Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4258–4262, July 1983
Biochemistry

Molecular cloning of the cDNA coding for rat ornithine transcarbamoylase

(cDNA cloning/X-linked gene/ornithine transcarbamoylase deficiency/carboxyl-terminal peptide sequence)

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Communicated by Robert W. Berliner, April 8, 1983

ABSTRACT **Ornithine transcarbamoylase is a mitochondrial matrix enzyme composed of three identical subunits encoded on the X chromosome. The subunit is synthesized on cytoplasmic polysomes as a precursor that is cleaved during transport into mitochondria. We report here the isolation and characterization of cDNA clones containing sequences corresponding to the mRNA encoding the ornithine transcarbamoylase subunit. cDNA was synthesized using rat liver mRNA enriched by polysome immunoadsorption for the low-abundance messenger species encoding the enzyme subunit. After insertion of cDNA into plasmid pBR322 and cloning in *Escherichia coli*, identification of the desired plasmids was accomplished by (*i*) differential colony hybridization using cDNA probes synthesized from mRNA of various tissues; (*ii*) differential blot hybridization using cDNA probes synthesized from mRNA enriched for or depleted of the ornithine transcarbamoylase message; (*iii*) hybrid-selected translation assays; and (*iv*) most definitively, structural analysis, which matched 25 consecutive amino acid residues determined by sequential Edman analysis of the carboxyl-terminal portion of the purified enzyme subunit with coding sequence present in the insert of one of the plasmids.**

Ornithine transcarbamoylase [OTCase; carbamoylphosphate:L-ornithine carbamoyltransferase, EC 2.1.3.3], the enzyme that catalyzes the second step of the urea cycle in mammals, has several important biological and clinical features (1–3). (*i*) The enzyme, a trimer of identical 36-kilodalton (kDa) subunits, is localized to the mitochondrial matrix but is encoded in the nucleus. Like most nuclear-encoded mitochondrial proteins studied to date, its subunit has been shown to be synthesized on free cytoplasmic ribosomes as a precursor with an apparent molecular mass (40,000) several thousand daltons greater than that of the subunit of the active matrix enzyme (4, 5). The NH_2-terminal portion of the OTCase precursor is removed during its energy-dependent translocation across the mitochondrial membranes by two sequential proteolytic cleavage events (6–8). The details of these events, including the "signal" in the precursor that targets it to mitochondria, the specificity of the cleavage sites, and the characteristics of the removed residues are unknown. (*ii*) The structural locus encoding the enzyme is on the X chromosome, as shown by analyses of OTCase deficiency in mice and man (9, 10). Because this locus is differentially inactivated in females (11), the OTCase gene is a candidate for a molecular analysis of the process of X chromosome inactivation. (*iii*) Deficiency of OTCase activity in man generally results in ammonia intoxication and early death of affected male infants (2, 3). Prenatal diagnosis in pregnancies at risk has not been possible because no unusual metabolites have been detected in amniotic fluid and because OTCase activity is not detectable in amniotic fluid cells. Therefore, a diagnostic approach using molecular genetic techniques would be useful.

To approach these biological questions and clinical needs, we have undertaken a molecular analysis of mammalian OTCase genes. In this paper, we describe the isolation and characterization of a cloned cDNA segment encoding a portion of the rat OTCase subunit.

MATERIALS AND METHODS

Carboxypeptidase Cleavage. OTCase (2 nmol) purified from rat liver was digested for 5 hr at 37°C in 100 μl of 0.2 M $NaHCO_3$ containing 1 unit of carboxypeptidase A or B or of a mixture of both. The reaction was stopped by addition of 10 μl of 6 M HCl, and the products were subjected to amino acid analysis on a Beckman 121 M analyzer. In some experiments, OTCase was first denatured in 10% trichloroacetic acid; the precipitated enzyme was washed in acetone and dissolved in 8 M urea. Digestion with carboxypeptidase was then carried out after dilution of the urea to 1.5 M with 0.2 M $NaHCO_3$.

