Molecular Cloning of cDNA for Rat and Human Carbamyl Phosphate Synthetase I*

Mark W. Adcock and William E. O'Brien
From the Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

Recombinant plasmids with inserts complementary to the mRNA for carbamyl phosphate synthetase I were identified from a rat liver cDNA library by hybrid-selected mRNA translation. Four clones, the largest being 3100 base pairs, were identified for the rat liver enzyme. Using the rat liver cDNA as a probe, two homologous recombinant plasmids of approximately 1200 base pairs in length were isolated from a human liver cDNA library. Northern blot analysis of rat liver mRNA and baboon liver mRNA revealed the presence of a 5000-base mRNA homologous to both rat and human cDNA probes. No homologous mRNA was observed in mRNA from rat heart or rat kidney as is consistent with the known tissue distribution of this enzyme. The induction of carbamyl phosphate synthetase and argininosuccinate synthetase mRNA during the fetal and postnatal development of the rat was studied by dot blot analysis of isolated mRNA. The mRNA for both enzymes appeared between 17 and 19 days of fetal life and reached approximately 40% of adult levels during this period. This initial increase was followed by a rapid decline just prior to birth. The mRNA levels slowly increased during postnatal life, not reaching adult levels until after the 20th day of neonatal life. Using the human cDNA clones, the human carbamyl phosphate synthetase gene was mapped to chromosome 2 utilizing a panel of Chinese hamster X human somatic cell hybrids. Analysis of one hybrid with a human-Chinese hamster translocation provided a provisional assignment to the short arm of chromosome 2.

The ammonia-dependent carbamyl phosphate synthetase I (EC 6.3.4.16) is located in the mitochondrial matrix of liver where it functions to convert ammonia, bicarbonate, and ATP into carbamyl phosphate, the initial step in the urea cycle. This enzyme has been purified from numerous mammalian species including human, rat, and bovine (1-3) and has been estimated to make up 15-20% of total mitochondrial protein in the liver of these species (4). As has been shown for other mitochondrial proteins that are encoded by nuclear genes, carbamyl phosphate synthetase is synthesized as a precursor 5000-6000 daltons larger than the final product (5, 6). The amino acids in the leader peptide are removed by a protease during the vectorial transport of the molecule into the mitochondria (7, 8).

During early fetal development of the rat, the genes encoding the urea cycle enzymes are not expressed. Just prior to birth, all of the urea cycle enzymes begin to accumulate in the liver of the neonatal animal (9-11). Changes in dietary protein intake (12, 13) and steroid hormone levels (14) also affect the activities of all five urea cycle enzymes. This coordinate induction of a set of genes requires a different approach for the mammalian organism than the operon concept in procaryotes since the genes for at least three of the five enzymes are on separate chromosomes (15-17).

In order to investigate a molecular basis for coordinate regulation of the enzymes of the urea cycle and to investigate the structural requirements for vectorial transport into the mitochondrion, we have isolated cDNA clones for carbamyl phosphate synthetase from both rat and human. Preliminary accounts of this work have been reported previously (18, 19).

EXPERIMENTAL PROCEDURES

Isolation of Poly(A)* RNA—Wistar rats (150-200 g) were obtained from local suppliers. Poly(A)* RNA prepared according to the protocol of Chirgwin et al. (20) was fractionated on a 10-30% linear sucrose gradient. Fractions containing carbamyl phosphate synthetase mRNA were identified by in vitro translation using the rabbit reticulocyte system as described by Pelham and Jackson (21). Products of the translation were immunosorbent utilizing IgG sorb (The Enzyme Center, Boston, MA) and separated on SDS containing polyacrylamide gels. Three antiserum preparations were employed; one was prepared in our laboratory from human enzyme (supplied by Dr. Duane Pearson, National Aeronautical and Space Administration, Houston, TX) (1), one anti-rat enzyme supplied by Dr. Carol Lusty, Public Health Research Institute of the City of New York, and a second anti-rat enzyme from Dr. N. Hoogenraad (22), La Trobe University, Bundoora, Victoria, Australia.

Construction of cDNA Clones—Twenty micrograms of the sucrose gradient purified mRNA were used to prepare cDNA essentially as described by Maniatis et al. (23) except that reverse transcriptase was employed for the synthesis of both strands. The double-stranded cDNA was fractionated by electrophoresis in 1.2% agarose gels and molecules less than 600 bp in length were discarded. The cDNA molecules were inserted into the PstI site of pBR322 as described by Norgaard et al. (24) and the resulting chimeric plasmids were used to transform Escherichia coli K12 strain RRI as described by Dagert and Ehrlich (25). Approximately 800 ampicillin-sensitive clones were selected for analysis in the hybridization selection assay.

