Biochemical and Biophysical Research Communications **254,** 127–137 (1999)
Article ID bbrc.1998.9079, available online at http://www.idealibrary.com on IDEAL®

Spatial and Temporal Expression of the Cystathionine β-Synthase Gene During Early Human Development

Isabelle Quéré,* Véronique Paul,* Christelle Rouillac,† Charles Janbon,‡ Jacqueline London,* Jacques Demaille,§ Pierre Kamoun,* Jean-Louis Dufier,† Marc Abitbol,† and Jean-François Chassé*

**CNRS URA 1335, Faculté de Médecine Necker, Hôpital Necker Enfants Malades, 156 rue de Vaugirard, 75015 Paris, France; †Centre de Recherche Thérapeutique en Ophtalmologie, Faculté de Médecine Necker-Enfants Malades, 156 rue de Vaugirard, 75015 Paris, France; ‡Service de Médecine Interne B, Hôpital saint Eloi, 2 avenue Bertin Sans, Montpellier, France; and §Centre de Recherches de Biochimie Moléculaire, CNRS ERS 155, route de Mende, BP5051, 34033 Montpellier Cedex, France*

Received February 5, 1998

We report the cystathionine-β synthase (CBS) gene expression pattern during early human embryogenesis (3 to 6 weeks post conception) by in situ hybridization and in fetal and adult tissue by Northern Blot analysis. Probes were chosen to recognize either the common sequence to all known CBS mRNAs or the sequences of two different major exons 1 issued of we have previously identified. We demonstrate by in situ hybridization that CBS is continuously expressed from the earliest stages studied (22 days post conception) during embryogenesis in the tissues of developing embryos which will after birth present clinical abnormalities in homocystinuria patients. It is expressed at an especially high level in the neural and cardiac systems until the liver primordium appears. In embryonic central nervous system, the whole neural tube and primary brain vesicles are labeled. Secondary brain vesicles labeling are dependent on the neuroepithelium differentiation. The ventricular layer of the rhombencephalon, cranial nerve nuclei and then after cerebellar cortex derived from rhombencephalon ventricular layer are strongly labeled. Thalamus and other derivatives of the diencephalon plate, the neuroblastic layer of the retina, lens and dorsal root ganglia are labeled. After 35 days post conception, CBS mRNAs was detected in endocardial cells and in cells derived from the neural crest of the heart and in particular developing mesodermic regions such as the primitive hepatocytes of the liver, mesonephros vesicles, various endocrine glands and developing bones. We could not detect tissue specificity of different probes at this embryonic stage. Northern blot analysis consistently detected mRNA species in fetal 25 weeks post conception brain, liver and kidney. The common cDNA probe revealed the 2.5 and 3.7 kb mRNA species from brain, liver and kidney. The exon 1b probe detected only the 2.5 kb mRNA and the exon 1c probe the 3.7 kb mRNA in these three tissues. In adult tissue, the 1b probe detected only the 2.5 kb mRNA and the 1c probe only the 3.7 kb mRNA in the liver. © 1999 Academic Press

Key Words: **cystathionine-β synthase gene; development; homocysteine; human embryo.**

Abbreviations used: Cystathionine-β Synthase (EC 4.2.1.22), CBS; homocysteine, Hcy; 5-10 methylenetetrahydrofolate reductase (1.5.1.20), MTHFR; methionine synthase (2.1.1.13), MS; central nervous system, CNS.

Methionine is necessary for the growth and development of mammals. It is thought to be essential because of its use for the synthesis of both proteins and S-Adenosine-Methionine. S-Adenosine-Methionine is the methyl donor in numerous, biologically significant, transmethylation reactions. Homocysteine (Hcy) is an essential intermediate compound in this methionine metabolism in which it occupies a major regulatory branch point. It may be either converted to cystathionine and then to cysteine through the transsulfuration pathway or remethylated in liver mammals to give methionine. Pathological conditions, mainly cystathionine-β synthase (CBS) deficiency, that cause homocysteine accumulation also results in S-Adenosylhomocysteine accumulation. S-Adenosylhomocysteine is a potent inhibitor of the various methyltransferases. When methylation is inhibited, synthesis of creatine, sarcosine, lecithin and methylation of protein and nucleic acids are affected (1).

The first enzyme of the cysteine biosynthetic pathway, cystathionine-β synthase (CBS-EC 4.2.1.22), catalyzes the condensation of homocysteine with serine forming cystathionine. Also this enzyme can produce endogenous hydrogen sulfide from cysteine in liver,

0006-291X/99 $30.00

kidney (2, 3) and hippocampus (4). The human CBS gene consists of 19 exons (3 alternative exons 1 [exons 1a, 1b, 1c] and 16 other exons) with a 3′-alternative splicing region (5). The two 2.5 kb mRNAs called 'a' and 'b' containing exon 1a and 1b can lead to the same CBS protein subunit of 551 amino acids. The 'b' mRNA is the major transcript of the human CBS gene. The 63-kDa human CBS polypeptide is assembled into a tetramer of 250-kDa binding pyridoxal phosphate (PLP) and heme. The presence of heme is an absolute requirement for the correct folding of the enzyme and the formation of the PLP binding domain (6). A third 2.7 kb cDNA sequence called 'c', only observed in Northern blots of human adult pancreas RNA, contains the exon 1c. The end of exon [A] and the beginning of exon 5 [TG] forms an 'ATG' codon. Translation from this putative start codon would result in a protein of 451 amino acids. This putative protein has not been detected. A fourth cDNA of 3.7 kb has been also observed but not sequenced.

Genetic alterations in CBS (7), in 5-10 methylenetetrahydrofolate reductase (8) and in methionine synthase (9) can cause elevated plasma homocysteine levels leading to homocystinuria. The most common type of inherited homocystinuria (80%) is an autosomal-recessive trait related to a genetic deficiency of CBS. At least 80 different mutations (10; 7) have been found in homozygous patients who present various different clinical signs. The clinical manifestations of homocystinuria include mental retardation, seizures, ectopia lentis, osteoporosis, major skeletal development abnormalities, pale skin and fair brittle hair. Vascular complications resulting in heart attack, stroke or pulmonary embolism frequently causes early death of these patients (11). The clinical and biochemical phenotypes associated with CBS deficiency can be divided into two clinical types: pyridoxine-responsive and pyridoxine-nonresponsive, based on homocysteine levels following pyridoxine administration. Pyridoxine-responsive patients have milder hyperhomocysteinemia and milder clinical manifestations suggesting a dose effect of homocysteine on the clinical presentation.

