GENETICS

Regulation of Ceruloplasmin Gene in Mammals

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 137, No. 5, pp. 553-558, May, 2004
Original article submitted July 9, 2003

A site of rat DNA (about 1800 b. p.) adjacent to the first ceruloplasmin gene contains, apart from regulatory sequences common for all eukaryotic promotors, *cis*-elements, which are potential binding sites for soluble nuclear receptors of some hormones. Sequences characteristic of genes expressed in liver cells and mammary gland cells during lactation were detected. Full-length fragment of this locus of ceruloplasmin gene (1800 b. p.) was synthesized by PCR and used in gel shift experiments. It was found that soluble proteins extracted from purified nuclei of mammary gland cells during lactation and from the liver of adult and newborn rats, contain proteins specifically interacting with the PCR product. A fragment of chromosome gene containing exons encoding the central part of rat ceruloplasmin was cloned in pTZ19 bacterial vector. Gel shift assay showed that the cloned fragment contained binding sites for specific transcription factor YY1, whose level in nuclear protein fractions varied during ontogeny (according to immunoblotting data). Monoclonal antibodies detected protein YY1 in the complex of cloned DNA-nuclear proteins. Possible mechanisms of tissue-specific regulation of ceruloplasmin gene varying during ontogeny are discussed.

Key Words: *ceruloplasmin; tissue-specific regulation of ceruloplasmin gene activity*

Ceruloplasmin (CP), a polyfunctional Cu^{2+}-transporting glycoprotein, contains almost 95% extracellular Cu^{2+} [8]. Haploid genome contains the only copy of CP gene. However, apart from serum CP numerous molecular CP forms were detected: tissue-specific secretory proteins, intracellular membrane-bound proteins, and proteins anchored to the outer surface of the plasma membrane [2,3,10]. These CP, in accordance with their nature and location, transfer Cu^{2+} between cells, protect cells from active O_2 metabolites, and participate in bidirectional transmembrane transport of Fe^{2+} [7-10]. Activity of CP gene varies during ontogeny and during inflammation [5,6]. The existence of various molecular forms of CP, on the one hand, and modulation of CP gene activity under the effect of different factors, on the other, suggest an intricate mechanism of regulation of this gene expression. However, only *cis*-elements responsible for gene expression in the liver and response to estrogens and hypoxia were identified and located in the CP gene promotor area [6,13]. Since disturbances in CP gene expression can be the cause of various pathologies [11], the mechanisms realizing fine regulation of this gene activity require detailed investigation.

We studied the relationship between CP gene activity and the presence of factors specifically reacting with potential *cis*-elements of CP gene in cell nuclei in different organs.

MATERIALS AND METHODS

Rat organs with different expression of CP were used in the experiments: liver of 4-day-old rats (low acti-

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0007-4888/04/1375-0485

vity), female liver 1 day after delivery (maximum activity), and mammary gland on day 14 of lactation (reducing activity).

Amplification of the promotor zone of CP gene was carried out using primers selected in accordance with Oligo V3 protocol on the basis of GenBank data (5'-ACTTGCTTGTTATACATAAGGATGC3' and 3'-TCACGTGAAAATAAAAACGTATCAA5'). DNA isolated by phenol extraction from rat liver nuclei served as the matrix DNA [12]. The resultant PCR product was used for identification of specific DNA complexes with nuclear proteins by the method detecting changes in electrophoretic mobility of DNA (gel shift analysis). *EcoRI* hydrolysate of rat chromosome DNA fractionated by electrophoresis in 0.8% agarose gel was used for cloning of the CP gene site. DNA fragments located in gel zones hybridized with [^{32}P]CP-cDNA were eluted and fractions with high content of CP gene sites were inserted into pTZ19 plasmid. Cloning of the resultant recombinant DNA was carried out in *E. coli* (strain *DH5*-α) cells [12]. Transformed bacterial clones were selected by ampicillin resistance, by α-complementation of β-galactosidase gene, and capacity to bind to [^{32}P]CP-cDNA labeled with [α-^{32}P]-dATP by the statistical priming reaction with the Klenow fragment of DNA polymerase I using Multiprimer DNA labeling system kit (Amersham). The fragment of cloned DNA was used in gel shift experiments.

