Primary Structure of Rat Ceruloplasmin and Analysis of Tissue-specific Gene Expression during Development*

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Ceruloplasmin is a blue multi-copper oxidase which is found in the plasma of vertebrate species. The protein is synthesized in the liver as a single polypeptide chain and is secreted into the plasma with 6 atoms of copper bound per molecule (1). Ceruloplasmin circulates in the plasma as an α₂-glycoprotein and contains greater than 95% of the copper found in this compartment. The complete amino acid sequence of human ceruloplasmin has been determined by protein sequence analysis (2) and confirmed by cDNA cloning (3, 4). This sequence data reveal that ceruloplasmin consists of three consecutive 350-residue homology units suggesting that the protein arose early in vertebrate evolution by duplication of a primordial gene encoding a copper oxidase (5). Ceruloplasmin contains three spectroscopically different copper centers (6) and has remarkable sequence similarity to other evolutionary diverse members of the multi-copper oxidase family including Neurospora crassa laccase (7) and cucumber ascorbate oxidase (8). Although there is no detailed information on the sites of copper incorporation in ceruloplasmin, analysis of these sequence homologies suggests conserved domains which may be involved in copper binding. There are currently no data available on the primary structure of ceruloplasmin from species other than human.

Ceruloplasmin plays a role in a number of biological processes including tissue angiogenesis (9), copper transport (10), iron metabolism (11) and antioxidant defense (12, 13). Consistent with the role of each of these processes in host defense ceruloplasmin is an acute phase protein, and the serum concentration increases during inflammation, infection, and tissue injury (14). Hepatic copper content and serum ceruloplasmin concentrations are also altered during the perinatal and postpartum period. In the fetus reduced biliary copper excretion is associated with elevated liver copper and low serum ceruloplasmin oxidase activity. Following parturition the serum ceruloplasmin concentration rises coincident with a reduction in hepatic copper content (15). The mechanisms of these developmental changes have not been determined.

Although the liver is the major source of plasma proteins, extrahepatic synthesis of such proteins has been demonstrated (16, 17). Thus the function and regulation of ceruloplasmin must be considered in the context of extrahepatic tissue expression. Previous studies demonstrated ceruloplasmin synthesis in rat sertoli cells (18) and human synovial tissue (19). More recently extrahepatic ceruloplasmin gene expression was detected in adult rat testes and choroid plexus (20), fetal rat choroid plexus (21), and pregnant rat uterus (22). In the current study, we cloned and sequenced rat ceruloplasmin and examined the tissue-specific sites of gene expression during development. We report here on the primary structure of rat ceruloplasmin and that the lung is the predominant site of extrahepatic expression during development.

EXPERIMENTAL PROCEDURES

Materials

Chemicals and reagents used in this study included phenol from Anaecm Chemicals, Montreal; guanidinium isothiocyanate from Fluka, Switzerland; seakem GTG and ME-agarose from FMC Bioproducts, MD; nitrocellulose from Schleicher & Schuell, and nylon (Hybond-N) from Amersham Corp. Additional chemicals were purchased from Sigma. All restriction enzymes were purchased from Promega Biotech (Madison, WI) and used according to the manufac-
turer's specifications. cDNA synthesis reagents were purchased from Invitrogen (San Diego, CA) and AMV reverse transcriptase from Life Sciences Inc. (St. Petersburg, FL). DNA and RNA modifying enzymes were purchased from Stratagene (San Diego, CA). 32P-labeled nucleotides were purchased from Du Pont-New England Nuclear.

Adult male and female Sprague-Dawley and Wistar rats were purchased from Sprague-Dawley (IN) (125-140 g) and Wistar-Dawley (IN) (125-140 g) by homogenization and proteinase K digestion (29). For maintenance on normal diets. Adult rats were mated overnight and pregnancy was determined by the presence of a vaginal plug. The gestational age of all fetuses was calculated on this basis.

