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**A cDNA clone for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human leader sequences and conservation of catalytic sites**

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**ABSTRACT**

We have cloned a DNA complementary to the messenger RNA encoding the precursor of ornithine transcarbamylase from rat liver. This complementary DNA contains the entire protein coding region of 1062 nucleotides and 86 nucleotides of 5'- and 298 nucleotides of 3'-untranslated sequences. The predicted amino acid sequence has been confirmed by extensive protein sequence data. The mature rat enzyme contains the same number of amino acid residues (322) as the human enzyme and their amino acid sequences are 93% homologous. The rat and human amino-terminal leader sequences of 32 amino acids, on the other hand, are only 69% homologous. The rat leader contains no acidic and seven basic residues compared to four basic residues found in the human leader. There is complete sequence homology (residues 58-62) among the ornithine and aspartate transcarbamylases from *E.coli* and the rat and human ornithine transcarbamylases at the carbamyl phosphate binding site. Finally, a cysteine containing hexapeptide (residues 268-273), the putative ornithine binding site in *Streptococcus faecalis*, *Streptococcus faecium*, and bovine transcarbamylases, is completely conserved among the two *E.coli* and the two mammalian transcarbamylases.

**INTRODUCTION**

Ornithine transcarbamylase (OTCase) is a mitochondrial matrix enzyme composed of three identical subunits. The OTCase subunit is encoded on the X chromosome (1) and translated on cytoplasmic polysomes (2,3) as a precursor containing an amino-terminal amino acid extension (leader sequence) (4). The precursor is transported across both mitochondrial membranes to the matrix space, cleaved to the mature-sized subunit by a matrix protease (5) and assembled into OTCase trimers (6). Using an mRNA highly enriched for the OTC message (7,8), we initially obtained a cDNA clone encoding the carboxy-terminal portion of OTCase (9). This cDNA clone was used to screen a human liver cDNA library, from which a nearly full-length cDNA encoding human OTCase was isolated (10). The human cDNA insert, in turn, was used to screen a rat liver cDNA library from which a cDNA encoding the entire rat OTC precursor was obtained. In this communication, we wish to report the nucleotide and amino acid sequences of the rat OTCase precursor, and to compare the mitochondrial

leader sequences of the same protein from two mammalian species. Furthermore, complete conservation of two catalytic sites of the enzyme between E.coli and the mammals is reported.

### MATERIALS AND METHODS

#### Preparation of rat liver cDNA library.

Total RNA was prepared as described by Chirgwin (11). Polyadenylated RNA was isolated by oligo(dT) cellulose chromatography according to the supplier's instructions (type 2, Collaborative Research). cDNA cloning was carried out according to the following protocol, modified from Land et al. (12). Briefly, cDNA was synthesized from 15 µg of rat liver mRNA in a final volume of 150 µl of reaction mixture consisting of: 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM magnesium acetate, 4 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.5 mM [ $\alpha$ -<sup>32</sup>P]dCTP (500 cpm/pmol), 40 µg/ml actinomycin D, 25 µg/ml oligo(dT)<sub>12-18</sub>, 500 U/ml RNasin (Promega Biotec), 0.15 mg/ml BSA, and 120 units of reverse transcriptase (Life Sciences, Inc.). After 1 h of incubation at 44°C, another 60 units of RNasin and 48 units of reverse transcriptase were added, and the mixture was incubated for an additional hour. The reaction was terminated by addition of EDTA and NaCl to 25 mM and 100 mM, respectively, and the nucleic acids were separated from the reaction mixture on a 10 ml column of Sephadex G-150 equilibrated in 20 mM ammonium bicarbonate. The cDNA yield from mRNA was about 60%. The fractions containing the cDNA-mRNA hybrid were lyophilized and about 20 dC residues were added to the cDNA by incubating it at 15°C for 15 min in a 0.6 ml reaction containing 5 µM [<sup>3</sup>H]dCTP (6,500 cpm/pmol), 0.2 mM DTT, 1 mg/ml BSA, 140 mM potassium cacodylate - 30 mM Tris-HCl, pH 7.2, 1 mM cobalt acetate, and 240 units of terminal transferase (Boehringer-Mannheim). The reaction was adjusted to 0.3 M NaOH and incubated overnight at room temperature to hydrolyze the mRNA. The tailed cDNA was desalted by passing the reaction mixture through a 1 ml spun column of Bio-gel P-10. The tailed molecules were selected on a 0.5 ml column of oligo(dG)cellulose (Collaborative Research) and ethanol precipitated. Second strand synthesis was performed according to Land, et al. (12). Double stranded molecules were separated on a linear sucrose density gradient (5-20% w/w) in 0.2 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. Centrifugation was for 16 h at 20°C in a Beckman SW41 rotor at 36,000 rpm. Fractions containing molecules longer than 1,100 bp were pooled and ethanol precipitated. Approximately 20 dC residues were added to the double stranded DNAs as described above and annealed with Pst I cut, (dG) tailed pBR322 (Bethesda Research Laboratories) at a 1:10 ratio (w/w) and at a final DNA concentration of 0.5 µg/ml (13). E.coli of strain

