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Isolation and nucleotide sequence of a cDNA clone encoding rat mitochondrial malate dehydrogenase

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ABSTRACT

We have determined the complete sequence of the rat mitochondrial malate dehydrogenase (mMDH) precursor derived from nucleotide sequence of the cDNA. A single synthetic oligodeoxynucleotide probe was used to screen a rat atrial cDNA library constructed in λgt10. A 1.2 kb full-length cDNA clone provided the first complete amino acid sequence of pre-mMDH. The 1014 nucleotide-long open reading frame encodes the 314 residue long mature mMDH protein and a 24 amino acid NH_2-terminal extension which directs mitochondrial import and is cleaved from the precursor after import to generate mature mMDH. The amino acid composition of the transit peptide is polar and basic. The pre-mMDH transit peptide shows marked homology with those of two other enzymes targeted to the rat mitochondrial matrix.

INTRODUCTION

Most mitochondrial proteins are encoded by nuclear DNA, synthesized as larger precursors on cytoplasmic polysomes, released into the cytoplasm, and subsequently imported into mitochondria (1,2). The size difference has been attributed to an NH_2-terminal extension (or "transit peptide") which mediates recognition of proteins targeted for this organelle. Upon binding and import, these extensions are proteolytically removed by specific proteases and subunits assemble to form active complexes. Import of mitochondrial proteins is a post-translational event. Signals dictating subcellular compartmentation must be contained within the structure of the mitochondrial precursor protein.

Recent studies employing fusions of transit peptide coding domains to non-mitochondrial gene segments demonstrate that proteins derived from these chimeric genes are imported into mitochondria (3,4). These data suggest that NH_2-terminal sequences are sufficient to target proteins to the organelle. The precise role of transit peptide in subcellular targeting is unknown, although structures of several fungal and mammalian mitochondrial pre-proteins have been derived from cDNA clones. There is no correlation between peptide length and sub-organellar localization (2). All published transit peptides contain abundant basic residues; however, there is no apparent consensus sequence.

We have initiated studies to analyze which structural features of the precursor to rat mitochondrial malate dehydrogenase (mMDH; E.C.1.1.1.37) determine its sequestration by mitochondria. Mitochondrial MDH is a matrix enzyme which catalyzes the formation of oxaloacetate from malate in the tricarboxylic acid cycle and in the malate-aspartate shuttle by which reducing equivalents are transported across the inner mitochondrial membrane. The mature matrix form is a dimer of identical 33,000 dalton subunits. mMDH, like most mitochondrial enzymes, is synthesized on cytoplasmic polysomes as a larger precursor (5,6). In vitro import of pre-mMDH into mitochondria has been reported (7). Eukaryotic cells contain a second malate dehydrogenase isoenzyme, cytoplasmic malate dehydrogenase (sMDH). Although sMDH is structurally homologous to mature mMDH, it is not sequestered by mitochondria and is found as an active homodimer in the cytosolic fraction of cells. Both enzymes have been extensively characterized enzymatically and physically (8). We believe that comparison of the structures of sMDH and pre-mMDH will help define features responsible for their heterotopic subcellular locations.

In this paper, we describe the isolation of the first cDNA clone encoding the precursor form of mMDH. The derived amino acid sequence predicts a 24-amino acid NH_2-terminal extension. This sequence is analyzed in light of the current understanding of transit peptide structure.

MATERIALS AND METHODS

RNA Preparation and in vitro Translations

Total cellular RNA was prepared from fresh tissue by disruption in the presence of guanidinium thiocyanate followed by centrifugation through cesium chloride gradients (9). Poly(A)-containing RNA was selected by chromatography on oligo(dT) cellulose (10). Cell-free protein synthesis was performed using wheat germ lysates (11) programmed with total cellular RNA (320 µg/ml) or poly(A)-containing RNA (20 µg/ml). Where noted, citrate synthase and oxaloacetate were added to prevent NH_2-terminal acetylation (12).