The biological basis or mechanism whereby homocystinuria due to CBS deficiency is associated with these developmental abnormalities is unknown. It has been suggested that hyperhomocysteinemia may be the pathogenic agent per se on the basis of clinical and epidemiological evidence (1, 12, 13). Recently, D,L-homocysteine administration during avian embryo development has been shown to induce congenital defects of the heart and neural tube (14). However the specificity of the phenotype of homocystinuria with neurological, bone and vascular disease argues against a generalized non-specific deleterious mechanism but for some degree of tissue specificity underlying the observed pathology. This relative specificity may be linked to the ability of a tissue or organ to metabolize homocysteine at a high rate, especially when tissues are dividing rapidly as during embryogenesis.

To better understand the mechanisms of developmental abnormalities associated to CBS deficiency, we have determined the spatial and temporal pattern of CBS gene expression in serial sections of normal human embryo by in situ hybridization and by Northern blot analysis in fetal and adult tissues to test hypothesis of CBS specificity during normal embryo development.

MATERIALS AND METHODS

Human embryos. Morphologically normal human embryos (n=21) between 3 and 6 weeks post ovulation were obtained from legal abortions triggered by Mifepristone (RU 486) at the Broussais Hospital in Paris. The fetuses were all obtained from legal abortions performed for medical reasons threatening the health of the pregnant women. Completely independent the medical staff at Broussais Hospital is completely independent of the research group. Maternal consent for the study was never asked before the abortion was decided and performed: the consent was obtained after the abortion was completed. The relevant Ethics Committee at the Hôpital Necker-Enfants Malades, Paris, approved the procedure. The embryos were microdissected from the whole trophoblast under a dissecting microscope. The developmental stage of each embryo was estimated according to the Carnegie classification established by R O'Rahilly, Carnegie Laboratories of Embryology, (15,16). Twenty one embryos were used: 4 stage 10 embryos (J22-J23), 1 stage 11 embryo (J24-J25), 4 stage 13 embryos (J28-J31), 3 stage 15 embryos (J33-J36), 2 stage 16 embryos (J37-J40) and 4 stage 17 embryos (J41-J43), 2 stage 18 embryos (J44-J47) and 1 stage 20 embryos (J50-J52). Two human 25 weeks fetuses were also studied. Fetuses and microdissected embryos were frozen using powdered ice, and stored at −80°C. Cryostat sections (15 μm) were mounted on slides previously coated with 2% 3-aminopropyl-triethoxylane solution in acetone. Sections were fixed for 30min in 2% paraformaldehyde with 0.1M phosphate buffer (pH 7.4), rinsed once in phosphate buffered saline, rinsed briefly in water and dehydrated with series of ethanol concentrations (50%,75%,95%). Sections were then air dried and finally stored at −80°C. This procedure was used to preserve mRNA in embryonic and fetal tissues.

DNA probes for in situ hybridization and Northern blotting. The oligomer probes were synthesized and purified by Genset, France. They were 3′ end labeled with [α-^{35}S]dATP (NEN) using terminal deoxyribonucleotidyl transferase (BRL, 15U/μl) to give a specific activity of approximately 7×10^8 cpm/μg. The probes were purified on biospin columns (Biorad) before use. The sequence of the "common cDNA" CBS sense oligoprobe was: 5′-ATGACCAAGTTCCTGAGCGACAGG-TGGATGCTGCAGAAGGGCT TTCTGAAGGAGGAGGAC (positions 1291 to 1350 -EMBL N° X82166), this sequence is common to all known CBS cDNA sequences. The sequence of the sense oligoprobe specific for exon 1b (17) of CBS was: 5′-CAGTCGGGGCAGCCCTCGCCCCTC-TTTTCCA TGTATCCGTCCA G (position 19:67 -EMBL N° X82166). The sequence of the sense oligoprobe specific for exon 1c of CBS was: 5′-TGAGACAGAGAATGGGAATTTGGAGGGTCTGTGGTGGC-CTCGTC (EMBL N° L00972, no published). The three corresponding antisense probes were synthesized (Fig. 1). The sense probes were used as negative controls.

Northern blot hybridization. Tissue-specific expression of the CBS gene was assessed by hybridization of oligonucleotide antisense probes, labeled by T4 polynucleotide kinase (Life Technologies), to northern-blot filters containing human poly(A)$^+$ RNAs (2 μg for each lane) from various fetal tissues (#7756-1) ranging from 19 to 25

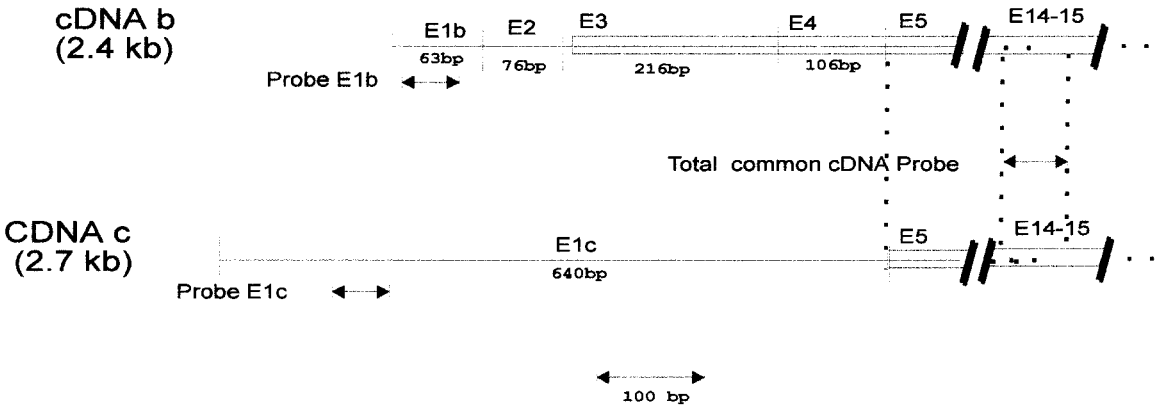

FIG. 1. General diagram of the three corresponding probes of the human cDNA CBS gene. The exons are separated by vertical lines and numbered. The coding region is shown as a filled bar. Specific probes for exon 1b, 1c and common cDNA used for hybridization are in dashed arrows. It should be noted that there are other cDNAs which differ in their 3′ end splicing or which is minor type. Another type of cDNA which has been not sequenced yet (3.7 kb, 6.0 kb).

weeks post conception and adult (#7760-1) tissues (Clontech, Palo Alto). The methods for hybridization and washing were as follows: each blot was incubated overnight (20 hours) with 5 ml of prehybridization buffer containing 50% formamide, 5× SSC, 50mM Na_2HPO_4 (pH 6.5), Dextran solution (× 1), 250 μg/ml salmon sperm DNA and 10 μg/ml poly(A^+). Hybridization was performed for 20 h at 42°C in the same solution except that 50 μg/ml salmon sperm DNA and 10^5 cpm/cm^2 of labeled probe were added. Each membrane was washed twice with 100 ml of 2×SSC/0.1% SDS for 30 min, once with 0.3×SSC/0.1% SDS for 30 min and once with 0.1×SSC/0.1% SDS for 30 min all at 65°C. The membrane was then used for autoradiography with X-OMAT X-ray film (KODAK) with an intensifying screen at −70°C.