Colony Screening by Translation of Plasmid-selected mRNA—A very similar approach to that described by Parnes et al. (26) was used to identify clones of interest. Plasmid DNA was prepared by centrifugation in CaCl2 gradients from pools of ten recombinants grown and amplified in 150 ml of M9 broth. One hundred and fifty micrograms of plasmid DNA was bound to a 4.5-cm2 disc of nitrocellulose. From this disc, seven smaller discs of 0.4-cm2 were punched out with a standard paper punch. One small disc was employed for each hybrid-selection assay.

Four small filter discs were pooled for prehybridization in 1 ml of 50% formamide, 0.4 M NaCl, 0.05 mM EDTA, 0.2% SDS, 20 mM PIPES, pH 6.4, 0.5 mg/ml of E. coli tRNA, and 1 mg/ml of poly(A) was added

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**The abbreviations used are: SDS, sodium dodecyl sulfate; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); bp, base pairs.
to each vial and incubation was carried out at 42 °C for 18 h. The four filters were transferred to a new vial containing 0.4 ml of hybridization solution (same as prehybridization buffer except calf liver tRNA was substituted for E. coli tRNA and poly(A) was eliminated) containing 30 μg of poly(A)* RNA prepared from rat liver. Vials were incubated with shaking at 42 °C for 18 h. Filters were washed twice with 0.5 ml of 50% formamide, 0.015 M sodium citrate, 0.5% SDS, 0.1 mM EDTA, and 10 mM Tris-Cl, pH 7.6. Bound poly(A)* RNA was eluted in 0.3 ml of 0.2 mM EDTA containing 70 μg/ml of calf liver tRNA by incubating at 75 °C for 6 min. The eluted RNA was precipitated with ethanol and collected by centrifugation.

Electrophoresis—For immunoabsorption, 2 μl of normal rabbit serum was added to the 100-μl in vitro translation reaction and incubated at 4 °C for 15 min. This mixture was transferred to a microfuge tube containing 10 μl of packed Staphylococcus aureus cells (IgG sorb, The Enzyme Center, Boston, MA), the pellet was resuspended, and incubated at 4 °C for 15 min. The IgG sorb was removed by centrifugation, the supernatant fluid was added to 900 μl of STE buffer solution (0.1% SDS; 0.1% Triton X-100, 2 mM Tris-Cl, pH 7.5, 1 μl of antiserum was added, and this solution was incubated at 4 °C for 60 min. After formation of the antigen-antibody complex, 100 μl of a 10% solution of IgG sorb in STE buffer solution was added and this suspension was incubated for 4 °C at 30 min. The IgG sorb-antibody complex was collected by centrifugation and washed three times with 1 ml of STE solution. The final pellet was suspended in 60 μl of 0.38 M Tris-Cl, pH 6.9, 4% (w/v) SDS, 40% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (w/v) bromphenol blue, and heated at 100 °C for 5 min. Samples were centrifuged and the supernatant fluid upon addition of 2.5 ml of 0.1% SDS containing 0.2 mM 35S-labeled in vitro translation products, the gel was prepared for fluorography (27).

Electrophoresis and transfer of RNA and DNA and other routine methods were carried out essentially as described by Maniatis et al. (23).

Developmental Studies in the Rat—Animals were killed by decapitation and livers were immediately removed and quick-frozen in liquid nitrogen. Total RNA was extracted in guanidinium isothiocyanate and isolated by centrifugation in CsCl gradients (23). Yields were typically 4–5 μg of RNA/mg of tissue.

Poly(A)* RNA was prepared using oligo(dT)-cellulose by batch absorption and elution (25). Poly(A)* RNA was prepared from 750 μg of total RNA, 1 1.5 ml of 0.2 mM 35S-labeled in vitro translation products, the gel was prepared for fluorography (27).

Identification of Carbamyl Phosphate Synthetase cDNA Clones—Colony screening was accomplished by preparing plasmid DNA from 12 pools of 10 recombinants each. Pools 11 and 12 contained the plasmid, pAS1, which carries a cDNA complimentary to argininosuccinate synthetase and served as an internal control. In this hybridization-selection experiment, positive translation signals were seen in two lanes (Fig. 1, lanes 11 and 12). Each of these pools contained the internal control, pAS1, which gave rise to the band at a molecular weight of 46,000 (designated AS on Fig. 1). Lane 12 contained an additional signal at approximately 165,000 daltons which corresponded to the migration of carbamyl phosphate synthetase. The 9 unknown recombinants in pool 12 were grown independently, plasmids were prepared by the alkaline mini-preparation method of Birnboim and Doly (34), and the plasmid-selection assay performed again. Only one of the nine recombinants gave a positive signal for carbamyl phosphate synthetase in the hybrid-selection assay (data not shown).