Methods

Construction and Screening of cDNA Libraries—cDNA libraries were constructed from newborn Sprague-Dawley rat lung and liver tissue by ligating nucleotides synthesized on an Applied Biosystems oligonucleotide synthesizer and radiolabeled with [32P]dATP (30) and incubating with T4 polynucleotide kinase (23). cDNA was then sized by electrophoresis in a 1% GTG-glycol (30). Blots were incubated in 0.25 M NaPO4, pH 7.2, 0.2% SDS, and 1 M NaPO4, pH 7.2, 0.2% SDS, and 1 M EDTA. Blots were washed at 42 °C, the blots were washed in 6 x SSC, 0.1 M NaPO4, pH 8.3, 0.5 M KCl, and 1 MM EDTA. The samples were sealed in glass capillary tubes and incubated for 1 h at 60 °C (40). After allowing samples to cool to room temperature for 10 min, each sample was adjusted to 0.05 M Tris-HCl, pH 8.3, 0.15 M KCl, 0.7 mM dNTP, 5 mM dithiothreitol, and 425 units/ml of AMV reverse transcriptase (Life Sciences). The transcriptase reaction was carried out for 2 h at 42 °C and the reactions were subsequently washed with 0.3 M sodium acetate, 0.01 M Tris-HCl, pH 7.5, 1mM EDTA. The primer extended products were precipitated with 10% trichloroacetic acid at 6 °C for 2 h, collected by centrifugation, and analyzed on a 6% polyacrylamide-urea gel using known sequence reactions as size standards.

RNA Isolation and Analysis—Individual organs from litter mates were isolated, pooled, and snap frozen in liquid nitrogen. RNA was isolated by digestion of tissue with guanidinium isothiocyanate followed by cesium chloride gradient centrifugation. All samples used in this study included rabbit anti-rat ceruloplasmin (46), rabbit anti-human ceruloplasmin (19), and rabbit anti-human albumin and anti-human factor B (Atlantic Antibodies).

RESULTS

Characterization of Rat Ceruloplasmin Primary Structure—The complete nucleotide sequence of rat ceruloplasmin including 15 bp of 5'-untranslated sequence, 19 amino acids of a leader peptide, 1040 amino acids of mature protein, and 512 base pairs of 3'-untranslated sequence is shown in Fig. 1. The nucleotide sequence from 70–3715 bp was obtained from newborn lung cDNA clones and the remaining sequence (Fig. 1, 1–68 bp) was deduced from the sequence of the rat ceruloplasmin gene.4 Comparison of this sequence to that of human ceruloplasmin reveals significant homology (nucleotide 84%; amino acid 93%) (Fig. 1). However, a region of 12 amino acids (475–486) in the human sequence diverges completely in the

1 The abbreviations used are: kb, kilobases; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; bp, base pair.

2 R. Fleming and J. D. Gillin, unpublished data.
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sequence indicates a conservation of the Cys-His-Lys Type I Cu" ligands (5) in the later two homology units (rat amino acids 674–679–684; amino acids 915–920–925). Six of seven potential N-linked oligosaccharide attachment sites in the human are conserved in the rat; one site (Asn-339) is not because the Asn-X-(Thr) sequence is changed to Asn-X-Pro. This same site is variably glycosylated in human ceruloplasmin accounting for the two isoforms detected in serum (5).

Rat Ceruloplasmin Gene Exists as a Single Copy—To estimate the copy number of the ceruloplasmin gene in the rat haploid genome, the rat ceruloplasmin cDNA clones were used to analyze rat genomic DNA by Southern blot hybridization. Hybridization of restriction endonuclease-digested genomic DNA with cRNA probes derived from various portions of the full-length cDNA revealed patterns which were characteristic of a single copy gene approximately 45 kb in length. An example of such a pattern using prCpl-4 is shown in Fig. 2.