In situ hybridization procedure. Hybridization was performed as previously described (18). The hybridization cocktail contained: 50% Formamide, 4× SSC (standard saline citrate), 1 × Denhardt's solution, 0.25 mg/ml yeast tRNA, 0.25 mg/ml sheared herring sperm DNA, 0.25 mg/ml poly(A)$^+$, 10% Dextran sulfate (Sigma), 100 mmol DTT and one of the ^{35}S dATP-labeled probes, at a concentration of 6×10^5 cpm/100 μl of final hybridization volume; 100 μl of hybridization solution was put on each section. Then the sections were covered with a parafilm coverslip and incubated in a humidified chamber at 43°C for 20h. After this hybridization, the slides were washed twice (2 × 10 min) in 1× SSC containing 10 mM DTT at 55° C, and twice (2 × 15 min) in 0.5× SSC containing 10 mM DTT at 55°C, and finally in 0.5× SSC containing 10 mM DTT for 15 min at room temperature. The sections were then dipped in water, dehydrated with a series of concentrations of ethanol and exposed to Amersham Hyperfilm betamax X-ray film for 4 days and then to Kodak NTB2 photographic emulsion for 2 months at +4° C.

RESULTS

In Situ Hybridization Analysis

Although the CBS gene seems to be ubiquitously expressed in embryonic tissues, it is clearly most expressed strongly in embryonic neural and cardiac tissues until the liver primordium appears at CS 15. The intensities of labeling by the "common cDNA" probe and the specific 'b' and 'c' probes differed but did not appear to vary in tissues according to stage of embryonic development.

CBS expression in the human embryonic central nervous system (CNS). The CBS gene was strongly expressed in the CNS from the earliest stages we have studied. At Carnegie stage 10 (22 days post conception), the whole neural tube scored positive as assessed by autoradiography (Fig. 2A) as well as at the cellular level (Fig. 2B). At 28 days (CS13), the three primary brain vesicles, which are the promesencephalon, the mesencephalon and the rhombencephalon neurons were equally intensely labeled. At CS15, when secondary brain vesicles are developed (35 days post conception); the labeling was not uniform through out the CNS. At CS 18 (44 days post conception), the rhombencephalic labeling was dependent on the neuroepithelium differentiation. In the rhombencephalic lips, cerebellar primordium labeling was very strong (Fig. 3B, 5A). The strongest labeling of the rhombencephalon was observed in the ventricular layer, then in the mantle layer and the weakest labeling was in the marginal layer. Within the ventricular layer, labeling was maximum in the inner part in close contact with the mantle layer (Fig. 4A). In the diencephalon plates, the three embryonic swellings i.e the thalamus, hypothalamus and epithalamus were labeled (Fig. 3B). The choroid plexuses, the tela choroidea, a layer of the pia mater covering the thin rhombencephalon roof plate were only poorly labeled. All the cells constituting the spinal cord were equally and strongly labeled whereas arachnoid and dura mater surrounding spinal cord were not. At CS (41 days post conception) the cranial nerve nuclei V, VII, VIII and X from the rhombencephalon and dorsal root ganglia contained large amounts of CBS mRNAs. The differentiating otic and the olfactive duct vesicles were also labeled (Fig. 3A). The neuroblastic layer of the retina and the lens at all the developmental stages examined contained CBS mRNAs (Fig. 4B). In 25-week-old fetal brain, strong labeling was observed in the cerebral and cerebellar cortices and in the subventricular germi-

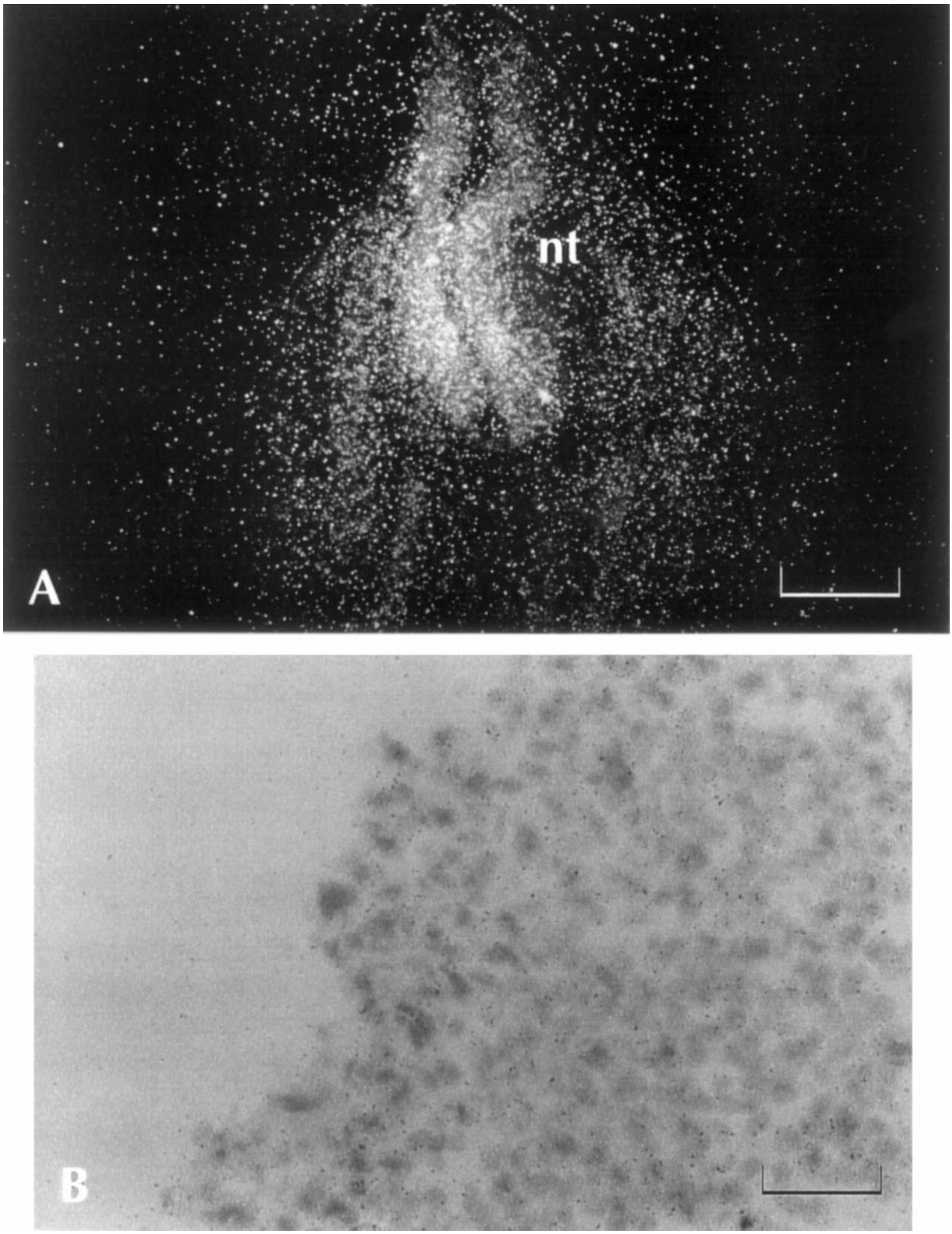

FIG. 2. Neural tube CBS gene expression in human embryo 23 days post conception (CS 10). (A) Labeling of the neural tube observed in dark field. (B) Histological aspect of the same neural tube observed in bright field. This represent a 4-fold magnification of the neural tube of (A). (A, bar = 200 μm; B, bar = 50 μm).