Preparation and Isolation of the COOH-Terminal Peptide. The *S*-cyano derivative (31 nmol) of native rat liver OTCase was prepared and cleaved by using 5,5′-dithiobis(2-nitrobenzoic acid) as described for the bovine enzyme (12). The COOH-terminal cysteine peptide was purified by reversed-phase HPLC on a μBondapak C_{18} column without prior removal of guanidine hydrochloride. After neutralization with triethylamine and drying, the peptide was dissolved in 25 mM $NaHCO_3$ (pH 8.0) and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin at a ratio of 1:30 (wt/wt). The resulting digest was separated on the same HPLC column. Several of the purified peptides were immobilized on aminopolystyrene, and their sequences were determined on a Sequemat Mini-15 solid-phase sequenator as described (13).

Preparation of RNA and DNA. Total RNA from rat liver and rat brain was isolated by the method of Shields and Blobel (14). Total RNA from the Morris rat hepatoma 3924A was supplied by J. Boulter. Poly(A)-containing fractions of these RNAs were prepared by using oligo(dT)-cellulose (Collaborative Research, Waltham, MA) chromatography. Rat liver mRNA enriched several hundred-fold for rat OTCase mRNA (i.e., from an initial abundance of ≈0.2% to a value of ≈40%) was prepared by the method of polysome immunoadsorption (15). mRNA depleted of OTCase mRNA was prepared from polysomes that did not bind to the protein A-Sepharose column during immunoadsorption of the polysome–anti-OTCase complexes. RNAs were analyzed for the presence of OTCase mRNA by using cell-free translation, immunoprecipitation with anti-OTCase antiserum

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Abbreviations: OTCase, ornithine transcarbamoylase; kDa, kilodalton(s); bp, base pair(s); TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

and formalin-fixed *Staphylococcus aureus*, and $NaDodSO_4$ gel electrophoresis as described (15). Plasmid DNA was prepared according to the method of Clewell and Helinski (16).

Enzymes. Reverse transcriptase from avian myeloblastosis virus was supplied by J. Beard (Life Sciences, St. Petersburg, FL). The Klenow fragment of polymerase I was obtained from New England BioLabs. S1 nuclease, terminal transferase (ribosubstitution grade), and restriction endonucleases were obtained from Bethesda Research Laboratories. Carboxypeptidases A and B were obtained from Sigma. TPCK-treated trypsin was obtained from Worthington. OTCase from rat liver was isolated by the method Hoogenraad *et al.* (17).

Construction and Screening of Recombinant Plasmids. Several hundred nanograms of immunopurified RNA, enriched for OTCase mRNA such that ≈40% of [^{35}S]methionine incorporated during cell-free translation was immunoprecipitable with anti-OTCase antiserum, was used for cDNA synthesis. Construction of double-stranded cDNAs, insertion into plasmid pBR322, and transformation of *Escherichia coli* strain HB101 were carried out as described (18), except that cDNA molecules were purified during each step in the synthesis by using a 0.5-ml Sephadex G-50 column.

Screening of Clones by Differential Hybridization and Hybrid-Selected Translation. Colony hybridization was carried out as described (19) using ^{32}P-labeled cDNA probes prepared by oligo(dT)-primed reverse transcription of poly(A)-containing RNA. Southern blot screening of plasmid DNAs was carried out as described (20). Plasmid DNAs were digested using *Bam*HI, electrophoresed through 1% agarose gels, transferred to nitrocellulose (Schleicher & Schuell) (21), and hybridized with radiolabeled cDNA. Restriction digests, gels, and nitrocellulose filters were prepared in duplicate, and equal numbers of cpm of ^{32}P incorporated into either OTCase-enriched or OTCase-depleted cDNA probes were used in parallel hybridization reactions.