The Ceruloplasmin Gene Is Expressed in the Rat Lung during Fetal Development—RNA blot analysis of samples from liver, lung, heart, brain, placenta, and GI tract of 20-day rat fetuses revealed a single 3.7-kb ceruloplasmin mRNA in liver and lung tissue (Fig. 3). Hybridization of the same RNA samples with a full-length cDNA for rat Cu/Zn/superoxide dismutase identified a single 0.7-kb transcript in each tissue. The abundance of Cu/Zn/superoxide dismutase mRNA in the tissues is identical to that previously reported (47) and thus this data supports the conclusion that equal amounts of total RNA were present in each lane. A developmental analysis of liver, lung, heart, brain, placenta, GI tract, kidney, spleen, rat including an apparent omission of 5 amino acids. This sequence divergence could have been the result of a cloning artifact or a tissue-specific difference since the cDNA was obtained from lung and the human sequence was obtained from liver (3,4). To resolve this problem we prepared a second cDNA library from newborn rat liver and isolated and sequenced ceruloplasmin cDNA clones. The sequences of the lung and liver clones were identical (data not shown) indicating that the species divergence is real and not the result of tissue-specific differences in protein sequence. The amino acid

FIG. 2. Southern blot analysis of the rat ceruloplasmin gene. Rat genomic DNA (Sprague-Dawley) was digested with a series of restriction endonucleases (10 µg/ enzyme), electrophoresed through 1% GTG-agarose, transferred to nylon membranes, and hybridized with a [32P]cRNA probe (prCpl-4) corresponding to 420–1063 bp of rat ceruloplasmin. Enzyme digests are as indicated, molecular weight markers are restriction fragments of a HindIII digest of phage DNA. Hybridization and washing as described in text (see "Experimental Procedures").

FIG. 3. RNA blot analysis of rat ceruloplasmin-specific mRNA in rat fetal tissues. RNA was prepared and hybridized as described in the text. Lanes 1–6 contain 10 µg of total RNA from the tissues indicated; the blot was probed with prCpl-4 and following hybridization was exposed to XAR film for 3 h in ~70°C. The blot was subsequently rehybridized with a rat Cu/Zn/superoxide dismutase (SOD) cDNA and the results are shown in the lower half of the figure.
skeletal muscle, and adipose tissue from day 16 to birth
revealed a single 3.7-kb ceruloplasmin transcript only in liver
and lung tissue at each gestational age, while control hybrid-
ization with a 28S rRNA probe indicated that equivalent
amounts of RNA were present at all time points in each tissue
data not shown). Trace ceruloplasmin mRNA was also de-
tected in embryonic yolk sac, maternal uterus (Fig. 4), and
lactating mammary gland (data not shown). Hybridization of
RNA blots with oligonucleotide probes corresponding to pro-
teins known to be specific to lung (surfactant A protein) or
liver (albumin and α-fetoprotein) confirmed the organ fidelity
of isolated samples (data not shown). An identical pattern of
liver and lung ceruloplasmin gene expression was also ob-
tained using fetal and newborn Wistar rats (data not shown).

The Temporal Pattern of Ceruloplasmin Gene Expression Is
Different in Lung and Liver—RNA blot analysis of liver and
lung tissue at various stages of development with cDNA
probes corresponding to the entire ceruloplasmin sequence
indicated no qualitative changes in ceruloplasmin gene
expression (Fig. 5). Because of inherent difficulties in quan-
tititating mRNA abundance by this method (i.e. reprobing
of blot in Fig. 5 with β-actin revealed more RNA in newborn
lung samples), the abundance of ceruloplasmin RNA was
quantitated by dot blot analysis of total RNA from lung and
liver tissue as illustrated in Fig. 6. These studies indicate that
the abundance of ceruloplasmin-specific mRNA is equivalent
in lung and liver throughout gestation increasing at term to
50-60% of that found in adult liver. Postnatally ceruloplasmin
gene expression in the lung decreases such that by 3-4 weeks
ceruloplasmin mRNA in this tissue is <10% of that seen in
adult liver. In contrast, hepatic ceruloplasmin mRNA, already
>60% of adult levels at birth, continues to increase postnatally
reaching 70% of adult levels by 3-4 weeks.

A Single Transcription Start Site Used in Lung and Liver—

Fig. 4. RNA blot analysis of ceruloplasmin mRNA in extra
embryonic tissue. A, 10 μg of RNA from term placenta, uterus at
day 6 and day 20 of gestation hybridized with prCpl-4. B, same blot
as in A hybridized with rat β-actin. C, 10 μg of RNA from adult rat
liver, newborn lung, and term uterus hybridized with prCpl-4.

