Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1203–1207, March 1986
Biochemistry

Molecular cloning of human ornithine aminotransferase mRNA

(gyrate atrophy/cDNA/λgt11/plaque immunoscreening/tryptic peptide microsequencing)

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Communicated by W. A. Hagins, October 10, 1985

ABSTRACT **The isolation and characterization of a cDNA clone for the mRNA of human ornithine aminotransferase (OATase; ornithine–oxo-acid aminotransferase; L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13), a nonabundant mitochondrial matrix enzyme that is severely deficient in a hereditary chorioretinal degenerative disease (gyrate atrophy), is described. Human liver, retina, and retinoblastoma (Y79) mRNAs were prepared and tested for the OATase mRNA content by *in vitro* translation, immunoprecipitation, and NaDodSO$_4$/PAGE. The retinoblastoma cells were found to be expressing this enzyme at a relatively high level. The primary translation product of the OATase mRNA is larger than the pure OATase protein on NaDodSO$_4$/PAGE by ≈4 kDa, suggesting a precursor protein. λgt11 cDNA libraries were prepared from the human mRNAs, and the recombinant clones were immunoscreened as plaques with two different preparations of rabbit anti-human OATase antibodies. A clone (λgtRB315) was isolated from the retinoblastoma library that reacts with both of the antibody preparations, and the DNA sequence of its 2.1-kilobase-pair cDNA insert was obtained. An open reading frame consisting of 1371 nucleotides is present in the sequence, and a putative translational initiation methionine codon is identified at position 55. A putative leader sequence consisting of 32 amino acid residues is identified, resulting in a precursor protein of 439 amino acid residues and a molecular mass of 48,534 Da and a mature protein of 407 residues and 45,136 Da. The amino acid sequences of seven tryptic peptides (115 amino acid residues) of the pure human OATase were obtained by microsequencing. When the tryptic peptide and cDNA-derived amino acid sequences were compared, homologies in 111 of 115 residues, including a match of 20 consecutive residues, were observed. An RNA blot hybridization of ^{32}P-labeled OATase cDNA to normal human retina and retinoblastoma mRNAs demonstrated an OATase mRNA species of ≈2.2 kilobases. The level of OATase mRNA in the normal human retina is ≈1/100th the level of rhodopsin mRNA and 1/5th to 1/10th the level present in the retinoblastoma cells.**

Ornithine aminotransferase (OATase; ornithine–oxo-acid aminotransferase; L-ornithine:2-oxo-acid aminotransferase, EC 2.6.1.13) is a nuclear-encoded pyridoxal phosphate-requiring mitochondrial matrix enzyme that catalyzes the interconversion of ornithine, glutamate, and proline (1). The enzyme is present in many mammalian tissues, including the liver (1, 2), kidney (1, 2), and retina (3, 4). OATase is believed to be important in the intracellular production of glutamate and proline from ornithine (5) and in shuttling excess dietary amino acid carbons to the gluconeogenic pathway via the tricarboxylic acid cycle (6, 7). The enzyme has been purified from various tissue sources, including rat liver (8), kidney (9), and brain (10), and recently from the human liver in a crystalline-pure form (11). The rat liver and kidney OATase have been shown to be synthesized as a 49-kDa precursor molecule in the cytoplasm, which is then processed and becomes associated with the mitochondrion (12). Different dietary and hormonal controls of OATase synthesis have been demonstrated in the rat liver and kidney (13, 14). Cloning of a cDNA complementary to the rat liver OATase mRNA has been reported (15). The OATase sequence, either from the protein or cDNA, has not been reported.

OATase has been a focus of interest to many eye researchers since it was discovered that patients with gyrate atrophy are severely deficient in this enzyme activity (16–18). Gyrate atrophy is a rare autosomal-recessive degenerative disease of the retina and the choroid of the eye, characterized by hyperornithinemia and progressive blindness. The OATase activity in the fibroblasts and phytohemagglutinin-stimulated lymphocytes from the gyrate atrophy patients was demonstrated to be severely decreased (19, 20). The deficiency of OATase activity in the fibroblasts of two such patients was recently shown by an immunoassay technique to be due to decreased concentration of the enzyme (21). To begin to study this hereditary eye disease at the gene level, we set out to construct a molecular probe of human OATase. Here we describe the molecular cloning of human OATase cDNA by using the expression cloning vector, λgt11 (22) and anti-human OATase antibodies prepared from purified human liver OATase (11), and its characterization.