Hybrid-selected translation analysis of plasmid DNA was carried out according to Parnes *et al.* (22) with several modifications. Plasmid DNA (20 μg) that had been digested with *Hha* I and extracted was boiled in H_2O (50 μl) for 5 min, and an equal volume of ice-cold 3 M NaCl/0.3 M Na citrate, pH 7.0, was added. The mixture was immediately pipetted onto three 1-cm nitrocellulose discs, which were air dried and baked at 80°C for 2 hr in a vacuum oven; 20 μg of rat liver mRNA was used for each hybridization. Translation was carried out in a 30-μl volume; 3 μl was applied directly to a $NaDodSO_4$ gel, and the remainder was immunoprecipitated with anti-OTCase antiserum.

DNA Sequence Analysis. DNA fragments containing inserted cDNA sequences were electroeluted from 5% acrylamide gels and radiolabeled at their 5′ termini by using bacterial alkaline phosphatase (Bethesda Research Laboratories) followed by T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP (Amersham) or at their 3′ termini by using the Klenow fragment of polymerase I and [α-^{32}P]dNTP (Amersham) (23). Fragments were then subjected to further digestion with a restriction enzyme and gel purification or to strand separation. Chemical cleavage reactions of the fragments were carried out according to Maxam and Gilbert (23), and the products were separated by using gels that were 0.4 mm thick and 36 or 90 cm long (24). In all cases, the sequence of complementary strands of DNA fragments containing inserted cDNA was determined.

RESULTS

Amino Acid Sequence of the COOH-Terminal Portion of the OTCase Subunit. Because OTCase cDNA clones generated by using oligo(dT)-primed first-strand synthesis were likely to contain COOH-terminal coding information, amino acid sequence information was obtained from the COOH-terminal portion of the mature OTCase subunit by two approaches. First, carboxypeptidase cleavage of the denatured subunit released only phenylalanine, thereby identifying phenylalanine as the COOH-terminal residue.

The second approach depended on isolating and determining the sequences of peptides from the COOH-terminal portion of mature OTCase. Amino acid analysis of rat liver OTCase showed the presence of two cysteine residues (25). As shown in Fig. 1*A*, when conditions for partial cleavage of the *S*-cyano derivative were used, a single peptide with mass of 6 kDa was released from the mature subunit of the homogeneous rat liver enzyme. This peptide was purified by HPLC (Fig. 1*B*). It was identified as the COOH-terminal peptide by its blocked NH_2 terminus and because phenylalanine was released from this peptide on carboxypeptidase digestion. Phenylalanine was also released after trypsin digestion, indicating that OTCase must terminate with either Lys-Phe or Arg-Phe. The COOH-terminal cysteine-containing peptide was digested with trypsin and the resulting peptides were analyzed by HPLC (Fig. 1*C*). Peptides T-4 and T-20 were subjected to 14 and 11 cycles, respectively, of automated sequential Edman degradation, followed by HPLC analysis of the phenylthiohydantoin-amino acids. The sequences of the entire T-4 peptide and the NH_2-terminal portion of peptide T-20 were obtained (see below).

Screening of the cDNA Bank Generated from OTCase-Enriched mRNA. Approximately 25% of the double-stranded cDNA generated from OTCase-immunopurified mRNA was used to transform HB101, and several hundred tetracycline-resistant ampicillin-sensitive colonies were obtained. Because as much as 40% of the mRNA used to generate the clones was OTCase mRNA as judged by translation, we assumed that a similar percentage of clones might contain OTCase sequences. Therefore, several dozen plasmids were digested with the frequent-cutting restriction endonucleases *Hae* III and *Hin*fI, in an effort to identify a recurring restriction pattern. No pattern was recognized, necessitating an effort to identify OTCase clones by using hybridization probes.