DH1 were transformed as described by Hanahan (14). 90% of the tetracycline-resistant colonies contained cDNA inserts.

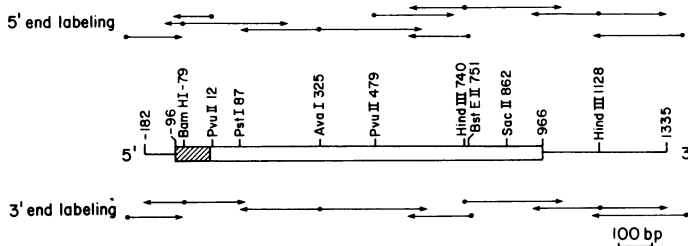
#### Additional methods

Published methods were used for the small-scale isolation of plasmid DNA from transformed *E.coli* cells (15), as well as for large scale plasmid DNA preparations on cesium chloride gradients (16). *E.coli* colonies containing chloramphenicol-amplified plasmids were screened (17) using a nick-translated nearly full length human OTCase cDNA probe (10). cDNA inserts from candidate clones were screened by Southern blot hybridization (18). Northern blot analysis was performed according to Thomas (19) except that 3 M NaCl, 0.3 M sodium citrate, pH 7, was used during transfer to nitrocellulose. 14 S rat liver mRNA and immunoselected rat liver OTCase mRNA were prepared as described (7,8). DNA was sequenced according to Maxam and Gilbert (20). The methods used to obtain amino acid composition and sequence data from tryptic peptides derived from homogenous rat liver OTCase were previously described (9,10).

### RESULTS AND DISCUSSION

#### Nucleotide sequence of cloned cDNA to OTCase mRNA

Screening of 7,500 rat cDNA clones yielded four independent OTCase clones with cDNA inserts in the range of 400-1,500 nucleotides, the longest of which (1446 bp insert) was designated pR021. A limited restriction map of the insert and a diagram of the strategy taken in DNA sequencing are shown in Fig. 1. Fig. 2 shows the nucleotide sequence of the pR021 insert, which corre-



**Figure 1.** Partial restriction map and sequencing strategy of rat OTCase cDNA.

The boxed region encloses the open reading frame; the hatched region designates the sequence coding for the amino-terminal leader. Restriction endonuclease fragments were radiolabeled at their 5' termini using bacterial alkaline phosphatase followed by T4 polynucleotide kinase (Bethesda Research Laboratories) and [ $\gamma$ - $^{32}$ P]ATP (Amersham) or at their 3' termini by using the Klenow fragment of polymerase I (Boehringer-Manheim) and [ $\alpha$ - $^{32}$ P]dNTP (Amersham). The two labelings outside of the cDNA insert used the MspI sites in pBR322 adjacent to the PstI site.