MATERIALS AND METHODS

Reagents and Strain. Restriction enzymes, *Eco*RI linker, T4 kinase, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (XGal) and isopropyl β-D-thiogalactopyranoside were from Bethesda Research Laboratories. DNA polymerase I, calf intestinal alkaline phosphatase, and S1 nuclease were from Boehringer Mannheim. *Escherichia coli* ligase, T4 DNA ligase, and *Eco*RI methylase were from New England Biolabs. Avian myeloblastosis virus reverse transcriptase was from Life Sciences (St. Petersburg, FL), and bovine pancreas trypsin was from Cooper Biomedical (Malvern, PA). Ribonuclease H was a generous gift from R. Crouch and S. Kanaya. λgt11 bacteriophage and *E. coli* Y1090 were the generous gifts of R. Davis, T. Huynh, and R. Young.

mRNA Preparation. Total RNAs were prepared from human liver, retina, and retinoblastoma cells (Y79) by the guanidine thiocyanate method (23). Polyadenylylated [poly(A)] RNA was obtained from the total RNAs by oligo(dT)-cellulose (Collaborative Research, Waltham, PA) chromatography (24).

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Abbreviations: kbp, kilobase pair(s); XGal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; OATase, ornithine aminotransferase.

***In Vitro* Translation and Analysis of Products.** The poly(A) RNAs (0.3 μg each) were used for *in vitro* translation in a rabbit reticulocyte lysate system (New England Nuclear) containing [^{35}S]methionine in a 25-μl reaction mixture (25). A portion of the translation product was immunoprecipitated with rabbit anti-human OATase antibody by using the *Staphylococcus aureus* indirect immunoprecipitation method (26). The translation products, before and after immunoprecipitation, were analyzed on a NaDodSO$_4$/polyacrylamide gel (10%) by electrophoresis and autoradiography of the fixed dried gel.

Preparation of Antibody. Anti-human OATase antiserum was obtained from a rabbit after three intradermal injections of pure human OATase (70 μg each) combined with complete Freund's adjuvant 2 weeks apart. The IgG fraction of the antiserum was prepared by precipitation in 1/3 saturated ammonium sulfate and DEAE-cellulose chromatography as described (27). *E. coli* sonicate (BNN97) was coupled to activated CNBr–Sepharose 4B (1 mg/ml) according to manufacturer's protocol (Pharmacia), and the IgG fraction was passed through a column containing this material to absorb out the anticoliform antibodies. The purity and activity of the purified IgG fraction were tested by the immunoassay technique described below.

Preparation of λgt11 cDNA Libraries. Four micrograms each of human liver, retina, and retinoblastoma (Y79) poly(A) RNA was used to construct the λgt11 cDNA libraries as described by Huynh *et al.* (28).

Detection of OATase λgt11 cDNA Clone with Antibody. The recombinant clones were screened by the plaque immunoassay technique (29). The purified IgG used in the screening showed no reaction with the original λgt11 vector plaque formed in Y1090.

RNA Blot Analysis. One microgram each of human poly(A) RNAs in a 25-μl mixture containing formamide and formaldehyde was denatured at 60°C and electrophoresed in a 0.8% agarose gel containing 2.2 M formaldehyde (30). The RNAs were transferred onto a GeneScreen filter (New England Nuclear) with 10× SSC (1× SSC = 0.15 M NaCl/0.015 M Na citrate) to prepare the RNA blot. The radioactively labeled probe was the *Eco*RI-cut gel-purified OATase cDNA insert labeled with [^{32}P]dCTP (Amersham) by nick-translation (31). The hybridized blot was washed at high stringency (0.1× SSC, 52°C) and autoradiographed.