Significant OTCase enzymatic activity has been detected only in hepatic tissue and, in particular, is not present in rat hepatomas or rat brain. This pattern of expression is reflected at the mRNA level, as shown by cell-free translation studies (data not shown). This suggested that OTCase clones might be identified by differential hybridization using cDNA probes constructed from these mRNAs. The results of such a colony-screening experiment are shown in Fig. 2*A*; clone 279 (indicated by an arrowhead) hybridized with radiolabeled hepatic cDNA but not with hepatoma or brain probes. A total of 12 clones hybridized in this manner (≈4% of the total) and were considered candidates for containing OTCase sequences.

An additional colony-screening experiment was carried out using cDNA probes made either from hepatic mRNA or from hepatic mRNA specifically depleted by immunoadsorption of OTCase mRNA. As shown in Fig. 2*B*, clone 279 failed to hybridize with the OTCase-depleted probe. The clone at the lower left corner of Fig. 2*B* also failed to hybridize with the OTCase-depleted probe but, because it had hybridized with the hepatoma probe (Fig. 2*A*), it was not further analyzed. The majority of candidates previously identified by differential hybridization using the tissue probes (data not shown) produced differential signals with hepatic and OTCase-depleted probes.

In a further analysis, plasmid DNA from several dozen clones, including those showing differential colony hybridization, was subjected to Southern blot analysis using radiolabeled cDNA probes prepared from either OTCase-enriched or OTCase-depleted mRNA. The enriched mRNA used in this experiment

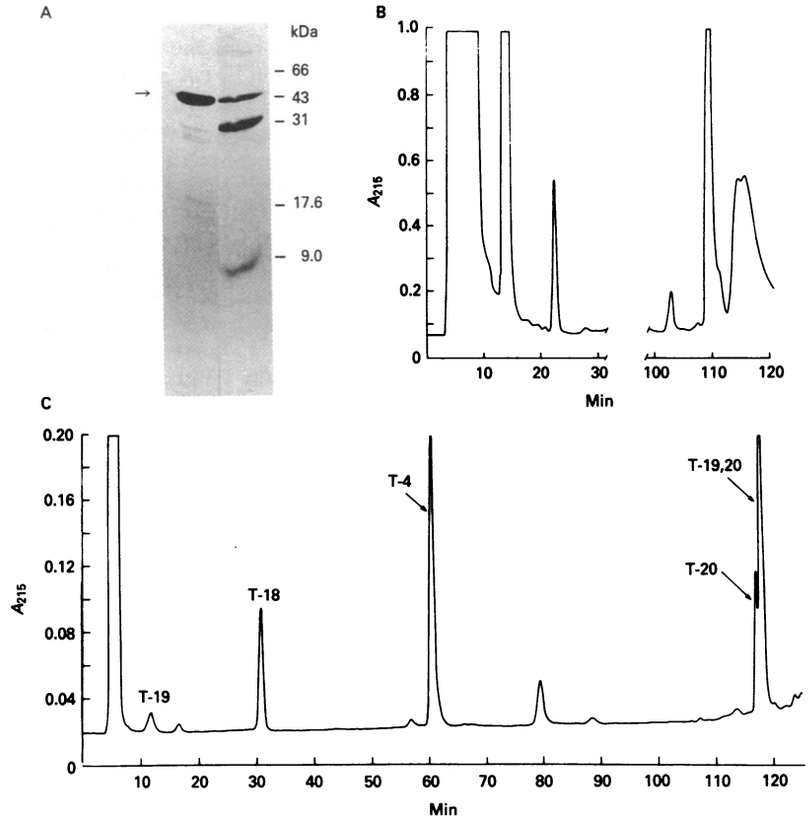

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis and HPLC of the COOH-terminal cysteine peptide and its tryptic digest. Purified OTCase was subjected to cyano cleavage. (*A*) $NaDodSO_4$/polyacrylamide gel showing OTCase (20 μg) before (left lane) and after (right lane) the cleavage reaction. (*B*) HPLC profile of the cleavage products. The peak occurring at 110 min (the 6.0-kDa species noted in *A*) was isolated and identified as the COOH-terminal peptide. (*C*) HPLC profile of the products of tryptic cleavage of the COOH-terminal cysteine peptide. T-19, T-18, T-4, etc., designate individual tryptic peptides; the complete amino acid sequence of peptide T-4 and a portion of the sequence of peptide T-20 were determined (see Fig. 5).