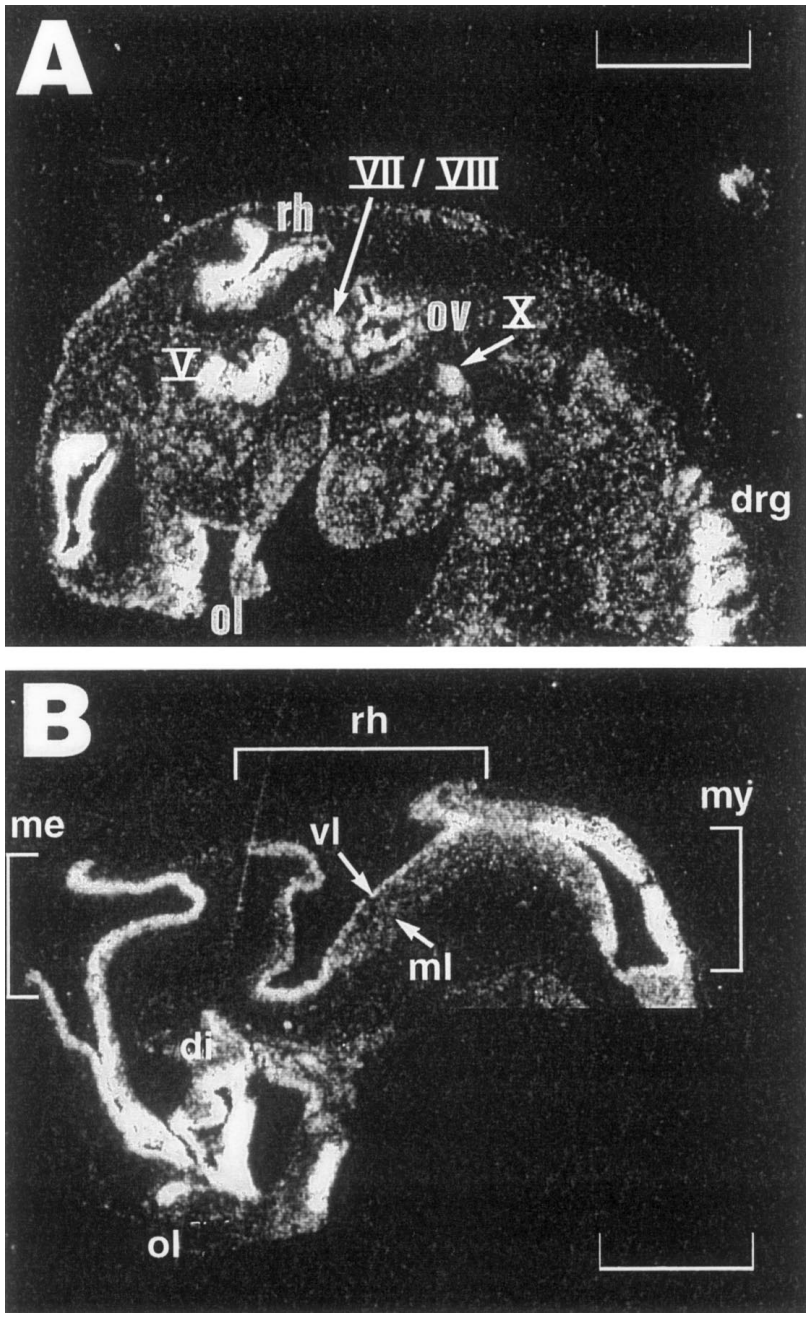

FIG. 3. Autoradiography showing the distribution of CBS mRNA in parasagittal sections of the central nervous system of 6-week-old human embryo after in situ hybridization. (A) Dorsal root ganglia and cranial nerve nuclei of the central nervous system display significant labeling. (B) Secondary brain vesicles of the central nervous system display significant labeling (Bar = 1,4 μm for A and B sections). (A) ov. otitic vesicles, ol. olfactory extremity; drg. dorsal root ganglia; V, VII, VIII, X. cranial nerve ganglia. (B) rh. rhombencephalon; me. mesencephalon; di. diencephalon; mi. Myelencephalon; VL. Ventricular layer; ML. Molecular layer; li. liver; h. heart; mv. mesonephros vesicles; f. developing femur; (A) ov. otitic vesicles, ol. olfactory extremity; drg, dorsal root ganglia; V, VII, VIII, X. cranial nerve ganglia.

native zones. In the cerebellum, CBS gene expression was weak in the deep cerebellar nuclei and the strongest labeling was observed in the cerebellar cortex. No expression was detectable in the white mater (Fig. 5A). In the cortex, both the granular and the molecular layers were labeled (Fig. 5B) but cellular labeling was more intense in the molecular layer.

Spatial distribution of the CBS mRNAs in other human embryonic tissues at Carnegie stages 10-15 (from day 22 to 36). At these early stages of development, abundant CBS transcripts were detected in the CNS but also in other tissues. At CS 10, the first somites condensations expressed the CBS gene although to a clearly lower level than in the neural tube (Fig. 2A). At