DNA and Protein Sequencing. Restriction fragments of the purified 2.1-kilobase-pair (kbp) *Eco*RI OATase cDNA insert were cloned into either M13 mp10 or M13 mp11 (32). The procedure of Dale *et al.* was used to progressively shorten some of the large inserts (33). The phage clones were sequenced by the dideoxy-chain-termination method (34). Two hundred micrograms of crystalline pure human OATase was digested with trypsin (56 ng/ml) overnight, and the tryptic peptides were purified by HPLC (μBondapak C_{18}, Waters Associates). Seven tryptic peptides were selected and used for the determination of amino acid sequence with a Model 470A gas phase sequencer (Applied Biosystems, Foster City, CA) (35, 36).

RESULTS

In preparation for the construction of λgt11 cDNA libraries, we isolated mRNAs from various human tissues and determined the abundance of OATase mRNAs in each. Total RNAs were isolated from human liver, retina, and retinoblastoma Y79 cells; poly(A) RNA (mRNA) was obtained by oligo(dT)-cellulose chromatography; and the mRNAs were used for *in vitro* translation in a rabbit reticulocyte lysate system (25). The *in vitro* translation products were analyzed by NaDodSO$_4$/PAGE with or without immunoprecipitation with anti-human OATase antibody (26) (Fig. 1). All of the

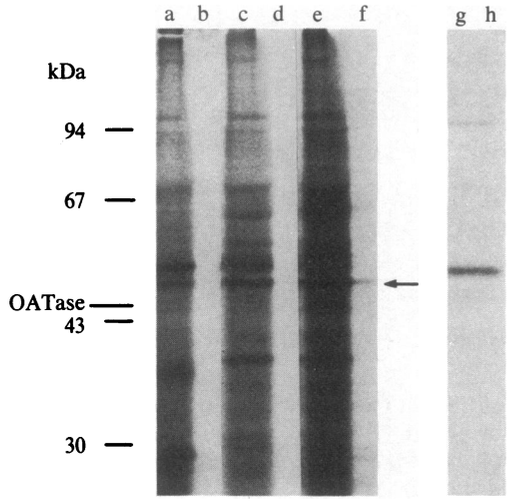

FIG. 1. NaDodSO$_4$/PAGE of ^{35}S-labeled *in vitro* translation products of human mRNAs. Lanes a, c, and e, fluorography of ^{35}S-labeled translation products of human liver, retina, and retinoblastoma poly(A) RNAs, respectively. Lanes b, d, and f, ^{35}S-labeled translation products of human liver, retina, and retinoblastoma poly(A) RNAs immunoprecipitated with anti-human OATase antibody, respectively. Lanes g and h, *in vitro* translation products obtained without added poly(A) RNA before and after immunoprecipitation with anti-human OATase antibody, respectively. Arrow indicates the immunoprecipitated human OATase among the translation products of retinoblastoma poly(A) RNA. The position of pure human OATase in the gel is also indicated. The molecular size markers are phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

mRNA preparations yielded distinct polypeptide products upon translation, including high molecular weight species. The level of immunoprecipitated OATase in the translation products of liver and retina mRNAs was barely detectable, in agreement with the low level of expression generally observed for the "housekeeping" enzymes such as OATase (1, 2, 13). On the other hand, a distinctly visible band of immunoprecipitated OATase was present for the retinoblastoma mRNA translation products, indicating a relatively high level of OATase expression in this tissue. On the basis of this finding, the retinoblastoma cells appeared to be the best tissue source for the cloning of an OATase cDNA.

The OATase protein immunoprecipitated by anti-human OATase antibody from the *in vitro* translation products of mRNAs appeared to have an approximate molecular mass of 48 kDa on NaDodSO$_4$/PAGE. Since the size of purified human OATase is 44 kDa on NaDodSO$_4$/PAGE (11), it appears that the primary translation product of OATase mRNA is a precursor molecule that undergoes subsequent processing as in the case of the rat liver and kidney OATase (12).