Fig. 5. RNA blot analysis of ceruloplasmin mRNA in liver
and lung. 10 μg of total RNA from day 18 (fetal), newborn (day 1),
and adult (>6 weeks) Sprague-Dawley liver and lung tissue hybridized
with prCpl-4 as discussed in text. An identical pattern is seen using
probes corresponding to all portions of the full-length cDNA sequence
(data not shown).

Fig. 6. Kinetics of ceruloplasmin gene expression during
development. At each developmental time point indicated total
RNA was isolated from liver and lung tissue, immobilized on nitro-
cellulose, and hybridized with prCpl-4 as described in text. Quantifi-
tion was carried out by liquid scintillation counting using an equiva-
 lent amount of immobilized yeast rRNA as a control for backgrounds
at each time point. The figure shows the results of a single experiment
which was repeated in triplicate with each sample in four separate
experiments. There was less than 10% variation between triplicate
samples in each experimental time point and less than 15% variation
different animals at the same time points. The RNA from
individual fetuses was pooled in each experiment. The results are
displayed as a percent of adult liver defined as 100% for this analysis.
Development times are as indicated assuming a 21-22 day gestational
period. NB, first postnatal day; YA, 3 weeks; A, adults >6 weeks old.

Fig. 7. Determination of the initiation sites for transcrip-
tion of the ceruloplasmin gene in rat lung and liver. A synthetic
oligonucleotide complimentary to 70-109 bp of the rat ceruloplasmin
sequence was used as a primer in the reverse transcriptase reaction.
Details of the primary extension analysis are as described in the text.
Reactions were carried out using 3 μg of poly(A+) RNA from day 21
fetal lung or adult liver with 30,000 cpm of oligonucleotide (lung,
liver) or adult liver with 300,000 cpm of oligonucleotide (liver-AP).
Primary extended DNA bands are indicated by the open arrows. The
lowest arrow indicates the size of the primer probe alone (40 bp). A
single transcription start site is seen in newborn lung and liver tissue.

An oligonucleotide (40-mer) corresponding to 70-109 bp of
rat ceruloplasmin was used in primer extension to characterize
the start site of the ceruloplasmin mRNA in lung and liver
tissue. Analysis of the primer-extended products from rat lung
and liver revealed that both tissues use a single identical start
site for ceruloplasmin gene transcription (Fig. 7). In addition,
a second primer-extended product was also detected in adult
rat liver hybridized with an increased amount of oligonucle-
otide primer (Fig. 7, lane 3). This second product is not consistently detected under these conditions and appears to be an artifact of incomplete sample denaturation. This band is not detected in lung tissue even with excess primer during the transcriptase reaction (data not shown). As expected, no primer-extended product was detected when yeast tRNA was used as a control (Fig. 7, lane 5). When analyzed with sequence reactions as size markers, the primer-extension data predict that the complete ceruloplasmin mRNA contains an additional 15 bp of 5′-untranslated sequence beyond the 5′ end of the sequence data as shown (Fig. 1). This data is consistent with data from genomic cloning.

The Expression of the Ceruloplasmin Gene in Newborn Lung Tissue Is Associated with Production of Ceruloplasmin Protein—To determine if the expression of ceruloplasmin mRNA in fetal and newborn lung tissue results in the production of ceruloplasmin protein, we isolated liver, lung, and brain tissue from newborn rats and immunoprecipitated ceruloplasmin from media (extracellular) and tissue lysate (intracellular) following biosynthetic labeling with [35S]methionine. As can be seen in Fig. 8 both lung and liver tissue synthesize and secrete a 132-kDa protein consistent with the single chain size of ceruloplasmin. Ceruloplasmin was not immunoprecipitated from newborn brain, a tissue previously shown not to contain detectable ceruloplasmin mRNA. Ceruloplasmin immunoprecipitated from tissue lysates of both lung and liver is seen as 2 bands approximately 4 kDa different in molecular mass (Fig. 8, intracellular, lanes 1 and 2). This doublet reflects the intracellular addition and processing of carbohydrate and is eliminated by preincubation of tissue for 6 h in tunicamycin (data not shown). The single band seen in media reflects the mature secreted form of the protein consistent with observations in hepatocyte cultures. In the experiments shown in Fig. 8 total counts/min in lysate and media were measured by trichloroacetate precipitation and ceruloplasmin was immunoprecipitated from equal counts/min in each tissue. Similar results were obtained using fetal tissues from days 18 and 20 of gestation and in each case the immunoprecipitation of newly synthesized ceruloplasmin was completely blocked by preaddition of purified rat ceruloplasmin but not rat albumin (data not shown).