Immunopurification and Analysis of pre-mMDH

Translation reactions were incubated with monospecific antiserum (13) and immunoreactive material was recovered by binding to S. aureus cells. After dissociation of bound material under reducing, denaturing conditions, cells were removed by centrifugation. The supernatant was analyzed by gel electrophoresis through 15% acrylamide slab gels containing SDS (14) followed by fluorographic exposure (3-5 d).

Immunopurified precursor was isolated from denaturing 12.5% polyacrylamide tube gels and subjected to automated Edman degradation using a Beckman 890C sequencer (0.3 M Quadrol program).

Clone Isolation and Analysis

Synthetic oligonucleotides were prepared on an Applied Biosystems Model 380A solid phase DNA synthesizer. Oligonucleotide probes were labeled using polynucleotide kinase and γ-[^{32}P]ATP (>5000 Ci/mmol) (15).

A rat atrial cDNA library constructed in λgt10 was a generous gift of Roger Wiegand and Mark Day, Monsanto Company.

Screening strategy followed guidelines presented by Ullrich (16). Phage plaques were plated at 12,000 pfu per 150 mm petri dish on a lawn of E. coli strain HB101. After 7 h growth, replicate filters were prepared on Millipore HATF nitrocellulose (0.45 µm). After processing (15) filters were prehybridized in 6X SSC (1X SSC is 0.15 M NaCl/0.015 M Na_3Citrate, pH 7.0)/5X Denhardt's (1X Denhardt's is 0.02% each bovine serum albumin/polyvinyl pyrrolidone/Ficoll)/20% formamide/100 µg sonicated salmon sperm DNA overnight at 50°C.

Hybridization conditions used the same mix with inclusion of 2-3 x 10^5 cpm/ml [^{32}P]-labeled 47mer. Filters were hybridized overnight at 37°C and washed. The final wash was 5 min at 42°C in 0.1 X SSC/0.01% SDS. Plaques corresponding to autoradiographic signals were isolated and screened again under identical conditions. Selected plaques were tested for hybridization to a mixture of 17-base oligonucleotides designed from a different region of mMDH.

DNA from plaques which hybridized to both the 47mer and mixed 17mer probes was isolated and insert size was determined by Southern analysis (17). A 1.2 kb cDNA insert was subcloned into the M13 vector mp18. Single stranded phage DNA templates and synthetic oligonucleotide primers were used for sequencing by dideoxy chain-termination.

Northern Blot Analysis

Poly(A)$^+$ RNA was resolved according to size by electrophoresis through a 1.2% agarose gel containing 6% formaldehyde. RNA was transferred onto nitrocellulose paper (Schleicher and Scheuell, 0.45 µm) and processed according to manufacturer's directions. Prehybridization was at 37°C in 50% formamide/5X SSC/2X Denhardt's/50 µg/ml sonicated salmon sperm DNA. Overnight hybridization conditions were identical with inclusion of 3 x 10^5 cpm/ml pmMDH probe which was labeled with α[^{32}P]deoxy ATP (19). The blot was sequentially washed in 0.1X SSC/0.01% SDS at room temperature, 46°C and 65°C.

RESULTS

The biogenesis of mMDH was initially studied using cell-free translation of mRNA isolated from different tissue sources. A wheat germ lysate translation mixture was programmed with exogenous mRNA in the presence of [^{35}S]-methionine. Immunoprecipitation was performed with monospecific antiserum raised against porcine mMDH. A single immunoreactive species with a molecular mass of 36,500 daltons was detected (Fig. 1). Primary translation products of similar size were recovered by immunoprecipitation of wheat germ lysates containing RNA from pig heart, pig liver (not shown), rat heart, and rat liver. Addition of 40 µg mature porcine mMDH to immunoprecipitation mixtures prevented immunoprecipitation of the 36,500 dalton protein (Fig. 1, lanes 6, 7). This competition demonstrates that the binding to antibody was specific and supports the hypothesis that this species is the precursor to mature mMDH (M_r