The human liver, retina, and retinoblastoma mRNAs were used for the synthesis of cDNAs with reverse transcriptase. A "filling in" method of cDNA second-strand synthesis was used (37) because this method appeared to give longer cDNA products. The cDNAs were size-selected for species at least 1 kbp long and cloned into the *Eco*RI site of λgt11 after addition of *Eco*RI linkers. The base number of clones obtained for the human liver, retina, and retinoblastoma λgt11 cDNA libraries was ≈100,000 each. Eighty-five to 95% of the clones appeared as colorless plaques when plated out on XGal plates, indicating that the λgt11 galactosidase genes in these clones have been interrupted by inserts (22). The presence of inserts was confirmed by an analysis of random colorless clones, which were shown to contain cDNA inserts ranging in size from 500 bp to >3 kbp (data not shown).

The three λgt11 cDNA libraries were screened for the clone containing the human OATase cDNA with rabbit anti-human OATase polyclonal antibodies. The antibodies were purified IgG fractions that had been cleaned of anti-coliform antibodies by passages through affinity column containing *E. coli* extracts (38). Anti-human OATase antibodies were prepared separately from two different rabbits and used to cross-screen the λgt11 cDNA libraries. The actual screening consisted of a filter plaque immunoassay technique using ^{125}I-labeled protein A (22, 39). The two anti-OATase antibodies gave detectable positive reactions with as little as 5 ng of pure antigen by this immunoassay technique (data not shown).

In a preliminary screening of ≈10,000 clones from each of the three human λgt11 libraries, 10 clones were detected that showed a positive reaction with one or both of the two rabbit anti-human OATase antibodies. Upon purification of the phage clones, one clone, λgtRB315, was isolated from the retinoblastoma λgt11 library that continued to show strong positive reactions with both of the rabbit anti-human OATase antibodies. The λgtRB315 clone was amplified, and its DNA was isolated and analyzed by agarose gel electrophoresis after digestion with *Eco*RI enzyme. The clone was shown to contain a 2.1-kbp cDNA insert (data not shown). To facilitate the use of this clone for DNA sequencing and as a radioactively labeled probe, the 2.1-kbp cDNA insert was subcloned into the bacterial plasmid pBR322; the plasmid clone was named pHOAT.

The putative human OATase cDNA was cut out of pHOAT, radioactively labeled, and used as a hybridization probe to total poly(A) RNAs (mRNAs) of normal human retina and retinoblastoma in an RNA blot analysis to identify the OATase mRNA. A very faint band of mRNA of ≈2.2 kb was demonstrated in both the normal retina and retinoblastoma mRNAs in the RNA blot analysis (data not shown). The level of OATase mRNA in the total mRNA population of normal retina was shown to be extremely low, in agreement with the previous *in vitro* translation analysis of mRNAs (Fig. 1) and the reported low abundance of OATase in mammalian cells (1, 2). An approximate quantitation of the level of OATase and rhodopsin mRNAs in the normal human retina mRNA population by a densitometric analysis of their hybridization levels on an autoradiogram indicated that the OATase mRNA is 1/100th to 1/200th as abundant as the rhodopsin mRNA (data not shown). The RNA blot analysis also demonstrated that the level of OATase mRNA in the retinoblastoma mRNA population is 5–10 times higher than that in the normal retina mRNA population, again in agreement with the previous *in vitro* translation analysis of mRNAs (Fig. 1) and the fact that the OATase cDNA clone was indeed isolated from the retinoblastoma λgt11 library. The approximate size of the OATase mRNA (2.2 kb) indicated that our OATase cDNA, which is 2.1 kbp long, may be very close to being a full-length copy of the mRNA.