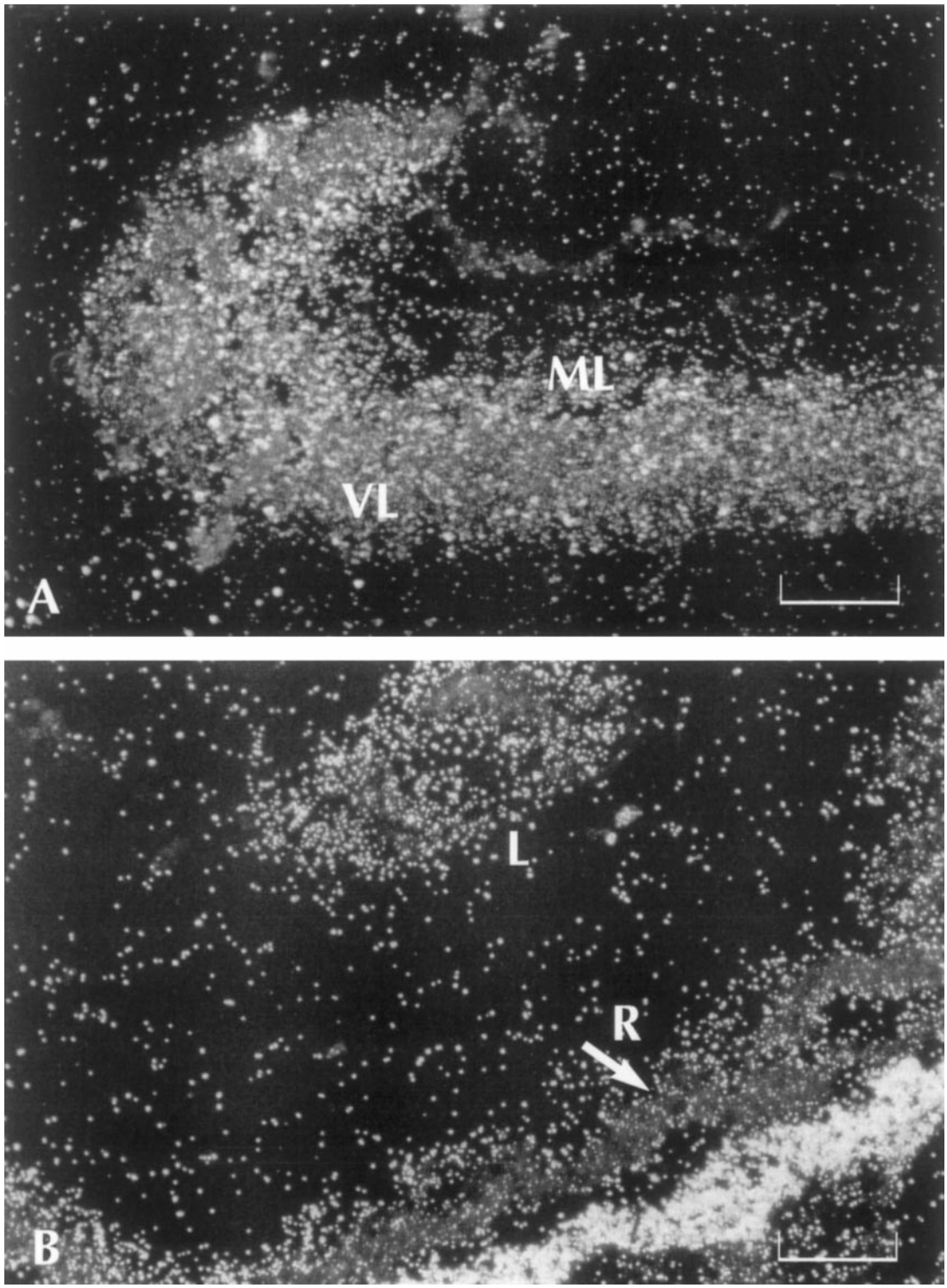

FIG. 4. Distribution of CBS mRNA in the rhombencephalon and the eye in a 6 week-old embryo after in situ hybridization. (A) Labeling of the rhombencephalon at the cellular level observed in dark field showing predominant labeling in the inner part of the ventricular layer; (B) Labeling of the retinal layer and of the lens at the cellular level observed in dark field; (A, bar = 200 μm; B, bar = 100 μm) ML. molecular layer; VL. ventricular layer; l, lens; r, retinal layer.

this stage which corresponds to 22 days post conception when vascularization commences in the embryo, only low levels of CBS mRNA was found in the developing vasculature (angioblasts and angiocysts) of the extraembroynic mesoderm and the dorsal aortae. In the heart, CBS gene expression was found in the endocar-