A Human Type II Cell Line and Human Fetal Lung Tissue Express the Ceruloplasmin Gene—Following our finding of ceruloplasmin gene expression in newborn rat lung, we examined human fetal lung tissue and human lung cell lines for ceruloplasmin gene expression. The left half of Fig. 9 illustrates an RNA blot with 10 μg of total RNA from human GI (CaCo), liver (Hep 3B), placenta (BeWo), fibroblast (HeLa), and lung (SAP-1) cell lines. The blot was hybridized with a human ceruloplasmin cDNA, and both ceruloplasmin transcripts detected previously in human liver (4) were found in liver and lung cell lines. The right half of Fig. 9 depicts RNA from a human fetal lung explant culture (20-week gestational age). In explant culture at 20 weeks of gestation no ceruloplasmin mRNA is detected at day 0 (lane 6), but following 4 days in culture, a time associated with the appearance of differentiating alveolar epithelial cells, both ceruloplasmin transcripts are seen (lane 7). Analysis of two additional samples of human fetal lung tissue confirmed these observations (data not shown).

DISCUSSION

The derived amino acid sequence of rat ceruloplasmin reveals extensive homology (~93%) to human ceruloplasmin along the entire length of the proteins with the exception of a 12-amino acid region which occurs at the site of "autolytic" cleavage of the human protein (5). This sequence difference may explain the absence of such cleavage products with rat ceruloplasmin and suggests that any proposed role for such fragments must take into account a relatively late evolutionary divergence. In addition to coding region homology, the first 46 bp of 3′-untranslated sequence of rat and human ceruloplasmin were found to be greater than 95% homologous (data not shown). Previous reports suggest that the 3′-untranslated regions of mRNAs encoding structurally homologous proteins can be markedly conserved among species (48). A search of primate and rodent data banks against this region indicated no sequence homology with other structurally unrelated proteins.

The high degree of sequence conservation between human and rat ceruloplasmin indicates selective pressure to maintain a tertiary structure essential (presumably) for copper-binding. The sites of copper ion binding in ceruloplasmin have not been clearly defined. Limited crystallographic data and sequence homology to other multi-copper oxidases suggests that a cluster of Cys-Met-His in both the second and third domains of human ceruloplasmin serve as ligands for the type I (blue) Cu +2 (II) ions (5). Consistent with this concept these residues are entirely conserved in rat ceruloplasmin. More recently three-dimensional structural data for crystallized ascorbate
Ceruloplasmin gene expression and its role in pulmonary development.

In early fetal development, ceruloplasmin mRNA is detected in the yolk sac but not in other fetal tissues. The lack of ceruloplasmin mRNA in fetal tissues suggests a role in the regulation of gene expression. Ceruloplasmin mRNA is detected in lung tissue in fetal rats, suggesting a role in fetal lung development. The abundance of ceruloplasmin mRNA in fetal lung tissue is equal to that in the liver, supporting the notion that these regions are the site of copper binding in ceruloplasmin.