Rat 5'	AT-TCTTAGCTTGCCAGTGGCCCC-ACTGCCTGCCTGCCGGAAGCTCTAGACC	-128
Human N <sub>47</sub>	T T TTAG CG G AA TG T GT T A GC	
	met leu ser asn leu arg ile leu leu asn lys ala	-21
ATAGATTCCTCCTCCACTCTAGCAAGAGAAG	ATG CTG TCT AAT TTG AGG ATC CTG CTC AAC AAG GCA	-61
A G TT A A TT A	T C	
	phe asn	
ala leu arg lys ala his thr ser met val arg asn phe arg tyr gly lys pro val gln	GCT CTT AGA AAG GCT CAC ACT TCC ATG GTT CGA AAT TTT CGG TAT GGG AAG CCA GTC CAG	↓ -1
T T T G AC T	G A C A C A A	
phe asn gly asn phe	cys gln leu	
ser gln val gln leu lys gly arg asp leu leu thr leu lys asn phe thr gly glu glu	AGT CAA GTA CAG CTG AAA CCC CGT GAC CTC CTC ACC CTG AAG AAC TTC ACA GGA GAG GAG	20 60
A A G G	T T A A	
asn lys		
ile gln tyr met leu trp leu ser ala asp leu lys phe arg ile lys gln lys gly glu	ATT CAG TAC ATG CTA TGC CTC TCT GCA GAT CTG AAA TTC AGG ATC AAA CAG AAA GCA GAA	40 120
A A T	A A T	
lys	G	
tyr leu pro leu leu gln gly lys ser leu gly met ile phe glu lys arg ser thr arg	TAC TTC CCT TTA TTG CAA GGG AAA TCC TTA GGG ATG ATT TTT GAG AAA ACA ACT ACT ACA	60 180
T	C	
thr arg leu ser thr glu thr gly phe ala leu leu gly gly his pro ser phe leu thr	ACA AGA CTG TCC ACA GAA ACA GGC TTC GCT CTT CTG GCA GGA CAT CCT TCT TTT CTC ACC	80 240
T T	T A	
	G CT	
	cys pro	
thr gln asp ile his leu gly val asn glu ser leu thr asp thr ala arg val leu ser	ACA CAA CAC ATT CTT TGG CCG GTG AAT GAA AGT CTC ACA GAC ACA GCT CCG TGT TTA TCT	100 300
T T	G G	
	A G	
ser met thr asp ala val leu ala arg val tyr lys gln ser asp leu asp ile leu ala	AGC ATC ACA GAT GCA GTG TTA GCT CGA GTG TAT AAA CAA TCA GAT CTG GAC ATC CTG CCT	120 360
G A	T C	
ala	thr	
lys glu ala thr ile pro ile val asn gly leu ser asp leu tyr his pro ile gln ile	AAG GAA GCA ACC ATC CCA ATT GTC AAC GGA CTG TCA GAC CTG TAT CAT CCT ATC CAG ATC	140 420
A T	T G	
	T T C	
	ser	
leu ala asp tyr leu thr leu gln glu his tyr gly ser leu lys gly leu thr leu ser	CTG GCT GAT TAC CTT ACA CTC CAG GAA CAC TAT GGC TCT CTC AAA GGT CTC ACC CTC ACC	160 480
C A	A	
	ser	
trp ile gly asp gly asn asn ile leu his ser ile met met ser ala ala lys phe gly	TGC ATA GGA GAT GGG AAC AAT ATC CTG CAC TCC ATC ATG ATG AGT GCT CCA AAA TTC GGG	180 540
T T C G	C A G	
cys phe	A	
met his leu gln ala ala thr pro lys gly tyr glu pro asp pro asn ile val lys leu	ATG CAC CTT CAA GCA GCT ACT CCA AAG GGT TAT GAG CCA GAT CCT AAT ATA GTC AAG CTA	200 600
G	G G AC T G	
	ala ser val thr	
ala glu gln tyr ala lys glu asn ser thr arg leu ser met thr asn asp pro leu glu	GCA GAG CAC TAT GCC AAG GAC AAT ACT ACC AGG TTG TCA ATG ACA AAT GAT CCA CTG GAA	220 660
A	T C	
	gly lys leu leu	
ala ala arg gly gly asn val leu ile thr asp thr trp ile ser met gly gln glu asp	GCA GCA CGT GGA GGC AAT GTA TTA ATT ACA GAT ACT TGG ATA AGC ATG GCA CAA GAG GAT	240 720
G A	G A G	
his	arg glu	
glu lys lys lys arg leu gln ala phe gln gly tyr gln val thr met lys thr ala lys	GAG AAG AAA AAG CCT CTT CAA GCT TTC CAA GCT TAC CAG GTT ACA ATG AAG ACT GCT AAA	260 780
G C	A	
val ala ala ser asp trp thr phe leu his cys leu pro arg lys pro glu glu val asp	GTG CCT GCG TCT CAC TGC ACC TTT TTA CAC TGC TTC CCT AGA AAG CCA GAA GAA GTA CAT	280 840
T C	C	
asp glu val phe tyr ser pro arg ser leu val phe pro glu ala glu asn arg lys trp	GAT GAA GTG TTT TAT TCT CCC CGG TCA TTA GTG TTC CCA GAG GCA GAA AAT AGA AAG TGG	300 900
C	T A C	
	C	

