant action. The availability of hormonally responsive non-hepatic cell lines able to synthesize caeruloplasmin, such as Ishikawa and ECC1, will offer opportunities to study the regulation of caeruloplasmin biosynthesis.

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REFERENCES

- Ortel, T. L., Takahashi, N. & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4761-4765
- Frieden, E. (1980) *Ciba Found. Symp.* **79**, 93-124
- Marklund, S. L. (1986) *J. Free Radicals Biol. Med.* **2**, 255-260
- Gitlin, J. D. (1988) *J. Biol. Chem.* **263**, 6281-6287
- Sternlieb, I. & Scheinberg, I. H. (1961) *Ann. N.Y. Acad. Sci.* **94**, 71-76
- Takahashi, N., Ortel, T. L. & Putnam, F. W. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 390-394
- Sato, M., Schilsky, M. L., Stockert, R. J., Morell, A. G. & Sternlieb, I. (1990) *J. Biol. Chem.* **265**, 2533-2537
- Neifakh, S. A., Monakhov, N. K., Shaposhnikov, A. M. & Zubzhitski, Y. N. (1969) *Experientia* **25**, 337-344
- Wright, W. W., Musto, N. A. & Bardin, C. W. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7565-7569
- Skinner, M. K. & Griswold, M. D. (1983) *Biol. Reprod.* **28**, 1225-1229
- Fleming, R. E., Whitman, I. P. & Gitlin, J. D. (1991) *Am. J. Physiol.* **260**, L68-L74
- Jaeger, J. L., Shimizu, N. & Gitlin, J. D. (1991) *Biochem. J.* **280**, 671-677
- Chu, F. F. & Olden, K. (1985) *Biochem. Biophys. Res. Commun.* **126**, 15-24
- Gitlin, J. D., Gitlin, J. I. & Gitlin, D. (1977) *Arthritis Rheum.* **20**, 1491-1499
- Angela, R. A., Grimes, A., Schreiber, G. & Mercer, J. F. B. (1987) *J. Biol. Chem.* **262**, 2875-2878
- Thomas, T., Schreiber, G. & Jaworski, A. (1989) *Dev. Biol.* **134**, 38-47
- Thomas, T. & Schreiber, G. (1989) *FEBS Lett.* **243**, 381-384
- Fleming, R. E. & Gitlin, J. D. (1990) *J. Biol. Chem.* **265**, 7701-7707
- Shvartsman, A. L., Voronina, O. V., Gaitskhoki, V. S. & Patkin, E. L. (1990) *Molek. Biol. (U.S.S.R.)* **24**, 657-662
- Yang, F., Friedrichs, W. E., Cupples, R. L., Bonifacio, M. J., Sanford, J. A., Horton, W. A. & Bowman, B. H. (1990) *J. Biol. Chem.* **265**, 10780-10785
- Holinka, C. F., Hata, H., Kuramoto, H. & Gurdip, E. (1986) *J. Steroid Biochem.* **24**, 85-89
- Tabibzadeh, S., Kafka, K. L., Kilian, P. L. & Satyaswaroop, P. G. (1990) *In Vitro Cell Dev. Biol.* **26**, 1173-1179
- Morell, A. G., van den Hamer, C. J. A. & Scheinberg, I. H. (1969) *J. Biol. Chem.* **244**, 3494-3496
- Diater, E., Pampfer, S., Yeung, Y. G., Barad, D., Stanley, E. R. & Pollard, J. W. (1992) *J. Clin. Endocrinol. Metab.* **74**, 1-9
- Pampfer, S., Tabibzadeh, S., Chuan, F. C. & Pollard, J. W. (1991) *Mol. Endocrinol.* **5**, 1931-1938
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680-685
- Cleveland, D. W. (1983) *Methods Enzymol.* **96**, 222-229
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350-4354
- Kraft, R., Tardiff, J., Krauter, K. S. & Leinwand, L. A. (1988) *BioTechniques* **6**, 225-228
- Yang, F., Naylor, S. L., Lum, J. B., Cutshaw, S., McCombs, J. L., Naberhaus, K. H., McGill, J. R., Adrian, G. S., Moore, C. M., Barnett, D. R. & Bowman, B. H. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3257-3261
- Morell, A. G. & Scheinberg, I. H. (1958) *Science* **127**, 588-590
- Pollard, J. W., Pampfer, S., Daiter, E. & Arceci, R. J. (1991) in *Uterine and Embryonic Factors in Early Pregnancy* (Strauss, J. F. & Lyttle, C. R., eds.), pp. 107-118, Plenum Press, New York
- Tabibzadeh, S. (1991) *Endocr. Rev.* **12**, 272-290

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