had been subjected to sucrose gradient fractionation after immunopurification and consisted of ≈80% OTCase mRNA as judged by translational analysis. A composite photograph of the results of Southern blot screening is shown in Fig. 3. Many of the plasmids tested, including one containing rat serum albumin sequences, hybridized with the enriched probe, but only plasmids 279, 221, and 170 hybridized much less strongly with the OTCase-depleted probe. These three clones, as well as plasmid 202, were subsequently shown to share sequences with each other. When the insert of plasmid 279 was radiolabeled and used in RNA blot analysis, it identified a species of rat hepatic mRNA that migrated to ≈14 S, corresponding to the sedimentation coefficient of OTCase mRNA in sucrose gradients (data not shown).

Data from several of the plasmids was also used in a hybrid-selected translation assay (Fig. 4). Two specific polypeptides were detected after translation when plasmid 170 was used (Fig. 4*A*, lane 2), one of which had a molecular mass of 40 kDa and

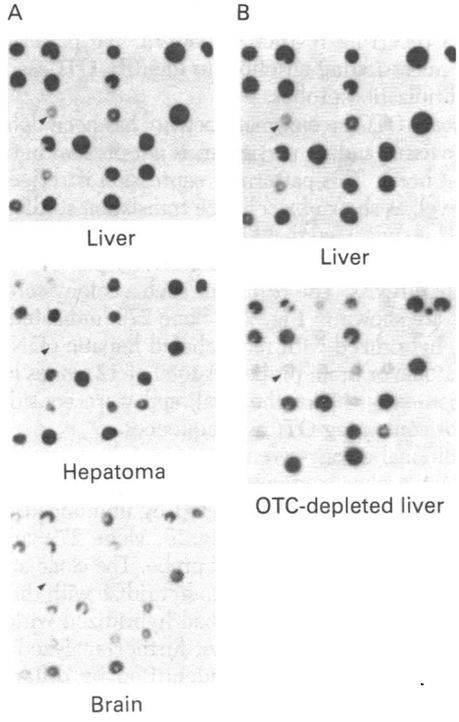

FIG. 2. Colony hybridization using ^{32}P cDNA probes. The same group of clones was analyzed in *A* and *B*. (*A*) cDNA probes were prepared from poly(A)-containing RNAs from liver, hepatoma, and brain. (*B*) cDNA probes were prepared from either total poly(A)-containing hepatic RNA or from poly(A)-containing hepatic RNA depleted of the OTCase message. Arrowheads indicate plasmid 279.

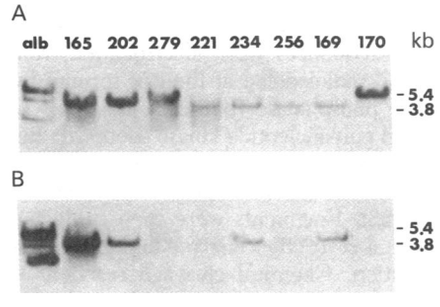

FIG. 3. Southern blot hybridization of *Bam*HI-digested cDNA-containing plasmids 165, 202, 279, 221, 234, 256, 169, and 170. Probes were prepared from hepatic mRNA enriched for (*A*) or depleted of (*B*) OTCase message by polysome immunoadsorption. Equal numbers of cpm of ^{32}P in the OTCase-enriched or OTCase-depleted cDNA probes were used, and the two filters were hybridized, washed, and exposed identically. Representative portions of the autoradiographs are shown. The plasmid designated "alb" was identified in our cDNA bank and contained ≈300 bp corresponding to the 3′ coding and untranslated sequence of rat serum albumin mRNA.