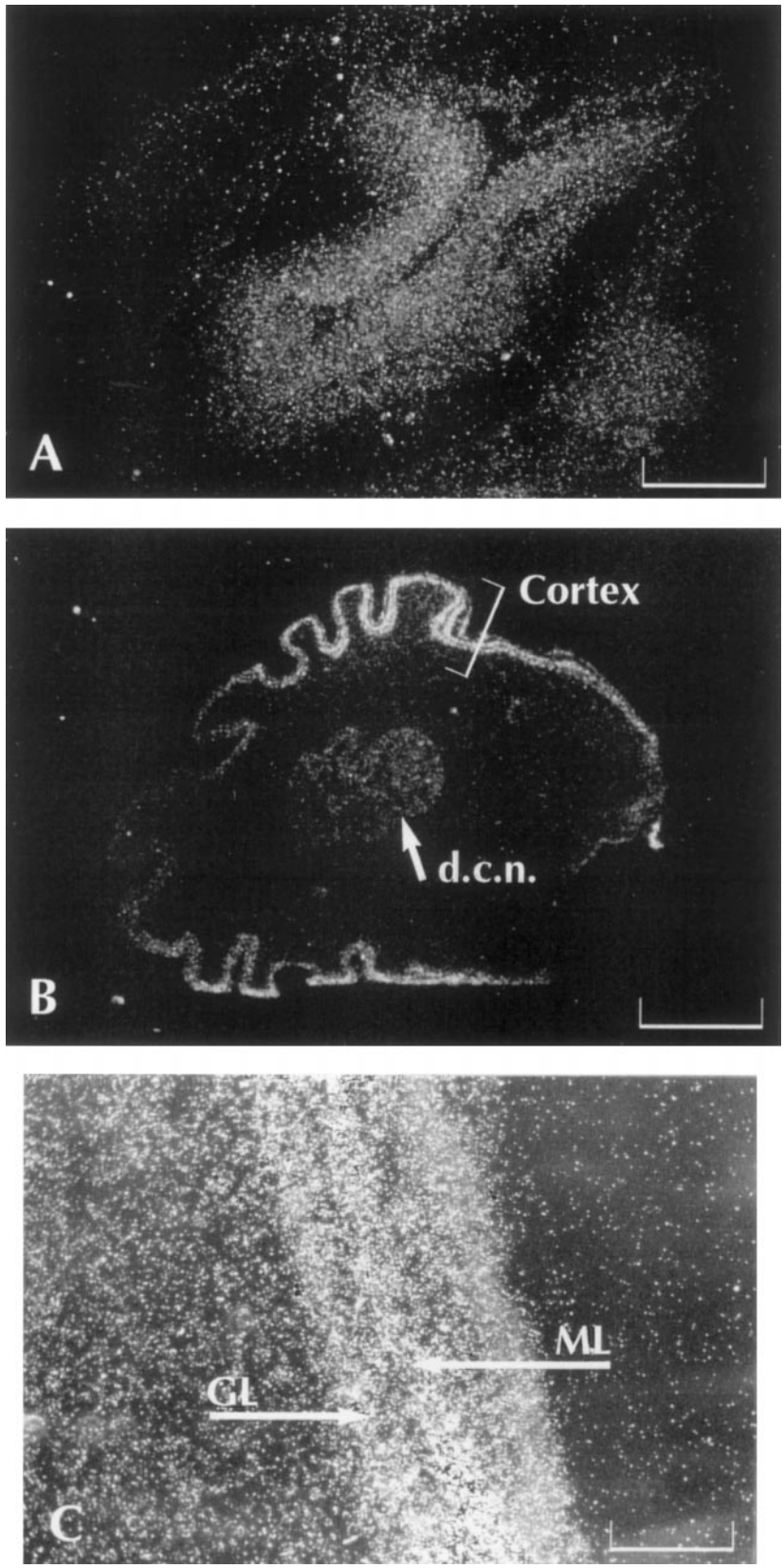

FIG. 5. Distribution of CBS mRNA in the rhombencephalon and in the fetal cerebellum of human embryo and fetus (A) Labeling of the rhombencephalon observed in dark field in a 6-week-old embryo. (B) Frontal section of a 25 week-old human fetal cerebellum showing CBS mRNA strong labeling in the cerebellar cortex and lower level of labeling in the deep cerebellar nuclei. (C) Histological aspect of the labeling in the cerebellar cortex of the same cerebellum section as observed in the upper section observed in dark field. (A, bar = 200 μm; B = 5,5 mm; C = 100 μm) (B) dcn, deep cerebellar nuclei; ML. Molecular layer, GL. Granular layer.

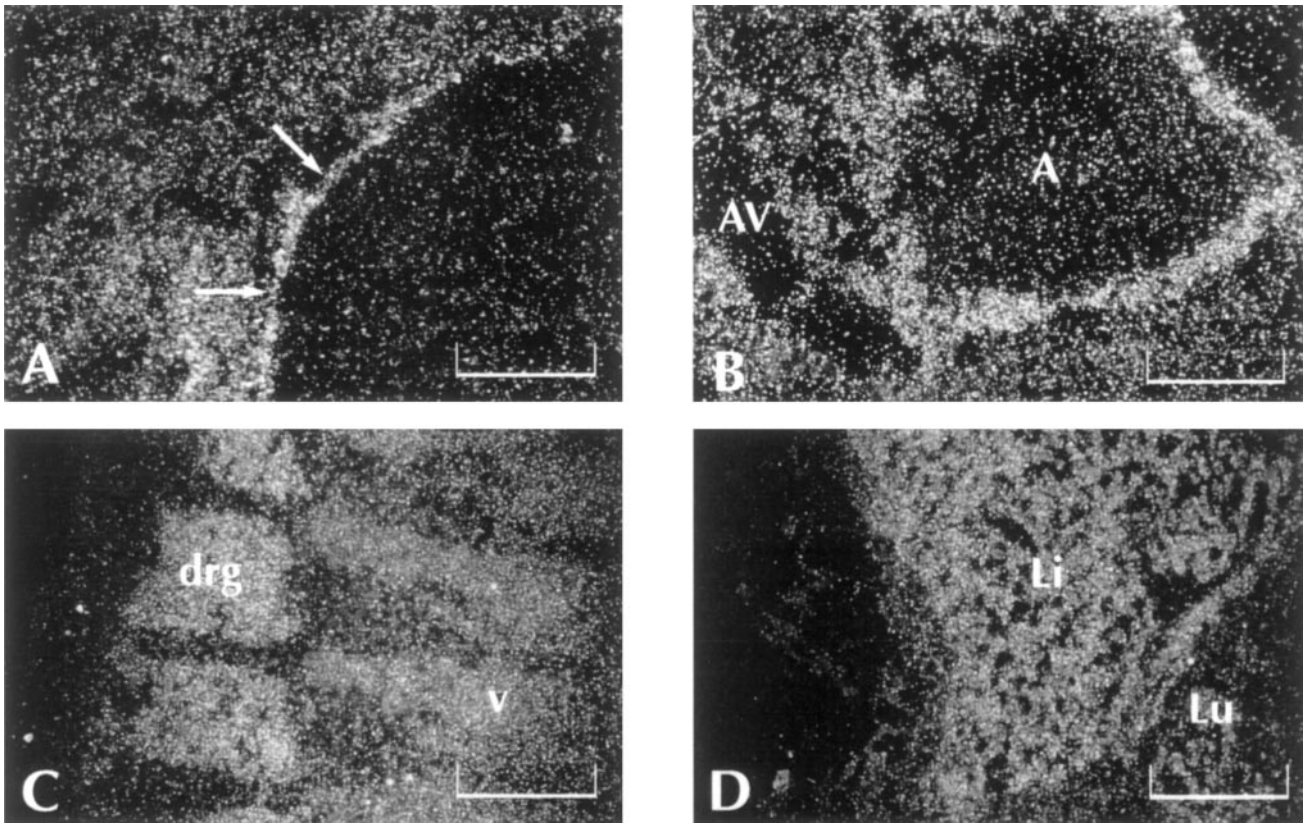

FIG. 6. Distribution of CBS mRNA in various tissues out of the central nervous system. (A) Labeling of the endocardial cells lining the cardiac cavities in a 36 day-old embryo (CS15) as indicated by the arrows. (B) Labeling of the cells in the myocardial wall of the atrioventricular channel in a 36 day-old embryo (CS15). (C) Labeling of the dorsal root ganglia and of the developing vertebral bodies and arches in a 5 week-old embryo. (D) Intense labeling of the liver in a 6 week-old embryo and weak labeling of the lung observed in dark field. (A, bar = 100μm; B, C, D, bar = 200μm). A. artrial chamber; AV. atrioventricular canal; drg: dorsal root ganglia; v. vertebrae bodies; li. Liver; Lu. Lung.

dium cells (Fig. 6A) including the endocardial tissue lining the atrioventricular channel and the future aorticopulmonary septum, and the atrial and ventricular cavity (Fig. 6B). CBS mRNA was also detected in cells derived from the neural crest forming the myocardial walls of the bulbus cordis at CS 15. CBS mRNA was detected in many somitic derivatives and at lower level in many tissues originating from the mesoblast: the developing vertebral bodies and arches were labeled whereas the developing fibrous intervertebral disks were not (Fig. 6). The original core of each disk, which is composed of cells originating from the notochord, was not labeled.

Spatial distribution of CBS mRNA on CS16 to 18 (from J37 to J44) in other tissues. These stages correspond to the start of the organogenesis and different tissues are particularized. From Carnegie Stage 15 (J37-J40), the CBS gene mRNA was most strongly found in the central nervous system and in the liver. High levels of CBS mRNA begin to be detected in particular developing mesodermic regions including liver (Fig. 6D) and mesonephros vesicles. Analysis of the cellular distribution of labeling revealed that primitive hepatocytes were labeled. In the heart, CBS mRNA was found in the endocardial tissue and in the atrial and ventricule myocardial wall. Examination of CS18 human embryos revealed CBS mRNA in various endocrine glands including the thymus and thyroid primordium and the adrenal cells. CBS mRNA was also detected in developing bones of the head and in pharyngo-larynx cartilages, ribs, vertebrae and in areas where bone ossification is thought to be initiated. The weak labeling observed in autoradiography in the lung was not confirmed at the cellular level (Fig. 6D).