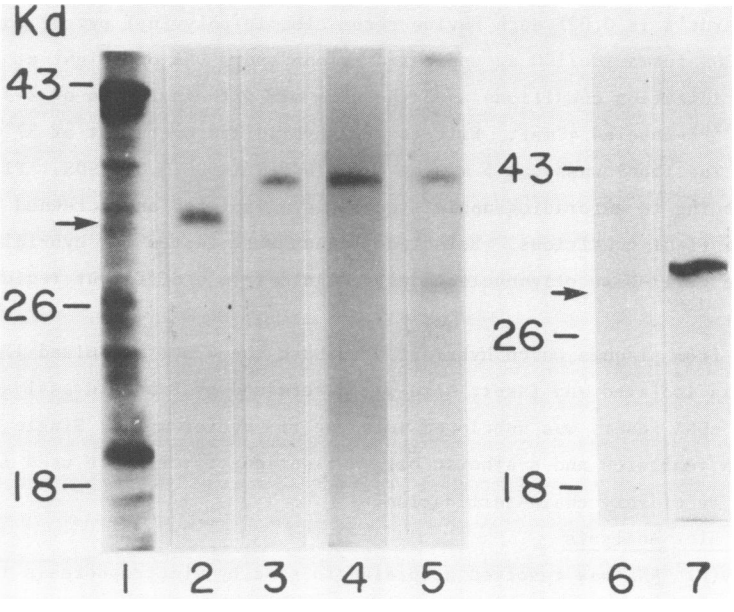

Figure 1. In vitro synthesis of precursor form of mMDH. Total RNA prepared from different tissues was translated in a wheat germ lysate system for 90 min at 25°C in the presence of [^{35}S]-methionine. Products were immunoprecipitated as described in "Methods". Molecular weight standards are marked (in kilodaltons). Arrow represents migration of mature mMDH. Lane 1. Total products from translation of pig heart cellular RNA. Lane 2. Immunoprecipitation of sMDH from pig heart RNA translation (1 day exposure). Lanes 3, 4, 5. Immunoprecipitation of pre-mMDH from translations of RNA isolated from pig heart, rat heart, and rat liver (3 day exposure). Lanes 6, 7. Immunoprecipitation of pig heart pre-mMDH with (6) and without (7) inclusion of 40 µg cold porcine mMDH in the immunoprecipitation reaction.

= 34,500). Size estimations from electrophoretic mobility of pre-mMDH in denaturing gel systems suggest that pre-mMDH is ∿2,000 daltons larger than the mature protein (6,7). For quantitation of mRNA levels, the amount of radioactivity incorporated into pre-mMDH was compared to total label incorporation into protein. The mRNA representation was calculated to be 0.02% in heart and <0.01% in liver.

Pre-mMDH was immunopurified from cell-free translation reaction mixtures containing porcine heart mRNA and [^{3}H]-leucine. The protein was subjected to Edman degradation to obtain a partial NH_2-terminal sequence. No radioactivity was recovered, although the sequence of the unlabeled carrier protein was detected, indicating that the cleavage reactions had occurred. This suggested that the NH_2-terminus of pre-mMDH is blocked. When acetylation was prevented by inclusion of an "acetyl-CoA trap" (12) in the translation mixture, pre-mMDH was susceptible to Edman degradation. These results demonstrate that pre-mMDH is modified by NH_2-terminal acetylation in the wheat germ system. Unblocked porcine pre-mMDH was labeled with [^{3}H]-leucine and subjected to automated sequential Edman degradation. Radioactivity was recovered from cleavage cycles 2, 5, 13, 30, 40, 42, 43, and 44 (Fig. 2). Mature porcine mMDH has