A detailed developmental survey indicated that the lung is the predominant extrahepatic site of ceruloplasmin gene expression. In early fetal development, ceruloplasmin mRNA is detected in the yolk sac consistent with the role of this tissue in fetal plasma protein synthesis. A recent report suggests that a 4.7-kb ceruloplasmin transcript is detected in the fetal brain (choroid plexus); however, we did not detect ceruloplasmin mRNA in fetal or newborn brain tissue using the methods described here. Ceruloplasmin mRNA is detected in low abundance in poly(A') RNA from choroid plexus tissue isolated from newborn brain (data not shown), suggesting that these differences are due to the sensitivity of the technique involved. The abundance of ceruloplasmin mRNA in lung tissue is equal to that of the liver during all stages of fetal development. The presence of ceruloplasmin mRNA in lung tissue is probably not simply the result of expression in differentiating fetal endothelial or epithelial tissue because kidney and GI tract (all portions) did not contain detectable ceruloplasmin mRNA. Pulmonary ceruloplasmin gene expression occurs prior to the alveolar phase of lung development in the rat (51). Interestingly, either dietary copper deficiency or the administration of copper-chelating drugs during early pregnancy result in a profound pulmonary emphysematous lesion in fetal rats (52, 53). Biochemical studies suggest that these effects are mediated by abnormal elastin metabolism related in part to a deficiency of copper-dependent lysyl oxidase (54). It is possible that ceruloplasmin plays a role in this process either by directly affecting elastin metabolism or by donating copper to lysyl oxidase (55). Alternatively, ceruloplasmin bound copper, known to be important in experimental angiogenesis (56) may be utilized in the development of sites in which angiogenesis is occurring casts some doubt on this role. Lastly, antioxidant defenses are known to increase in the rat and other species in lung tissue prior to birth in preparation for introduction to an oxygen-rich environment (57). Ceruloplasmin production in lung tissue in late fetal life may be part of this process either directly as an antioxidant (11, 12) or indirectly as a copper donor for Cu/Zn superoxide dismutase (55). The postnatal decrease in ceruloplasmin in lung is in contrast to what is seen for other antioxidants again casting some doubt on this proposed role.

Extrahepatic expression of other plasma proteins during development in the rat has been reported. α1-Macroglobulin (57-59) and α1-acid glycoprotein (60) are expressed in placenta and decidual tissue and transthyretin is abundantly expressed in fetal choroid plexus (61) but these proteins are not detected in rat lung tissue during development. Although albumin and α-fetoprotein mRNA are reported to be present at very low abundance relative to liver in several fetal rat tissues (62), under the conditions used here (10 μg of total RNA/tissue) we were unable to detect albumin or α-fetoprotein mRNA in any fetal or newborn tissue except liver. These data therefore support the concept that the developmental pattern of ceruloplasmin gene expression reported here is very different than that which has been reported for other plasma proteins. The finding of abundant ceruloplasmin mRNA and protein in fetal and newborn rat liver suggests that translational or post-translational mechanisms, perhaps involving copper incorporation into the ceruloplasmin apoprotein, contribute to the abrupt increase in serum ceruloplasmin concentration following parturition. The precise contribution of pulmonary ceruloplasmin to serum levels cannot be determined from the experiments reported here and additional experiments will be necessary since it is possible that copper metabolism and incorporation into ceruloplasmin is different in these two tissues. The consistent finding of greater ceruloplasmin protein synthesis in newborn lung than liver is in contrast to the mRNA data, further suggesting that translational mechanisms of gene expression may be important. During pregnancy and immediately postpartum, ceruloplasmin mRNA was detected in uterus and lactating mammary gland suggesting a possible role for these tissues in the changes in serum ceruloplasmin concentration seen during pregnancy and lactation.

The mechanisms of tissue-specific ceruloplasmin gene expression in lung and liver tissue during development require further study. Southern blot analysis, cDNA sequence, and primer extension data suggest differential transcription of a single gene utilizing identical exons at a single start site in both tissues. Hepatic gene expression during development involves a complex interplay of transcriptional and posttranscriptional events (63) and potential mechanisms would include: 1) tissue-specific selective loss (absolute or functional) of a trans-acting transcriptional activator. This might involve a transcriptional regulating protein present in both lung and liver and recognizing defined cis-acting sequences such as recently described for C/EBP protein (64). Alternatively such factors may recognize tissue-specific cis-acting sequences as reported for other proteins expressed in liver and extrahepatic sites (65). 2) Active repression of ceruloplasmin transcription in lung tissue mediated via common or unique cis-acting sequences as described for α-fetoprotein (66). 3) A change in cell-specific ceruloplasmin mRNA content (via either transcription or mRNA turnover) in lung tissue related to changes in lung cell type during development resulting in positional effects on cell-specific expression as described recently for glutamine synthetase (67) or a direct loss of cell type expressing the gene. These mechanisms will be distinguishable by molecular cloning and analysis of the ceruloplasmin gene and by in situ hybridization of lung tissue.

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