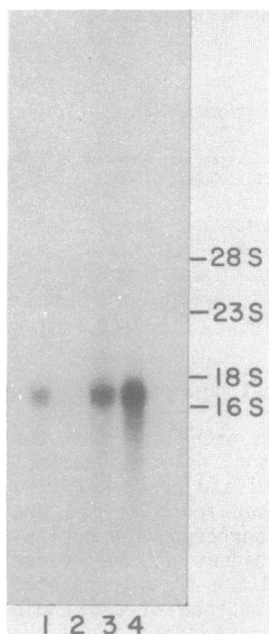
thr ile met ala val met val ser leu leu thr asp tyr ser pro val leu gln lys pro	320
ACA ATC ATG GCT GTC ATG GTA TCC CTG CTG ACA GAC TAC TCA CCT GTG CTC CAG AAG CCA	960
	G T CA gln
lys phe ter	
AAG TTC TGA TG-----CTG-CAAGAGGACGAAAAACCCAAAAGACAAAAAATCTGTTCTTTAGCAGCAGAA	1027
A T TTGTGTTA T T ----- G AG ----- GG T A	
TAACTCAGTTTATGTAGAAAAGAGAAGA--ATTGAAATTGTAACACATCCCTAGTCGGTGAT-----AATTATGTA	1099
G TG GG CA CT AAA CA ACA G GGCG CG ACC	
AT-TGCTTTGCTATTGCTGAGAATTGCTTAAAGCTTTTAGTTTAAGTGCTGG-GCATTTTATTATCTGCTTGACTTGAC	1176
A C A C T C T C C A AT C G A - G A TG	
TTAAGCACTCTCTTCAATTCACAACCTCTGAATGATATTGGGTTTCATATTAATTATCATACACATTTCTTCCACTA	1255
A T ---- C T G T C A A A C T A 3'	
AGCATAAA [CACTATGCTTACAATGCATACCATCTAAGTCATTAATGTAATCCATGCTTTATTCGCCCTA] <sub>12</sub> 3'	1335

**Figure 2.** Nucleotide and amino acid sequence of the rat and human OTCase precursors.

The top amino acid and nucleotide sequences in each line show the data obtained for the rat OTCase precursor. For comparison, only the nucleotides and amino acids which are different in the human OTCase precursor (10) are shown on the bottom of each line. The serine residue found at the NH<sub>2</sub> terminus of the mature rat OTCase subunit, and its first corresponding nucleotide base (adenine) were denoted +1, all amino acid residues of the leader peptide and nucleotide sequences encoding the leader and the 5' untranslated region (182 bp) are denoted by the (-) sign. The DNA sequence of pRO21 did not contain the poly(A) tail but other plasmids did (9) as indicated by the sequence enclosed in brackets. Several gaps, depicted by dashes, were introduced in the untranslated 5' and 3' regions to maximize the homology between the rat and human sequences.

sponds to the entire coding region of OTCase mRNA and adjacent portions of its untranslated sequences. The pRO21 clone did not contain the poly(A) tail but other previously sequenced rat OTC cDNA inserts did (9), as shown in the sequence enclosed in brackets. Northern blot analysis of total liver mRNA and of mRNAs enriched for OTCase mRNA yielded an estimated length for OTCase mRNA of  $1650 \pm 50$  nucleotides (Fig. 3). The sequence shown in Fig. 2 accounts for 1517 nucleotides and because mammalian messages may contain poly(A) tails 200 nucleotides in length (21), it seems likely that little information is missing from the 5' untranslated sequences in our rat cDNA clone. However, the 5' region may not be complete, since the human cDNA sequence (10) is very homologous but is 47 nucleotides longer.