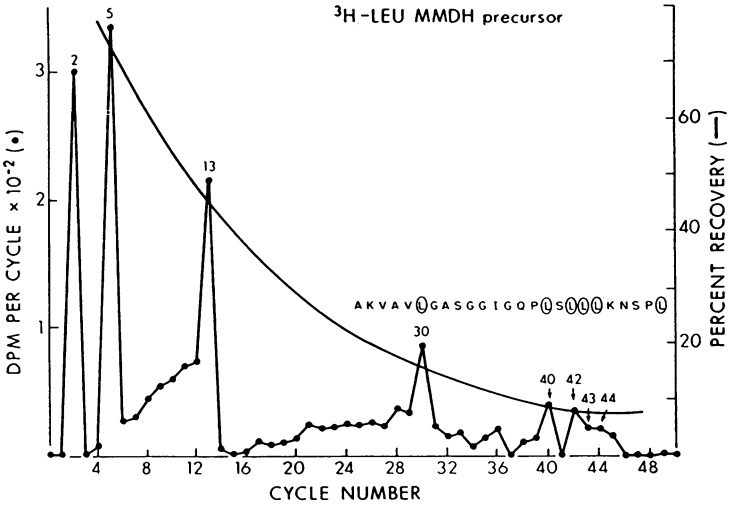

Figure 2. Automated Edman degradation of porcine pre-mMDH. Radioactive pre-mMDH was generated by in vitro translation of pig heart mRNA followed by immunoprecipitation with anti-mMDH. Immunoreactive material was recovered from preparative SDS-polyacrylamide gel electrophoresis and subjected to NH_2-terminal sequencing. Radioactivity recovered in each cleavage cycle has been normalized according to a 94% repetitive yield. The sequence of mature mMDH is inset to show overlap consistent with 24 amino acid extension.

leucine residues at positions 6, 16, 18, 19, and 20 (20). Therefore, the partial sequence of radiolabeled pre-mMDH is consistent with the sequence of the mature protein, if the primary translation product contains a 24 amino acid NH_2-terminal extension. This proposed extension would have leucine residues at positions 2, 5, and 13.

To determine the primary structure of the mitochondrial precursor, we have isolated a cDNA clone encoding pre-mMDH. A synthetic oligonucleotide probe was used to screen a rat atrial cDNA library constructed in λgt10. From the primary structure of rat mMDH (unpublished experiments), a region of low codon degeneracy was selected. A 47-base oligonucleotide probe was designed using the strategy of Ullrich (16). Codon usage tables (21) were consulted for selection of the third nucleotide in ambiguous codons. Comparison of the probe sequence with that of the selected pre-mMDH clone later proved that the probe matched 42 of 47 bases (89% homology, Fig. 3).

Hybridization of 200,000 plaques of a rat atrial cDNA library with the radioactive 47-mer followed by autoradiography yielded 49 positive signals. Upon rescreening with the 47-base oligonucleotide, 10 of the putative pre-mMDH clones remained positive. Only 5 of these reacted with a 17-base mixed oligo-

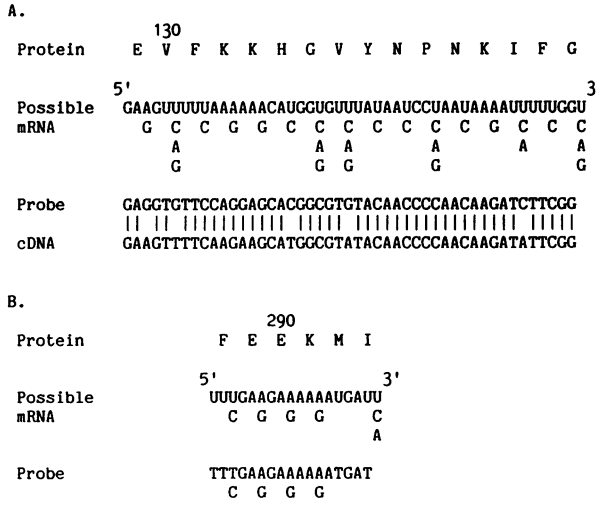

Figure 3. Oligonucleotide probes designed from rat mMDH protein sequence data. A) a 47-base sequence was derived from amino acid residues 129-144. A comparison of probe sequence to the actual cDNA sequence shows 89% homology. The probe proved suitable as a primer for template-directed DNA sequencing in the presence of dideoxynucleotides. B) A mixture of 17-base oligonucleotides was generated corresponding to all possible coding sequnces for amino acids 288-293. Although attempts to use this probe in library screening were unsuccessful, hybridization to clones selected by the 47-mer was used to identify clones containing the COOH-terminal portion of pre-mMDH.

nucleotide probe (Fig. 3), designed from a sequence near the COOH-terminal end of mMDH. Southern analysis of DNA isolated from these 5 clones demonstrated inserts at the λgt10 EcoRl site ranging from 600 to 1200 bp in length. All inserts gave strong signals after hybridization with the 47-mer probe; however, only two of these gave strong signals with the mixed 17-mer probe. The larger of these, a 1.2 kb EcoRl (designated pmMDH), was subcloned into mp18 and pUC19 for further analysis.