The nuclear protein fraction including transcription factors (TF) was obtained by saline extraction from the nuclei purified by centrifugation through 1.5 M sucrose [15].

Specific DNA complexes with TF were identified by gel shift analysis. Nuclear protein extract (5 μg) and 2-5 ng double-stranded [γ-^{32}P]-dATP-labeled oligonucleotide were used in the reaction of DNA binding to TF. The binding was carried out for 30 min at 20°C in 12 mM HEPES buffer (pH 7.9) containing 1 mM EDTA, 5 mM $MgCl_2$, 0.2 mM dithiotreitol, 10% Ficoll, 1 μg nonspecific DNA (sonicated chicken erythrocyte DNA). Unlabeled double-stranded oligonucleotides served as the competitors. The resultant complexes were separated in 4% PAAG in a buffer containing 23 mM Tris-Cl (pH 8.2), 23 mM boric acid, and 0.5 mM EDTA. The gels were dried under vacuum and autoradiographed.

We used double-stranded oligonucleotides containing consensus site for YY1 protein binding (YY1), a site from Alu-repeat consensus, and sequences for binding thyroid hormone receptors (α- and β-subunits, TRα and TRβ), 9-*cis*-retinoic and *trans*-retinoic acids. All oligonucleotides were synthesized by BioTeZ, Berlin-Buch Company, their sequences were taken from the Santa Cruz Biotechnology Catalogue. [^{32}P]DNA-labeled probes were obtained by statistical priming reaction using Multiprime DNA labeling system (Bo-