Northern Blots Analysis of Human CBS mRNAs in Adult and Fetal Tissues

Total RNA from human tissue samples were tested for hybridization with the various CBS probes by Northern blotting in order to investigate the distribution of human CBS mRNAs containing the nucleotide sequence corresponding exons 1b and 1c (5). Probes were chosen to recognize either the common sequence

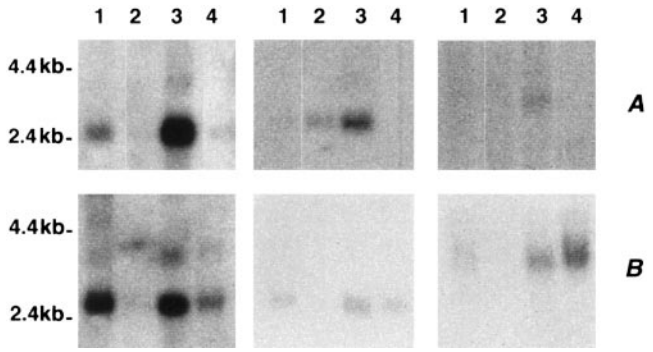

FIG. 7. Northern blot analysis of the CBS mRNAs in human adult and fetal tissues. RNA from various human adult and fetal tissues were tested by Northern blotting (Clontech) for hybridization with the "common cDNA" (left), specific oligonucleotide of exon 1b (middle) and specific oligonucleotide of exon 1c (right). Lanes 1 through 4 display: brain, lung, liver and kidney. Upper lanes A are adult tissues and lower lanes B are fetal tissues. Results were obtained after 3 days of exposure for the left figure, and 10 days for the two others.

to all CBS mRNAs (the "common cDNA") or the sequence of exon 1b (the major cDNA) and exon 1c (other major cDNA with a putative protein [5]). When the common cDNA is used as the probe, the intensity of the autoradiographic signal is assumed to be equal to the sum of all signals which would be obtained separately for each different mRNA species. The common cDNA probe revealed the 2.5 and 3.7 kb mRNA species in total RNA from fetal brain, liver and kidney (Fig. 7B left). The intensity for the 2.5 kb species was higher than that for the 3.7 kb species. The exon 1b probe detected only the 2.5 kb mRNA in these tissues (Fig. 7B middle). The exon 1c probe (Fig. 7B right) only revealed the 3.7 kb mRNA in fetal brain, liver and kidney (this probe detect the 2.7 kb in pancreas adult [5]). Thus the two probes reveal different mRNAs species in identical fetal samples. The common cDNA probe detected the 2.5 and 3.7 kb mRNA species in adult brain, liver and kidney (Fig. 1A left). In the adult liver, the 2.5 mRNA was more abundant than in the fetal liver. The exon 1b probe only detected the 2.5 kb mRNA in the liver and very faintly in the lung (Fig. 1A middle). The exon 1c probe (Fig. 1A right) detected only the 3.7 kb mRNA in the liver.

DISCUSSION

The CBS gene is expressed very early and continuously during embryogenesis in tissues that are mainly affected in homocystinuria. It is expressed at especially high level in the neural and cardiac systems until the liver primordium appears. It is found at lower level in developing bones. Embryonic changes of CBS gene expression during differentiation are detected by in situ hybridization in central nervous system (CNS) tissues. The high level expression of CBS gene in the neural tube is modulated as the tissue differentiates. As the rhombencephalon differentiates into the ventricular layer, the mantle and the marginal layer, expression of the CBS gene becomes highest in the inner part of the ventricular layer in close contact wit the mantle layer. These cells differentiate into Purkinje neuroblasts of the cerebellar molecular lamina, a site in which the strongest CBS labeling is observed in 25 weeks human fetal brain. At the same stage, neuroblasts of the deep cerebellar nuclei show only weak expression of CBS gene whereas neuroblasts of the granula lamina express CBS gene strongly. Thus the lower hybridization signal observed in adult brain Northern blots as compared with fetal blots may be due either to a dilution of the CNS RNA signals within the ensemble of total fetal RNA, either to the restriction of CBS expression to certain cell types of the brain rather than to a down regulatory mechanism. This is consistent with data showing a lower CBS activity in the brain structures in 125 day-old monkey fetus than in 45 day-old monkey fetus as the differentiation of cortex from underlying white mater becomes evident (18, 19). Furthermore, different CBS enzymatic activity was observed in different parts of the brain with the highest activity in the cerebellum cortex. Our result suggest that this high CBS enzymatic activity in the cerebellum cortex is at least partly due to substantial CBS enzyme synthesis resulting from a high level of CBS gene transcription. However our results do not agree wit those obtained in rat embryos. The CBS enzymatic activity is undetectable in the early stages of neurulation in rat embryos and is only detected in the embryonic fetal liver (20, 21). This discrepancy may result from possible differences between the detection threshold of enzymatic activity and that of in situ hybridization for RNA.

That CBS gene expression is higher in neural and cardiac tissues than in other tissues from the earliest stages of development is consistent with the common origin of some of the cells constituting these tissues. Neural crest cells that form vascular smooth cells of the aorticopulmorary septum are generated in a neural ectoderm site contiguous to the neural tube. Moreover, CBS gene expression is also observed at a high level in other tissues originating from the neural crest such as cranial nerve ganglia, dorsal root ganglia and pharyngeal cartilages. Melanocytes are also neural crest derivatives. Inhibition of melanin synthesis is responsible for pale skin and hair and has been described in homocystinuric patients. As high levels of CBS mRNA are observed in some neural crest derived cells, it can be hypothesized that CBS deficiency in these cells could lead to the inhibition of melanin synthesis as observed in skin and hair melanocytes of some homocystinuric patients (22).

Thus our results in early normal human embryo showed a clear strong expression of CBS mRNA in

neural, cardiac, lens and bone tissues affected during homocystinuria. We were enable to find any tissue specificity of the expression of the different exons we have studied. Northern blots showed differential expression between fetal and adult tissue. We consistently detect fetal brain, liver and kidney mRNA species. Whatever the probe used, none signal is detected in the fetal lung total mRNAs. The size of the hybridized fetal brain mRNAs varies according to the nature of probe used. The two distinct CBS mRNA species (2.5 and 3,7 kb) observed by northern blots in fetal brain may arise from alternative splicing or may correspond to a mature spliced isoform and to a premessenger transcript. In adult tissue, CBS mRNA labeling is restricted to liver and brain. These differences of CBS gene expression suggest the existence of complex transcriptional regulatory mechanisms acting throughout human development and specific transcriptional shifts occurring after birth. These data require further investigations.