The mature protein coding regions of the rat and human cDNA clones are exactly the same length, 966 nucleotides, and are 89% homologous at the nucleotide level; most nucleotide substitutions are silent, i.e., preserving the amino acid sequence. To analyze this homology further, the sequence was divided by the method of Perler *et al.* (22) into those sites that allow silent changes and those sites where changes lead to amino acid replacements. The



**Figure 3.** Northern blot analysis of OTCase mRNA from rat liver.

mRNA samples were denatured with glyoxal and electrophoresed in 1.1% agarose gel. The separated mRNAs were transferred to nitrocellulose and the blot was probed with radiolabeled rat OTCase cDNA (pOTC-1) (9). Lane 1, total rat liver mRNA, 5  $\mu$ g; lane 2, human skin fibroblast mRNA, 5  $\mu$ g; lane 3, 14 S fraction from sucrose gradient of total rat liver mRNA, 3  $\mu$ g; lane 4, immunoselected rat liver OTCase mRNA, 10 ng.

silent sites are 38% divergent (uncorrected for multiple mutations per Chan *et al.* (23)). This is consistent with the divergence seen between mammalian species for silent sites in other genes and may represent the unrestrained accumulation of mutations since the separation of mammalian species (Kafatos *et al.* (24)). On the other hand, the divergence at replacement sites is only 4%, suggesting that there is a high degree of selective pressure to maintain the mature OTCase amino acid sequence. This degree of divergence is consistent with that reported for replacement sites in other conserved mammalian proteins, such as insulin (23) and  $\beta$ -globin (24).

Analysis of sequences encoding the leader gives a different conclusion, however. Silent sites diverge between the rat and human by 32%, very similar to the mature protein coding sequences. In contrast, the replacement sites in the leaders diverge by 15%, considerably greater than the divergence in the mature sequences. This implies that the evolutionary constraints to maintain the leader peptide's primary amino acid sequence are much less than those acting on the mature OTCase. This divergence is similar in magnitude to that observed for both the signal sequence and C peptide of insulin between a number of mammals, including rat and human (23).

The untranslated regions of the rat and human cDNA clones are fairly

homologous. The 5' untranslated region of the rat is co-linear with that of the human if two single nucleotide deletions or insertions are introduced (Fig. 2). Overall, the 5' untranslated sequences are 69% homologous, excluding these gaps, consistent with the mean homology of  $75.0 \pm 6.5\%$  observed among mammalian species as calculated by Miyata *et al.* (25). The 3' untranslated region contains several regions of very good homology and regions with no obvious homology. Homologous regions were aligned and gaps introduced to maximize the apparent homology. Overall, the 3' untranslated regions are 77% homologous excluding gaps, and 69% homologous if gaps are designated as mismatches. These are again within the range of values seen among other mammalian genes (25).

#### Primary amino acid sequence of OTCase

Fig. 2 shows the amino acid sequence of the entire cytoplasmic precursor of the mitochondrial OTCase. The sequence of the rat protein is shown and only amino acids that are different in the human polypeptide are indicated. The amino acid sequence predicted from the cDNA sequence is supported by extensive protein sequence data. Thus, 41% of the sequence has been confirmed by amino acid sequences of tryptic peptides derived from homogenous mature rat OTCase (residues 1-56, 63-97, 190-206, and 275-299). In addition, 35% of the sequence has been confirmed by precisely matching the amino acid compositions of additional tryptic peptides (residues 110-178, 224-243, and 300-322) to ones predicted by DNA sequencing.

The homology between the amino acid sequences of mature rat and human OTCases is 93% (24 differences in 322 amino acids). It is apparent from the comparison of the two sequences in Fig. 2 that eight of the amino acid changes occur in two clusters involving residues 195-198 (four different out of four) and 209-214 (four different out of six). The remainder of the differences are scattered throughout, with long stretches of completely identical sequence. It is interesting to note that, although both polypeptides contain 322 amino acids with predicted molecular weights of 36,110 Da for the human enzyme and 36,207 Da for the rat enzyme, the human OTCase appears to be distinctly larger (by ~2,000 Da) on NaDodSO<sub>4</sub>/polyacrylamide gel electrophoresis.