**RESULTS**

Identification of Carbamyl Phosphate Synthetase cDNA—The plasmid-selected mRNA were separated on a 12.5% polyacrylamide gel containing SDS. Lane 12 contained a positive signal for carbamyl phosphate synthetase at 37 °C for 18 h. Electrophoresis and Southern transfer was conducted according to established procedures.

![Fig. 1. Autoradiogram of the translation products derived from screening pools of cDNA molecules by positive-selection translation.](image-url)
To confirm the identity of this isolate, we performed additional hybridization-selection experiments with the purified recombinant plasmid and employed antisera prepared in three different laboratories for the immunoprecipitation (Fig. 2). Each antibody precipitated a protein of 165,000 daltons that was synthetized from the hybrid-selected mRNA. In addition to the major band at 165,000 daltons, numerous products of smaller molecular weight are immunoprecipitated following the hybrid selection and in vitro translation. These smaller products are reproducible and are most likely a result of the degradation of the large carbamyl phosphate synthetase mRNA during the hybrid-selection protocol. Very analogous results were observed when this procedure was used for fatty acid synthetase (35). The combination of identical results with multiple antisera prepared independently against the rat and human enzyme and molecular weight of the immunoprecipitated polypeptide provide strong evidence for the identity of this recombinant molecule.

The initial cDNA clone, pCPSr1, contained an insert of approximately 790 bp. The insert from this clone was used to screen other rat liver cDNA libraries. From this screening three additional clones were identified. The restriction maps of these cDNA molecules are depicted in Fig. 3. The largest recombinant molecule contained an insert of 3100 bp which we estimate accounts for 60–70% of the mRNA sequence. No poly(A) region was found upon sequencing both ends of pCPSr4.

Isolation of Human cDNA Clones for Carbamyl Phosphate Synthetase—In order to isolate cDNA clones for human carbamyl phosphate synthetase, we screened a cDNA library prepared from human liver mRNA by Prochownik et al. (36). The probe was prepared from the pCPSr4 clone (Fig. 3) by digesting with EcoRI and PstI and isolating the 1300-bp EcoRI-PstI fragment. Four clones were identified upon screening approximately $5 \times 10^4$ recombinants. The restriction maps of two of the clones, designated pCPSh2 and pCPSh4, are shown in Fig. 3. The alignment and orientation of pCPSh2 and pCPSh4 was accomplished by hybridization to the pCPSr4 clone.

Analysis of Cellular RNA—Poly(A)$^+$ RNA obtained from rat liver, rat heart, and rat kidney was examined by Northern blotting (Fig. 4). A single species was observed in rat liver but no homologous mRNA was observed in mRNA prepared from rat heart or rat kidney even after extended autoradiography. This is consistent with the tissue distribution of enzyme activity (4) and provides further support for the identity of these recombinant clones. The migration of the carbamyl phosphate synthetase mRNA corresponded to a length of approximately 5000 bases. This length is sufficient to encode a polypeptide of 165,000 daltons. The rat cDNA probe also hybridized to an mRNA species of identical size from the liver of a baboon (data not shown). A similar analysis was con-
ducted utilizing the human clone, pCPSh2, and identical results were obtained (data not shown).

**Expression of Carbamyl Phosphate Synthetase mRNA during Fetal Development**—No enzyme activity for any of the urea cycle enzymes is observed prior to 17 days of fetal development in the rat (9-11). We followed the induction of carbamyl phosphate synthetase and argininosuccinate synthetase mRNA during fetal development by dot-blot analysis utilizing cDNA probes for these two enzymes (Fig. 5). We observed the initial appearance of mRNA for both enzymes between day 17 and 19 of fetal development. The level of mRNA for both proteins reached 30-40% of adult levels during this period. After this initial rise, a decrease of hybridizable mRNA was observed for both carbamyl phosphate synthetase and argininosuccinate synthetase prior to birth followed by a slow rise to adult levels in the postnatal period. Enzyme activity paralleled the mRNA levels for argininosuccinate synthetase. Carbamyl phosphate synthetase activity reached adult levels shortly after birth, whereas mRNA levels slowly increased to adult levels over a period of 20-30 days.

**Mapping the Human Carbamyl Phosphate Synthetase Gene**—In order to determine the chromosomal location of the carbamyl phosphate synthetase gene, DNA from a panel of Chinese hamster X human somatic cell hybrids was probed with the cDNA, pCPSh2. The analysis of the Southern blot of EcoRI cut DNA from each hybrid is shown in Fig. 6. Five

**FIG. 5.** Induction of carbamyl phosphate synthetase and argininosuccinate synthetase mRNA during development of the rat. Poly(A') RNA levels were determined by dot blot analysis as described under "Experimental Procedures." Carbamyl phosphate synthetase enzyme activity ( ), carbamyl phosphate synthetase mRNA ( ), argininosuccinate synthetase enzyme activity ( ), and argininosuccinate synthetase mRNA ( ).