Single-stranded templates were prepared from the mp18-pmMDH plaques. The nucleotide sequence of both strands was determined by extension of synthetic oligonucleotide primers within and adjacent to pmMDH according to the sequencing strategy shown in Fig. 4. The insert was 1194 nucleotides in length. Sequence analysis demonstrated an uninterrupted 1014 nucleotide long open reading frame flanked by 44 bases of non-coding sequence at the 5'end and 133 bases of non-coding DNA at the 3'-end (Fig. 5). The 3'-sequence does not contain the AAUAAA consensus recognition signal for polyadenylation or any of the poly(dA) tail. Thus, the pmMDH cDNA clone does not represent the entire mRNA sequence.

We determined the size of pre-mMDH mRNA by hybridization of radioactive pmMDH probes to a Northern blot. Autoradiography demonstrated the existence of a single mRNA 1.35 kb in length (Fig. 6). As our cDNA is 1.2 kb in length, it is nearly full-length.

Sequence analysis of the long open reading frame shows that it includes the 314 amino acid sequence of mature mMDH. This sequence is identical to that obtained in this laboratory by Edman degradation (unpublished experiments) except for substitutions of Lys for Arg_{205} and Leu for Ile_{275} in the protein sequence. The translation initiation site was assigned to an in-frame ATG codon 72 bases upstream from the GTC codon alanine at position 1 of mature

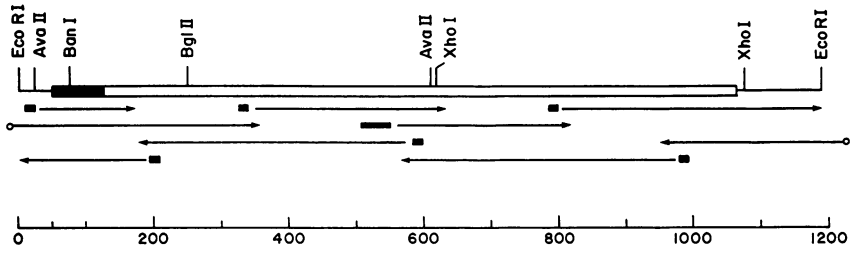

Figure 4. Sequencing strategy of pmMDH. The entire nucleotide sequence of pmMDH was determined in both orientations by the dideoxy chain-termination method. The coding region is represented by an open box. The region encoding the transit peptide is shaded. Arrows indicate the direction and extent of sequencing reactions. Synthetic oligonucleotide primers are represented by solid rectangles. Sequences extended from mp18 universal sequencing primer are indicated by open circles.