#### Conservation of catalytically important residues

The decapeptide comprising residues 53-62 (Fig. 4) represents a highly conserved region of the enzyme. Nine out of these ten residues can be found in both *E.coli* K-12 OTCases encoded by the arg I and arg F genes. In particular, the sequence ser-thr-arg-thr-arg centered around residue 60 in both the rat and the human OTCase possesses complete homology with OTCases but also

<u>E.coli</u> arg F,I	51	60 92	97 270	275
	...I F E K D S T R T R...	D T A R V L...	F L H C L P...	
rat, human OTCase	53	62 94	99 268	273
	...I F E K R S T R T R...	D T A R V L...	F L H C L P...	
<u>E.coli</u> pyr B	49	58 90	95 263	268
	...F F E - A S T R T R...	D T I S V I...	V L H P L P...	

Figure 4. Comparison of conserved sequences among the E.coli OTCases, mammalian OTCases, and the E.coli aspartate transcarbamylase

Only partial sequences possessing the longest, uninterrupted regions of homology are shown. Amino acid residues are numbered according to previously published sequences for E.coli OTCases encoded by the arg F and arg I genes (28), E.coli aspartate transcarbamylase encoded by the pyr B gene (28), and the human OTCase (10).

with aspartate transcarbamylase from E.coli (26-28). A similar consensus sequence X-ser-X-<sup>arg</sup><sub>lys</sub>-X has been shown to be the phosphate binding site in a number of proteins (29). Furthermore, it has been shown (30,31) that both human and bovine OTCases are inactivated by butanedione and phenylglyoxal, inhibitors that react with the arginine side chain, and that one arginine residue per subunit is modified in the inactivated enzyme. Carbamyl phosphate prevents both the inactivation and the loss of the arginine. Taken together, these findings support the conclusion that the arginine at residue 60 is the binding site for the phosphoryl group of carbamyl phosphate.

In addition to this conserved region, another fully conserved hexapeptide between the mammalian and E.coli OTCases is found near the carboxy terminus (residues 268-273). It is the phe-leu-his-cys-leu-pro sequence, which contains the only shared cysteine between OTCases from E.coli (28), Streptococcus faecalis (31), Streptococcus faecium (31), cow (31), rat, and human (10) (and presumably also yeast (32)). Marshall and Cohen (31) mapped the cysteine which reacted selectively with 5,5'-dithiobis(2-nitrobenzoic acid) to 51 residues from the carboxy terminus in bovine enzyme and 66 residues from the carboxy terminus in OTCase from Streptococcus faecalis. Both enzymes modified with this bulky group were inactive while the enzymes modified with the small cyano group were active but had a greatly increased  $K_m$  for ornithine. Similarly, modification of a unique cysteine in yeast OTCase (32) with p-chloromercuribenzoate or N-ethylmaleimide leads to complete loss of enzyme activity. The conservation of the cysteine hexapeptide among E.coli and mammalian OTCases and the fact that the cysteine residue in rat OTCase (52 residues from the COOH end) is unique support the conclusion of Marshall and Cohen (31) that this cysteine is either a part of the binding site for ornithine or, at least, is located close to it. Other regions of the mammalian OTCases contain amino



acid sequences that can be found in the E.coli OTCases, the most extensive of which is a completely conserved hexapeptide comprising residues 94-99 (Fig. 4). How they relate to structure and function remains to be determined.

#### Comparison of the two leader sequences

The leader peptide is devoid of acidic residues and is, therefore, positively charged similar to other mitochondrial leaders (10). The spacing of the four arginine residues is conserved between the rat and the human; however, the rat leader sequence is even more basic than the human one due to the presence of three additional lysines. The two OTCase leader sequences show significantly less homology than do sequences of the mature portion of rat and human OTCases. There are 10 differences in the 32 residues of the leader (69% homology) and another two differences in the adjacent first two residues of the mature subunits in contrast to 24 differences in 322 residues of the mature subunit (93% homology). Because of the differences in amino acid residues around the NH<sub>2</sub> terminus of the mature sequences, the cleavage site for the matrix protease is changed from pro-leu-gln + asn-lys-val for the human to pro-val-gln + ser-gln-val for the rat. Because both precursors are imported and correctly cleaved in vitro by intact rat liver mitochondria, it is apparent that the mitochondrial matrix protease is recognizing more than a stretch of primary amino acid sequence adjacent to the cleavage site.

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### Note Added

While this manuscript was being prepared, we became aware of another manuscript (McIntyre, P., Graf, L., Mercer, J.F.B., Wake, S.A., Hudson, P. and Hoogenraad, N., submitted to *EMBO J.*) describing the isolation and sequence of a different rat OTCase cDNA clone.