The definite identification of our cDNA clone was undertaken through DNA and protein sequencing. The DNA sequence was obtained from M13 clones of the cDNA fragments generated by various restriction enzyme cuts. The OATase cDNA contains 2073 nucleotides with a continuous open reading frame from position 1 to 1371 (Fig. 2). A putative translation initiation codon (ATG) is present at position 55 with an upstream sequence (CTTGAAG) similar to the consensus sequence CTTCCAG and a cluster of purine residues (″) reported to be often present upstream from a translation initiation codon (40–42). The sequence around the methionine codon (ACAATGT) is also similar to the reported consensus sequence AXXAUGG of eukaryotic initiation sites (43). A putative leader peptide of 32 amino acid residues encoded by nucleotides 55–150 was identified by a comparison of the OATase NH_2-terminal sequence with that of the human ornithine transcarbamoylase precursor protein, another nuclear-encoded mitochondrial matrix enzyme like the OATase (44). Two lysine residues encoded by nucleotides 145–150 appear to be just upstream of the putative cleavage point of the leader peptide. The OATase leader sequence is characterized by the same absence of acidic amino acid residues as observed for ornithine transcarbamoylase leader, while the mature OATase protein contains 12.5% acidic residues and 10.8% basic residues (Fig. 3) (44). An interesting conservation of 6 nonpolar amino acid residues was observed in the leader sequences of the two enzymes. The assignment of the putative translation initiation point and the cleavage position of the leader peptide results in an OATase precursor protein consisting of 439 amino acid residues with a molecular mass of 48,534 Da and a mature protein of 407 residues with a molecular mass of 45,136 Da. There are 702 bases of 3′ untranslated sequence including 55 adenine residues in the poly(A) tail. The polyadenylylation signal AATAAA is present 19 bases upstream from the start of the poly(A) tail (45).

Because the amino terminus of the purified OATase was found to be blocked, tryptic digestion of the OATase protein was performed. The tryptic peptides were purified by HPLC and used for amino acid microsequencing. The sequences of a total of 115 amino acid residues were obtained from seven pure tryptic peptides and compared to the amino acid sequence derived from the cDNA sequence. One hundred eleven of 115 residues are identical in the two sequences, including a match of 20 consecutive residues (Fig. 2). On the basis of these data, we conclude that the pHOAT is indeed the cDNA clone of the human OATase mRNA.

DISCUSSION

We have described in this paper the cloning of human OATase mRNA by using the λgt11 expression cloning vector (22). The isolation of a specific recombinant DNA clone by using the antibodies directed against the encoded protein is becoming a more and more popular approach to cloning (29, 46–50). This method proved to be useful for us in cloning the nonabundant human OATase mRNA. The low abundance of the OATase mRNA was demonstrated in the *in vitro* translation of mRNAs, the screening of the libraries, and the RNA blot hybridization experiment. Among the 10 clones we selected in the primary screening of ≈30,000 clones from the three human libraries, only 1 clone from the retinoblastoma library continued to show reactivity to two different preparations of anti-human OATase antibodies at the tertiary screening stage. The other 9 clones lost activity to the antibodies in the second screening and appeared to be "false positives."

The similarity in the size of the cDNA insert contained in λgtRB315 and the OATase mRNA in the RNA blot experiment indicated that our OATase cDNA may be very close to being a full-length copy of the mRNA. This prediction was borne out by the cDNA sequence, which indicates that our cDNA clone is probably missing some of the 5′ untranslated region of the OATase mRNA but appears to contain the entire translated region, thus enabling us to examine the putative complete amino acid sequence of the OATase precursor protein. Our conclusion that the cDNA contains the entire coding region of OATase starting with the methionine codon at position 55 is based on the following points: (*i*) the codon at position 55 is the first methionine codon in the open reading frame sequence (40); (*ii*) relatively typical consensus sequences of a translation initiation codon are present around the methionine codon at position 55 (40–43); (*iii*) the open reading frame sequence of 1317 nucleotides starting with the methionine codon at position 55 encodes a protein of 48,534 Da, which matches that of the OATase