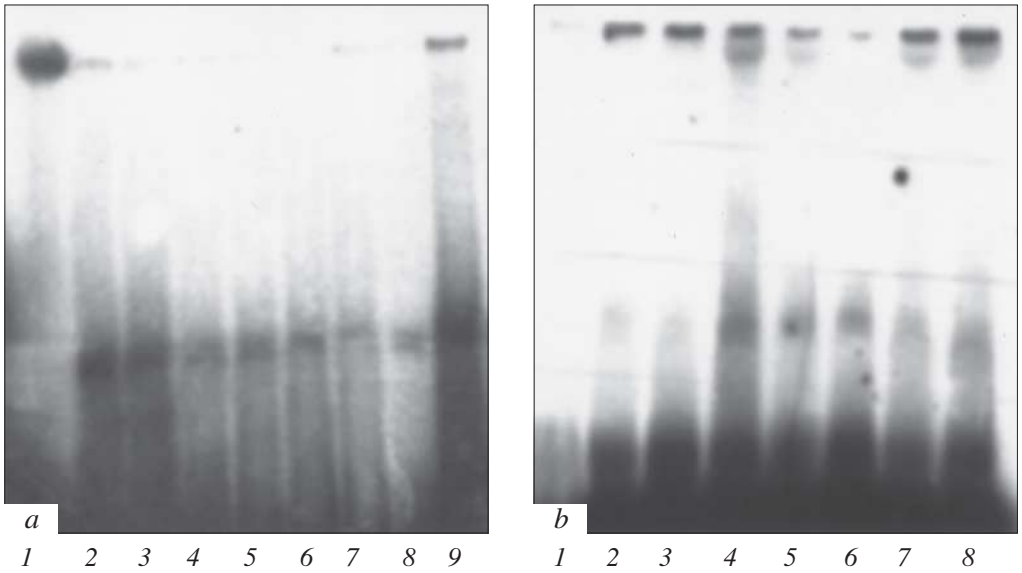

Fig. 1. Binding of nuclear factors from different rat organs to ceruloplasmin (CP) gene fragments. *a*) binding of adult rat liver nuclear factors to [^{32}P]-labeled oligonucleotides containing a site of Alu-repeat consensus AUB (*1*) and its displacement from complexes in the presence of a 4-fold excess of unlabeled oligonucleotides containing sites for binding α- (*2*) and β-subunits of thyroid hormone receptors (*3*), *trans*-retinoic acid (*4*), 9-*cis*-retinoic acid (*5*), unlabeled AUB (*6*), and 20-fold excess of unlabeled PCR product (*7*); *b*) binding of nuclear factors from different organs of rat to [^{32}P]YY1 and its displacement from the complexes by homologous unlabeled oligonucleotide and ~2300 b. p. site of CP gene. *1*) [^{32}P]YY1; *2*) [^{32}P]YY1 and newborn rat liver transcription factors (TF); *3*) [^{32}P]YY1, newborn rat liver TF, and 4-fold excess of unlabeled YY1; *4*) [^{32}P]YY1 and adult rat liver TF; *5*) [^{32}P]YY1, adult rat liver TF, and 4-fold excess of unlabeled YY1; *6*) [^{32}P]YY1, adult rat liver TF, and 2-fold excess of unlabeled CP gene site (~2300 b. p.); *7*) [^{32}P]YY1 and rat mammary gland TF; *8*) [^{32}P]YY1, rat mammary gland TF, and 4-fold excess of unlabeled YY1.

ehringer). Southern blot hybridization was carried out by the standard method [12]. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane by the semidry method [12]; immune complexes were detected by highly sensitive chemiluminescent method.

BLAST software based on the GenBank database was used in the study. The search for potential TF binding sites was carried out using MatInspector V2.2 and Sitehunter software [3] on the basis of SITE and FACTOR tables from TRANSFAC 4.0 database (www.transfac.gnf-braunschweig.de).

RESULTS

Computer analysis of a 1817 b. p. fragment of rat CP gene located upstream of the transcription start (GenBank, AC068577) showed that, apart from TATA and CAAT boxes, it contained potential binding sites for some TF typical of all eukaryotic promoters (Table 1, Nos. 4, 7, 10, 13, 21). Sequences homologous to binding sites for hormone nuclear receptor (RORalphal), progesterone and estrogen receptors were detected. The results of computer analysis are in full agreement with the data on the hormone involvement in activation of CP gene *in vivo* [8]. Scanning of CP gene 5' region revealed, apart from sequences specifically binding nuclear factors regulating gene transcription in the liver (Table 1, Nos. 6, 9, 16, 22) [6], a *cis* element specifically binding to nuclear factors characteristic of only lactating mammary gland (Table 1, Nos. 2, 8). This is in line with the data that CP found in breast milk is synthesized in mammary gland cells [2,4].

The rat CP gene promotor site was amplified by PCR. Electrophoresis of PCR product in 1% agarose gel revealed complete coincidence of the lengths of PCR product and CP gene site presented in the GenBank database (1817 b. p.). The presence of specific TF capable of binding to the studied promotor region of CP gene in liver cells of rats of different age and in lactating mammary gland was verified by gel shift assay. Double-stranded oligonucleotide containing the

TABLE 1. Location of Some *cis*-Elements Detected by Computer Analysis in the Sequence Flanking the 5'-Area of the Rat CP Gene

Nos.	Core sequence of *cis*-element*	*Trans*-factor specifically binding to *cis*-element		Position of the first nucleotide of core sequence of *cis*-element**
		reduction	completely	
1	tcgtaagGGTCag	RORA1	ROR-related orphan receptor alpha1	19
2	*CCAAAGT*	WAP	Whey acidic protein	51
3	aatAATCttgactac	GFI	Growth factor independence 1	197
4	ctTGACtacat	AP1	Activator protein 1	239
5	nnCAGTTAnnnn	PR	Progesterone receptor	372
6	ctatGTTTaaat	HFH1	HNF-3 Homolog	396
7	gaATGTa	OCT1	Octamer binding factor 1	436
8	CCAAAGT	WAP	Whey acidic protein	512
9	tgtgTGTTtgtgt	HNF-3B	Hepatocite nuclear factor 3beta	517
10	tatttgctGAAAGagact	C EBP	C\EBP binding site	525
11	tgaggcTGACcctt	ER	Estrogen receptor	623
12	gGTTAataaatgaag	HNF2	Hepatic nuclear factor 2	774
13	gacTGGCatcagagcagg	NF1	Nuclear factor 1	1103
14	gaATGTa	OCT1	Octamer binding factor 1	1114
15	aaCAGCagtg	AP4	Activator protein 4	1129
16	tgtgTGTTtgtgt	HNF3B	Hepatocite nuclear factor 3beta	1173
17	|ctTGACtacat	AP1	Activator protein 1	1188
18	ctTGACtacat	AP1	Activator protein 1	1328
19	ATGCAATTGTATAAA	TSHB	Thyrotropin beta subunit	1366
20	nnCAGTTAnnnn	PR	Progesterone receptor	1508
21	CCCCGTgg	USF	Upstream stimulating factor	1537
22	tgtgTGTTtgtgt	HNF3B	Hepatocite nuclear factor 3beta	1641

Note. *TF binding sites are written with capital letters; **position upstream from the transcription start.