We demonstrate CBS gene expression in the neural tube and heart normal tissues of the homologous early human embryos. In avians, homocysteine thiolactone causes neural tube and heart defects (14). In cases of homocystinuria due to CBS deficiency, there is no evidence of neural tube or cardiac dysmorphogenesis at birth. Neurological and cardiovascular abnormalities develop several weeks after birth in human homocystinuria patients. Furthermore, a study of CBS knock out mice (23) shows that embryo vitality is not affected and no dysmorphogenesis is observed at birth as in humans homocystinuric patients. By contrast, maternal folate deficiency leading to maternal hyperhomocysteinemia is associated with closure neural tube defects and cardiac dysmorphic syndromes. Thus maternal regulation or detoxification of hyperhomocysteinemia which is observed in CBS heterozygous maternal deficiency may explain the absence of major developmental dysmorphogenesis during embryonic and fetal life. If the mother detoxifies the increased homocysteine levels in homozygous CBS deficient fetuses, the biological consequences of homocysteine accumulation would only start to appear after birth. Our result showing CBS gene expression during embryonic development give an indirect argument for this hypothesis. Homocysteine derivatives, for example, homocysteine thiolactone (24), can cause cellular damage by acylation of lysine in proteins. Modification of collagen lysine residues or other manifestations of homocysteinylation in patients with homocystinuria can be similar to the protein glycation products in brain of Alzheimer's patients (25). These manifestations of homocysteinylation may have dramatic consequences in tissues for which transsulfuration is the main pathway of homocysteine metabolism. This situation occurs after birth in all CBS expressing tissues except in liver where the remethylation pathway is predominant.

Our work highlights the usefulness of expression mapping studies in early human embryonic tissues in order to improve our understanding of homocystinuria pathogeny. Further expression mapping experiments of methylenetetrahydrofolate reductase and methionine synthase genes on early postmortem human embryos should enable us to unravel some still mysterious pathogenic mechanisms underlying the different types of inherited homocystinuria.

ACKNOWLEDGMENTS

We thank Alexandra Kobetz and Dominique Marchand for technical assistance. This work was supported by CNRS, Centre National de la Recherche Scientifique, Fondation pour la Recherche médicale, Association Valentin Haüy pour le bien des aveugles and GRPMI Association, Groupe de Recherche pour la Médecine Interne. Véronique Paul's work is supported by a fellowship from the Ministère de la recherche et de l'Equipement (MRE), France. This work was supported by an Inserm grant N° 9304409.

REFERENCES

1. Mudd, S. H., Levy, H. L., Skovby, F. (1995) *in* The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Eds.), Disorders of Transsulfuration, 7th ed., pp. 1279–1327. Mc Graw–Hill, New York.
2. Stipanuk, M. H., and Beck, P. W. (1982) *Biochem. J.* **206,** 267–277.
3. Erickson, P. F., Maxwell, I. H., Su, L. J., Baumann, M., and Glode, L. M. (1990) *Biochem. J.* **269,** 335–340.
4. Abe, K., and Kimura, H. (1996) *J. Neurosci.* **16,** 1066–1071.
5. Chassé, J. F., Paul, V., Escañez, R., Kamoun, P., and London, J. (1997) *Mamm. Genome* **8,** 917–921.
6. Kery, V., Bukovska, G., and Kraus, J. P. (1994) *J. Biol. Chem.* **269,** 25283–25288.
7. Aral, B., Coudé, M., London, J., Aupetit, J., Chassé, J. F., Zabot, M. T., Chadefaux-Vekemans, B., and Kamoun, P. (1997) *Hum. Mutat.* **9,** 81–82.
8. Goyette, P., Frosst, P., Rosenblatt, D. S., and Rozen, R. (1995) *Am. J. Hum. Genet.* **56,** 1052–1059.
9. Leclerc, D., Campeau, E., Goyette, P., Adjalla, C. E., Christensen, B., Ross, M., Eydoux, P., Rosenblatt, D. S., Rozen, R., and Gravel, R. A. (1996) *Hum. Mol. Genet.* **5,** 1857–1874.
10. Kraus, J. P. (1994) *J. Inher. Metab. Dis.* **17,** 383–390.
11. Mudd, S. H., Havlik, R., Levy, H. L., *et al.* (1981) *Am. J. Hum. Genet.* **33,** 883–887.
12. Eskes, T. K., and Steegers-Theunissen, R. P. (1994) *Eur. J. Obstet. Gynecol. Reprod. Biol.* **53,** 544–554.
13. Steegers-Theunissen, R. P., Boers, G. H. J., Trijbels, F. J. M., Finkelstein, J. D., Blom, H. J., Thomas, C. M., Borm, G. F., Wouters, M. G., and Eskes, T. K. (1994) *Metabolism* **43,** 1475–1480.
14. Rosenquist, T. H., Ratashak, S. A., and Selhub, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 15227–15232.
15. O'Rahilly, R., and Müller, F. (1987) Developmental Stages in Human Embryo Including a Revision of Streeter's Horizons' and a Survey of the Carnegie Collection. Carnegie Institution of Washington, Publication No. 6.37.
16. O'Rahilly, R., and Müller, F. (1994) The Embryonic Human

Brain: An Atlas Developmental Stages. Wiley-Liss, Inc., New York.

17. Chassé, J. F., Paly, E., Paris, D., Paul, V., Sinet, P. M., Kamoun, P., and London, J. (1995) *Biochem. Biophys. Res. Commun.* **211,** 826–832.
18. Volpe, J. J., and Laster, L. (1970) *J. Neurochem.* **17,** 425–437.
19. Volpe, J. J., and Laster, L. (1972) *Biol. Neonate* **20,** 385–403.
20. VanAerts, L. A., Poirot, C. M., Herberts, C. A., Blom, H. J., De Abreu, R. A., Trijbels, J. M., Eskes, T. K., Peereboom-Stegeman, J. H., and Noordhoek, J. (1995) *J. Reprod. Fertil.* **103,** 227–232.
21. Reish, O., DeWayne, T., Berry, S. A., Tsai, M. Y., and King, R. A. (1995) *Am. J. Hum. Genet.* **57,** 127–132.
22. Abitbol, M., Menini, C., Delezoide, A. L., Rhyner, T., Vekemans, M., and Mallet, J. (1993) *Nat. Genet.* **4,** 147–154.
23. Watanabe, M., Osada, J., Aratani, Y., Kluckman, K., Reddick, R., Malinow, M. R., and Maeda, N. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1585–1589.
24. Jakubowski, H. (1997) *J. Biol. Chem.* **272,** 1935–1942.
25. Harrington, C. B., and Colaco, C. A. L. S. (1994) *Nature* **370,** 247–248.