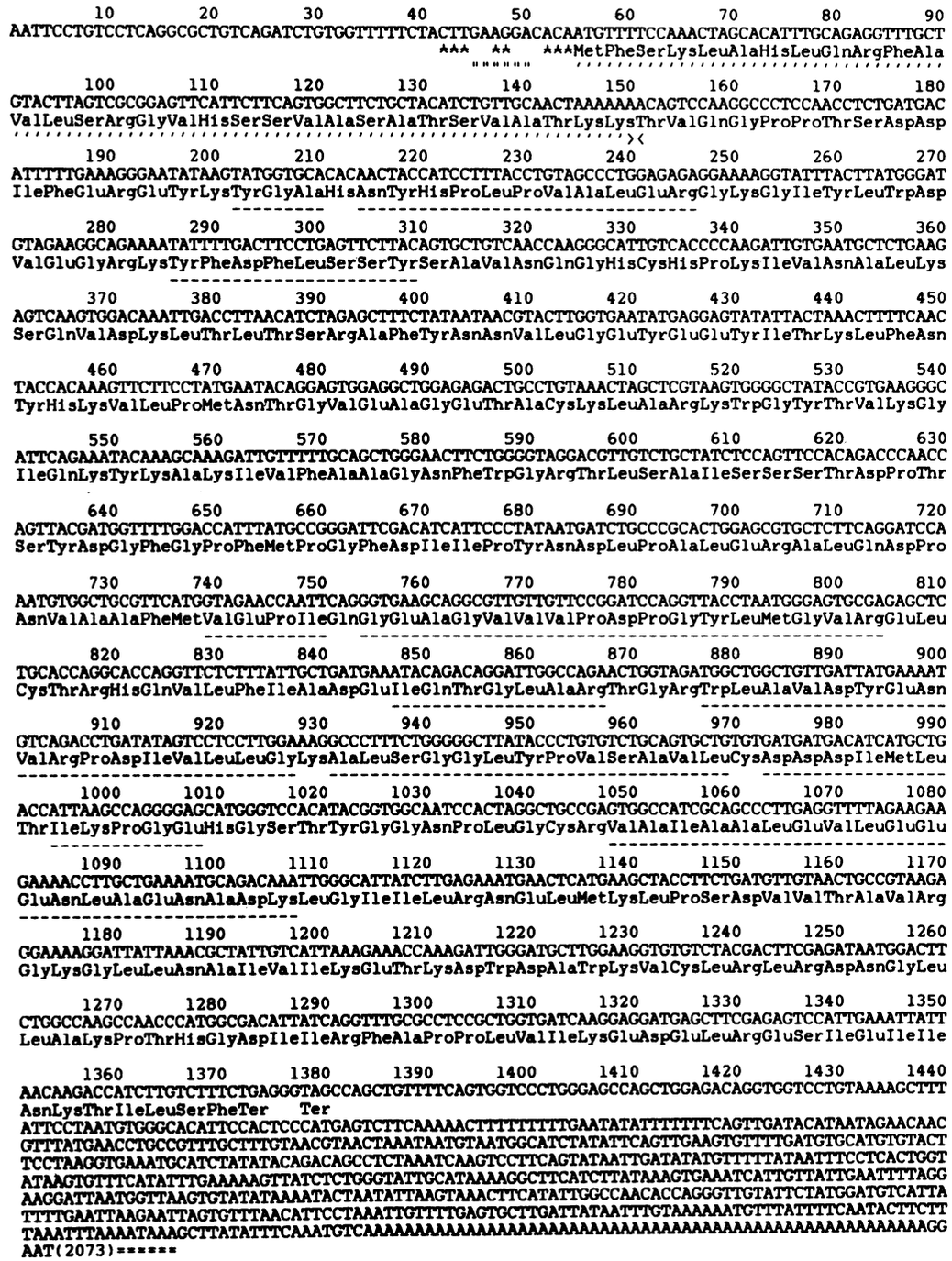

FIG. 2. cDNA and protein sequence of the human OATase. The cDNA contains 2073 bases, including 55 adenine residues in the 3′ poly(A) tail. An open reading frame exists from position 1 to 1371 followed by 702 bases of 3′ untranslated sequence. (*), Residues forming the consensus-like sequences; (″), a cluster of purine residues just upstream of the putative translational initiation codon (ATG) at position 55; (′), putative leader sequence; (⟩⟨), putative cleavage site of the leader; (---), amino acid sequence identical with that of tryptic peptide; (=), polyadenylylation signal. See text for discussion.

precursor protein observed in the *in vitro* translation experiment (≈48 kDa) (Fig. 1); and (*iv*) the cDNA-encoded protein contains local sequences that match the tryptic peptide sequences of pure OATase (Fig. 2).