Alu-repeat consensus site binding to the hormone nuclear receptors served as labeled DNA [3]. For evaluation of binding specificity unlabeled double-stranded oligonucleotides containing sites for TRα and TRβ, 9-*cis*-retinoic acid and *trans*-retinoic acid were used as competitors. Adult rat liver nuclear proteins with labeled oligonucleotides containing the Alu-repeat consensus site formed a specific complex (Fig. 1). Oligonucleotides binding the nuclear receptors (9-*cis* retinoic and *trans*-retinoic acids and TRβ) and the PCR product effectively competed with the oligonucleotide containing the Alu-repeat consensus site for nuclear protein binding. Similar data (data not shown) were obtained with nuclear proteins isolated from newborn rat liver (low activity of CP gene). It seems that none of the studied TF is involved in the suppression of CP gene activity.

Another series of experiments was devoted to identification of potential sites of regulation of CP gene activity inside the gene. For this purpose we used a 2300 b. p cloned fragment of rat chromosome gene hybridizing mainly with the central fragment of CP-cDNA including exons 5-10 of CP-cDNA (Fig. 2). Gel shift analysis showed (Fig. 1, *b*) that the internal site of CP gene contains a binding site for tissue-specific TF protein YY1 [14]. From the data presented in Fig. 1, *b* we can admit that the content of YY1 factor in nuclear protein extracts from adult rat liver is higher than in extracts from newborn rat liver and rat mammary gland. The same conclusion can be made on the basis of the data presented in Fig. 3, *a*. The relative content of YY1 protein (65 kDa) is appreciably higher in adult rat liver nuclei. The content of ST1 factor (90 kDa) (protein reacting with the promotor region of eukaryotic genes and a component of the main transcription complex) [14] also depends on the organ

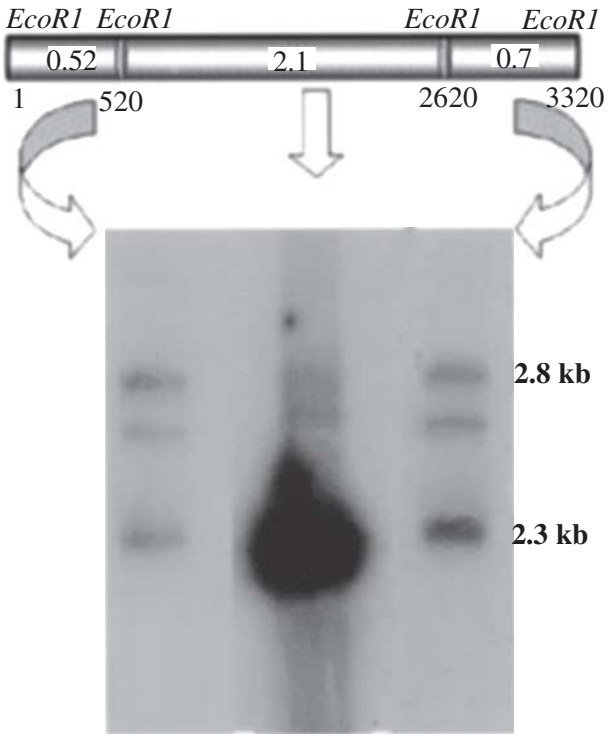

Fig. 2. Cloning of ceruloplasmin (CP) chromosome gene site. On the top: scheme of *EcoRI* fragments of full-length CP-cDNA, used for identification of the CP chromosome gene site after labeling with [α-^{32}P]-dATP. CP-cDNA [^{32}P]5' fragment (*1*), CP-cDNA [^{32}P] central fragment (*2*), and CP-cDNA [^{32}P]3' fragment (*3*) were used as probes. 10 μg of recombinant plasmid DNA treated with *EcoRI* endonuclease were added into all wells.