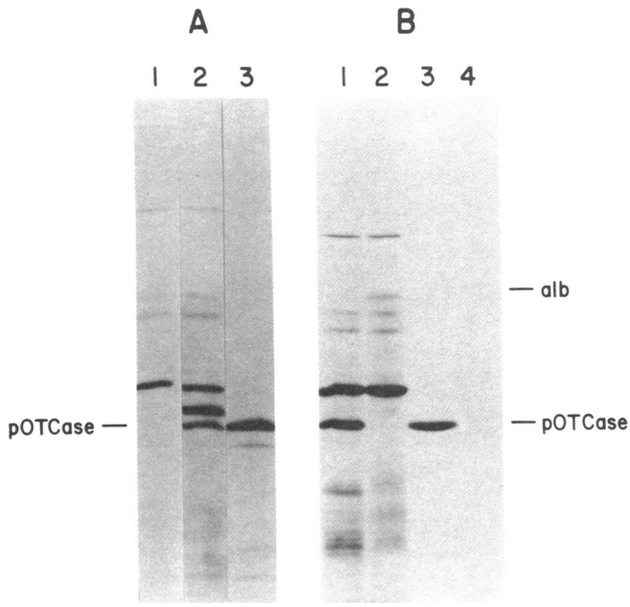

FIG. 4. Hybrid-selected translation of cDNA-containing plasmids. *A* and *B* are from different experiments. Fluorography of the dried gels was for 6 hr. (*A*) Lanes: 1, translation with no added mRNA, showing the [^{35}S]methionine-protein adduct; 2, translation of mRNA selected by plasmid 170; 3, immunoprecipitate of translation shown in lane 2. (*B*) Lanes: 1, translation of mRNA selected by plasmid 279 (pOTC-1); 2, translation of mRNA selected by plasmid containing rat albumin (alb) cDNA (described in Fig. 3); 3, immunoprecipitate of translation shown in lane 1; 4, immunoprecipitate of translation shown in lane 2. pOTCase, pre-OTCase.

was immunoprecipitable with anti-OTCase antiserum (lane 3). Only one specific polypeptide was detected when plasmid 279 was used (Fig. 4*B*, lane 1), and it was precipitable with anti-OTCase antiserum (lane 3). Findings identical to those with plasmid 279 were obtained with plasmid 202 (data not shown). When an albumin-containing cDNA plasmid was used, a specific polypeptide corresponding in size to albumin was detected (Fig. 4*B*, lane 2), but no polypeptide with the size or immunologic specificity of OTCase was noted (lane 4). These results indicate that clones 170 and 279 contain OTCase sequences; clone 170 appears to contain a second cDNA species as well.

DNA Sequence of Candidate Plasmids. The inserts of plasmids 202, 170, and 279 were subjected to DNA sequence analysis. The insert of plasmid 202 contained ≈350 base pairs (bp), including a poly(A) tail at one terminus. The sequence of the poly(A)-containing strand revealed numerous termination codons in all three potential translational reading frames. At the most upstream position, however, adjoining a deoxyguanosine tail, was the sequence C-C-A-A-A-G-T-T-C-T-G-A, putatively encoding the COOH-terminal sequence Pro-Lys-Phe. Plasmid 170 shared the entire untranslated sequence and poly(A) tail with plasmid 202 but, at the position of the putative COOH terminus, its sequence became totally divergent. Although this plasmid was not analyzed further, it is conceivable that the divergent sequence corresponded to a second cDNA that selects the non-OTCase mRNA identified by hybrid-selected translation (Fig. 4).

Plasmid 279 shared not only the untranslated sequence with plasmid 202 but also the putative COOH-terminal coding sequence. Of greatest importance, further upstream this plasmid contained the precise coding sequence for the two tryptic peptides T-4 and T-20 (Fig. 5). Moreover, this DNA sequence suggested that the two peptides adjoin one another. Thus, 25 consecutive residues were precisely predicted by the DNA sequence. The sequence also predicted the tetrapeptide Cys-Leu-Pro-Arg, whose amino acid composition precisely matched that determined from peptide T-18. The entire cysteine peptide is predicted to contain 52 residues, and the amino acid composition predicted by the DNA sequence matches that of the peptide exactly (data not shown).