Many nuclear-encoded proteins that are destined for the mitochondrial matrix are synthesized as precursors with NH_2-terminal extensions, which are thought to be important in directing the precursor protein to the mitochondria (51, 52). The NH_2-terminal leaders are cleaved off during the insertion of the protein into the mitochondrial matrix. A precursor–mature protein relationship has been demonstrated for the rat OATase (12), and our *in vitro* translation experiment indicated that a similar situation exists for the human OATase (Fig. 1). A comparison of the NH_2-terminal sequence of the human OATase to the leader sequence of another human mitochondrial matrix enzyme, ornithine transcarbamoylase (44), allowed us to identify the 32-amino acid putative leader peptide, the size of which (3398 Da) matches the difference between the precursor and the mature human OATase observed in the *in vitro* translation experiment (Figs. 1 and 2). It is noteworthy that paired basic residues such as the Lys-Lys present just upstream of the putative cleavage position of the human OATase precursor (Fig. 2) are also present at the sites of cleavage of other precursor proteins such as the corticotropin/endorphin precursor and the G34 gastrin (53). The OATase leader also contains features identical to those described for the ornithine transcarbamoylase leader such as the lack of a long "membrane-spanning" hydrophobic region, the lack of acidic residues, and the presence of uniformly distributed basic residues (44) (Fig. 3). Most interesting, however, is the conservation of six relatively nonpolar residues at specific positions in the two leader sequences (Fig. 3). These conserved residues, along with the absence of negative charge and/or the relative abundance of positive charge in the leader, may play a key role in the directed delivery of the

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   *     *     *              **
MFSKLAHLQRFAVLSRGVHSSVASATSVATKK  OATase
:   :  :   :   :   :
MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ  OTCase
      *        *      *  *
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FIG. 3. Comparison of the leader sequences of the human OATase and ornithine transcarbamoylase (OTCase). OATase, putative leader sequence of the human ornithine aminotransferase; OTCase, leader sequence of the human ornithine transcarbamoylase[44]; *, basic amino acid residue; :, conserved residue. Single letter amino acid designations are as follows: M, Met; F, Phe; S, Ser; K, Lys; L, Leu; A, Ala; H, His; Q, Gln; R, Arg; V, Val; G, Gly; T, Thr; N, Asn; I, Ile; C, Cys; P, Pro.

precursor protein to the mitochondria. The significance of the conserved residues should become clearer when more human leader sequences become available for comparison.

Although cloning of a rat OATase cDNA has been reported (15), no sequence was available for comparison with our OATase sequence. A computer search of the Protein Sequence Data Bank (Protein Identification Resources, National Biomedical Research Foundation, Georgetown University Medical Center) did not reveal any protein sequence with a significant homology to the human OATase. However, a separate comparison of the human OATase sequence to that of the mitochondrial form of the chicken aspartate aminotransferase (54) revealed a homology of 27% when gaps are allowed in the sequences to achieve maximum homology (data not shown). Aspartate aminotransferase exists as a cytosolic or mitochondrial isozyme, and the mitochondrial isozyme is similar to OATase in that it is also a nuclear-encoded, pyridoxal phosphate-requiring, mitochondrial matrix enzyme catalyzing a transamination reaction (54). Significantly, the region of highest homology between the human OATase and chicken aspartate aminotransferase sequences contains the active site including the pyridoxal phosphate-binding site of the aspartate aminotransferase molecule as delineated by a crystallographic study (55). The homology between the human OATase and chicken aspartate aminotransferase suggests a possible evolutionary linkage between these enzymes and that the tertiary structure of the active site of OATase may be similar to that described for aspartate aminotransferase (55).

Note Added in Proof. Mueckler and Pitot have recently published the rat OATase sequence (56), which shows 91% homology to the human OATase.

We thank R. Young , T. Huynh, and R. Davis for generous gifts of bacteriophage and bacterial strains; T. Shinohara for the rhodopsin cDNA; P. Russell for the Y79 retinoblastoma cells; J. Owens for help in the computer analysis of data; M. Cashel and J. Piatigorsky for helpful discussions; A. Korade for expert typing; P. M. Yuan of Applied Biosystems, and A. Ally and D. Filpula of Genex Corporation, and I. Gery and L. Merola for expert technical assistance.

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