and changes during rat ontogeny (Fig. 3, *a*). Using monoclonal antibodies to YY1 factor we showed that this protein directly binds to cloned fragment of the natural CP gene *in vitro* (Fig. 3, *b*). On the other hand, SP1 did not bind to ~2300 b. p. CP gene fragment (Fig. 3, *b*). Computer analysis detected no potential

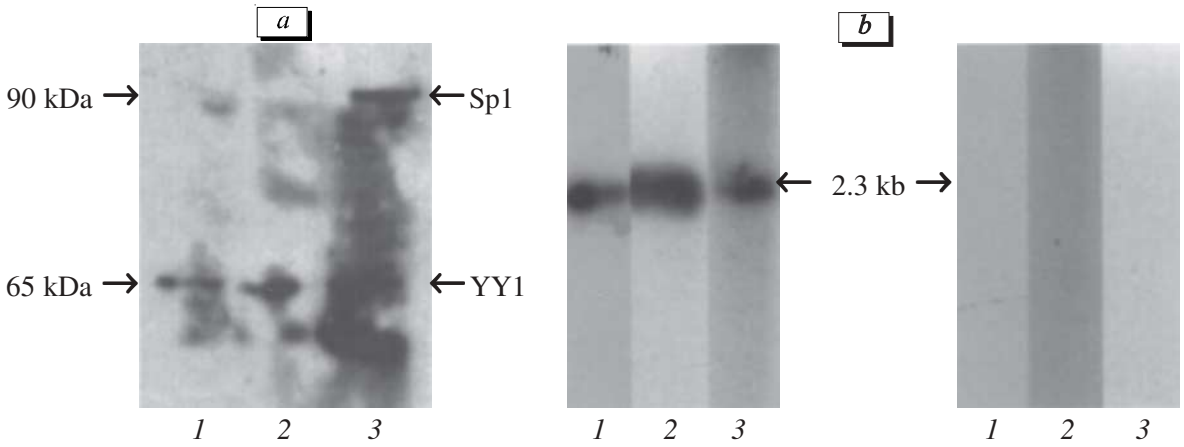

Fig. 3. Relative content of YY1 and SP1 factors in the nuclear factors from various organs of rat (*a*) and detection of these factors in complex with the transcription factors (TF; *b*). *a*) TF (30 μg) layered onto strips: TF from adult rat mammary gland (*1*), from newborn rat liver (*2*), and from adult rat liver (*3*). Monoclonal antibodies to YY1 and SP1 TF were used for TF identification, respectively; *b*) complexes: DNA-rat mammary gland TF (*1*), DNA-newborn rat liver (*2*), and DNA-adult rat liver TF (*3*). Left: reaction with monoclonal antibodies to SP1, right: reaction with monoclonal antibodies to YY1.

binding sites for YY1 *trans*-factor in either rat or human CP-cDNA. The structure of natural rat CP gene is un-known, and it is impossible to verify the presence of binding sites for YY1 protein in introns. However computer analysis detected 3 binding sites for YY1 protein in human CP gene (+)-strand fragment from 17,891 to 37,456 n. corresponding to the fragment of the rat CP chromosome gene including the cloned area. The first site starts from 21,464 n. and two neighboring sites start from 28,591 and 28,680 n., respectively. Since mammalian CP genes are highly conservative, our data suggest that YY1 protein binding to a specific site within intron can participate in the modification of the CP gene activity. Presumably, CP gene activity is negligible in low YY1 content. This hypothesis needs further verification.

The study was supported by the Russian Foundation for Basic Research (grants No. 00-04-49597 and No. 03-04-48748) and Integration company (contract No. I0064). Antibodies to YY1 and oligonucleotide were kindly provided by Prof. N. V. Tomilin (Laboratory of Chromosome Stability, Institute of Cytology, Russian Academy of Sciences), plasmids carrying full-length CP-cDNA were a gift from Prof. J. D. Gitlin (Washington University School of Medicine, USA).

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