**FIG. 6.** Chromosomal localization of the human carbamyl phosphate synthetase gene. DNA from each cell line was digested with EcoRI and separated by electrophoresis in 0.8% agarose. The DNA was transferred to nitrocellulose and probed as described under "Experimental Procedures." Lanes designated hamster and human are the parent cell lines prior to the cross. All other lanes contain DNA from the hybrids. The presence or absence of human chromosome is designated by + or − in the table. The designation +/- cell line 6.1 indicates that only the short arm of chromosome 2 is present translocated to a hamster chromosome. All hybridizing fragments co-segregated with chromosome 2. Thus, if there are pseudogenes hybridizing to the probe, they
are also present on chromosome 2. Hybrid 6.1 does not have an intact chromosome 2. G-banding and G-11 analysis have shown that this hybrid contains the entire short arm of chromosome 2 translocated onto a Chinese hamster chromosome (32). These data provide a provisional assignment to the 2cen-2pter region.

**DISCUSSION**

Using plasmid-selected mRNA translation, we have identified four cDNA clones for rat carbamyl phosphate synthetase I and two for the human enzyme. Since sequence data for this protein is not available, we employed three independent preparations of antisera to identify the product synthesized from the plasmid-selected mRNA. Northern analysis revealed that the cDNA clones hybridized to a single band of 5000 bases in poly(A)+ RNA prepared from rat and baboon liver. This is of appropriate length to encode a polypeptide of the size of carbamyl phosphate synthetase I in the human. In addition, no hybridizable mRNA species was observed in poly(A)+ RNA prepared from either rat kidney or rat heart. The tissue distribution of the mRNA homologous to this cDNA is also consistent with its identification as a carbamyl phosphate synthetase clone.

Early work demonstrated that virtually no enzyme activity was detected in hepatic tissue until the 18th day of fetal life (37). Enzyme activity steadily increases from this day until reaching adult levels 20-30 days postnatally. Raymond and Shore (9) measured translatable carbamyl phosphate synthetase mRNA during fetal development and noted a rapid rise to adult levels in this parameter just prior to birth with no change between birth and 6 days of neonatal life. These workers noted that the levels of primary translation product of carbamyl phosphate synthetase expressed in vitro was low compared to in vivo expression and speculated that this may be due to damage to the large mRNA during isolation.

Murakami et al. (10) examined this developmentally regulated gene by means of [[32P]methionine incorporation into immunoprecipitable protein in mechanically dispersed hepatocytes. They noted a rapid rise in incorporation between 16 and 20 days of fetal development followed by a rapid decline between birth and 2 days of neonatal life.

Both interpretations of these previous studies were complicated by the manipulations required to gather the data. Using the cDNA as a probe, we have been able to obtain a more direct measurement of carbamyl phosphate synthetase mRNA during this period of induction. Our results also demonstrated a rapid rise in mRNA between day 17 and 21 of fetal life followed by a decrease to day 3 of neonatal life. After day 3, a steady rise in carbamyl phosphate synthetase mRNA was noted until adult levels were reached. Our data correlate well with those of Murakami et al. (10) but are not consistent with those of Raymond and Shore (9) and suggest that using in vitro translation to measure mRNAs of such large size may lead to difficulties in interpretation of the data.

Carbamyl phosphate synthetase enzyme activity reached adult levels within 3 days after birth, much sooner than the RNA levels. This may be a reflection of the stability of enzyme protein synthesized within the mitochondrion. These data would suggest that the half-life of the protein is considerably longer than the half-life of the mRNA during this period of development. The levels of mRNA and enzyme activity for argininosuccinate synthetase, aspartate transcarbamylase, and dehydrogenase. A cDNA clone for this enzyme has been identified (41), therefore comparisons of these molecules at a DNA sequence level is now feasible. The yeast carbamyl phosphate synthetase gene has also recently been isolated (42) and sequenced (43).

The synthesis and vectorial transport of mitochondrial proteins in higher eukaryotes has been the focus of considerable efforts in numerous laboratories. Although the exact events of the processing are not known, it is clear that a leader peptide present on the amino terminus of the nascent polypeptide chain contains the information necessary to direct the polypeptide into the mitochondrion. The leader polypeptide is removed as part of the transport process. In order to delineate the sequences required to define a protein as mitochondrial, the primary sequence of the precursor peptide is required. Since mitochondrial proteins do not accumulate in the cytoplasm, it has been virtually impossible to obtain these data by protein sequencing. The availability of cDNA clones will enhance our ability to answer these questions.

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Carbamyl Phosphate Synthetase cDNA