The 3′ untranslated sequence of plasmid 279 adjoins a deoxycytidine homopolymeric tail at a position that is 22 bp upstream from the poly(A) tail present in plasmids 202 and 170. The additional sequence contained in plasmids 202 and 170 is shown in Fig. 5. Plasmid 279, designated pOTC-1, contains an additional 400 bp of insert sequence upstream from the cysteine codon designated residue 1 in Fig. 5 (DNA sequence not shown). This sequence contains termination codons in all three translational reading frames and, at its most upstream position, contains a poly(T) tail, adjoining at its 5′ end a deoxyguanosine tail. This suggests that a second cDNA is present in the insert of pOTC-1, encoded on the opposite strand.

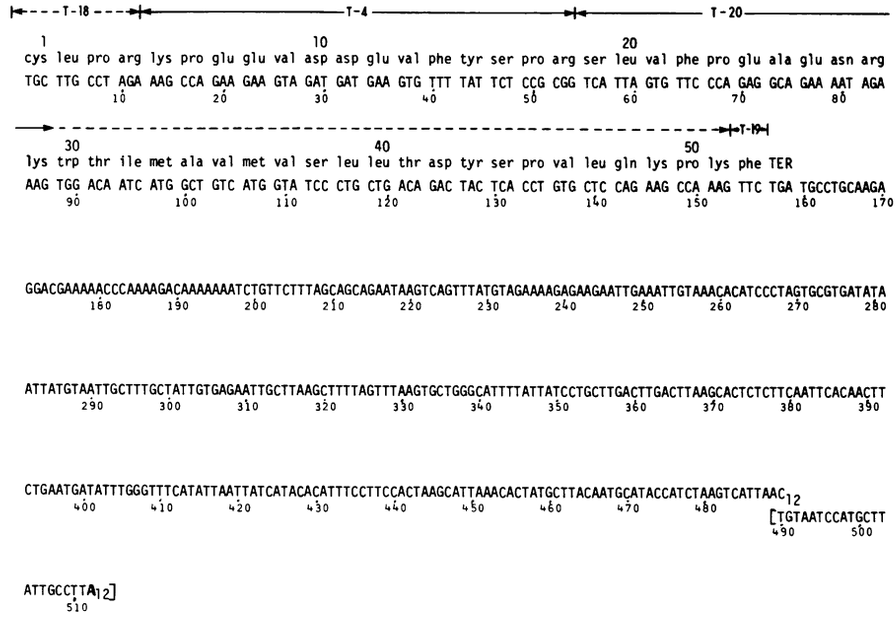

FIG. 5. Nucleotide sequence of pOTC-1 insert and amino acid sequence of the COOH-terminal cysteine peptide. DNA fragments from plasmid pOTC-1 containing inserted cDNA sequence were analyzed. The DNA sequence shown did not contain a poly(A) tail, but other plasmids containing the identical 3′ untranslated sequence did contain a poly(A) tail, as shown in the sequence enclosed in brackets. The COOH-terminal cysteine peptide was isolated from the purified OTCase subunit and cleaved with trypsin. The amino acid composition of the tryptic peptides, the complete sequence of peptide T-4, and the sequence of residues 19–29 of peptide T-20 were determined. The peptides are indicated by lines ending with arrows. Amino acid sequence results are indicated by solid lines.

DISCUSSION

A cloned cDNA segment encoding a portion of the rat OTCase subunit has been isolated. This was accomplished by (*i*) use of enriched OTCase mRNA for cloning and screening, (*ii*) identification of candidate clones by differential hybridization and hybrid-selected translation, and (*iii*) structural analysis of the enzyme subunit and cDNA inserts. Structural studies of the OTCase subunit proved to be essential because divergent plasmid insert sequences were encountered that, in the absence of amino acid sequence information, would have made it impossible to identify unequivocally plasmids containing OTCase sequences. In the presence of enzyme structural information, however, incontrovertible identification was possible—the perfect matching of DNA coding sequence and amino acid sequence at 25 consecutive residues.

Observations Concerning the Structure of the OTCase Subunit. Plasmid pOTC-1 contains the coding region for the COOH-terminal cysteine-containing peptide shown in Fig. 4 and all but 22 bp of the 3′ untranslated region of OTCase mRNA. The COOH-terminal residue predicted by DNA sequence analysis agrees with that determined from carboxypeptidase cleavage of the mature subunit, an indication that the primary translation product, preOTCase, does not undergo cleavage at the COOH-terminus during the events of subunit maturation.

Observations Concerning OTCase mRNA and Cloned cDNA. The 3′ untranslated sequence of the OTCase mRNA is A+T rich (67%), resembling many other eukaryotic mRNAs. However, in contrast to most reported eukaryotic mRNAs, it does not contain the sequence A-A-T-A-A-A characteristically observed within 25 bases of the poly(A) addition site and thought to play a role in polyadenylylation (26). The closest approximation to this sequence is A-T-T-A-A, located 23 bases upstream from the poly(A). This pentanucleotide may function as a polyadenylylation signal.

Plasmid pOTC-1 and the related plasmid 170 contain two apparently distinct cDNA segments—an OTCase cDNA joined with a cDNA corresponding to another mRNA. The point of joining in pOTC-1 has not been identified but should be definable by analysis of additional cloned OTCase sequences extending upstream from those in pOTC-1. On the other hand, the joining point in plasmid 170 has apparently been identified, and it brings together the region corresponding to the 3′ untranslated sequence of OTCase mRNA with another cDNA. How can two cDNAs become joined? Theoretically, reverse transcriptase might have left one template and copied another, but this can be excluded in the case of plasmid pOTC-1 by the inverted orientation of the two cDNAs—reverse transcriptase can copy RNA in one direction only. Joining of distinct cDNAs during homopolymeric tailing seems unlikely, because this might be expected to place a homopolymeric stretch at the joining point between the cDNA transcripts, and none was found. After bacterial transformation, joining might have resulted from homologous recombination between two plasmids present in the same bacterial cell, but this possibility seems remote because the strain of HB101 used in these experiments is reportedly recA$^-$ (27), a conclusion we have confirmed. A plausible mechanism to explain the observed configuration of pOTC-1 is the hybridization of two first-strand products at their 3′ termini, which would permit the primed second-strand synthesis of both transcripts, followed by homopolymeric tailing of the termini of the chimeric cDNA molecule.

Only 4% of the clones in our cDNA bank have been identified as containing OTCase sequences, compared with the estimate of 40% OTCase mRNA present in the material used to generate the clones. We have no explanation for this result. A similar discrepancy was observed when prothrombin mRNA enriched by polysome immunoprecipitation was used to generate cDNA clones (28).

The cDNA segment described here should be useful as a probe to identify the remainder of the OTCase coding sequence, as well as genomic sequences, the structures of which will be important to understanding the biological and clinical features of this enzyme.

We are grateful to Cindy Williamson for skillful technical assistance; to our colleague, Wayne Fenton, for helpful comments on the manuscript; to Ron Evans, John Rose, Michael Rosenfeld, Bill Summers, Geoff Wahl, Sherman Weissman, and Savio Woo for valuable advice and encouragement during these studies; and to Marilyn Feldman for secretarial expertise. This work was supported by National Institutes of Health Grants AM 09527 to L.E.R. and GM 31539 to W.K.; A.L.H. was the recipient of a postdoctoral fellowship from the National Institutes of Health.

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