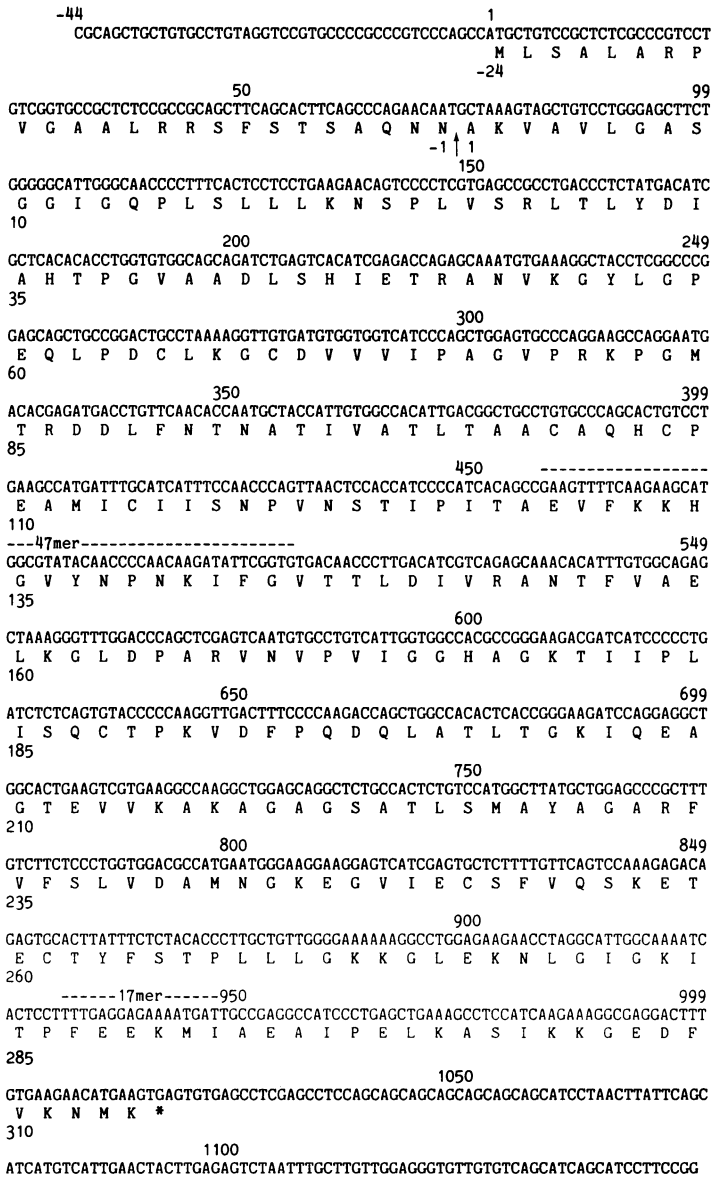

Figure 5. Nucleotide and amino acid sequences of rat pre-mMDH. The nucleotide sequence is numbered (superscript) with assignment of +1 to the first residue of initiator methionine codon. The protein sequence is numbered according to the amino acid sequence of the mature rat sequence. Cleavage to generate the mature protein occurs between amino acid residues +1 and -1 (arrow). Residues -24 to -1 constitute the transit peptide. Peptide regions used in design of oligonucleotide probes are indicated.

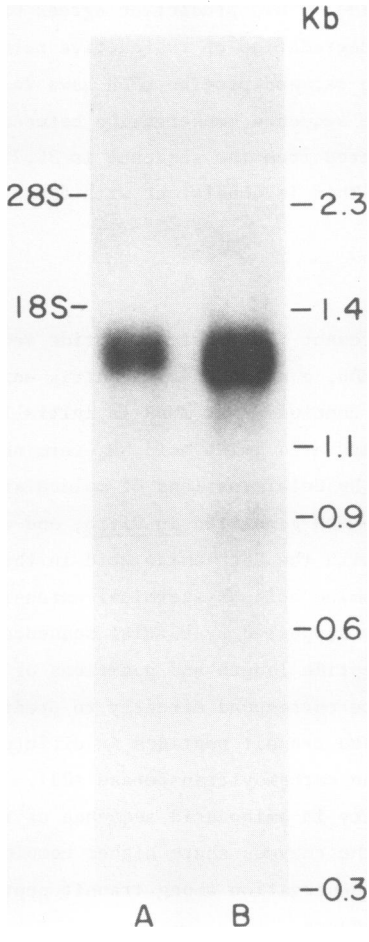

Figure 6. Northern blot analysis of pre-mMDH mRNA. Poly(A)$^+$ selected RNA was prepared from rat heart. Lanes A and B represent 1 µg and 3 µg RNA samples which were separated by size on a 1.2% agarose gel in the presence of 6% formaldehyde. Following electrophoresis, RNA was blotted onto nitrocellulose and hybridized with a probe derived from pmMDH. Positions of 18S and 28S ribosomal RNA species and double stranded DNA fragments are shown.

mMDH. We feel confident in this assignment as this is the only upstream ATG codon in our sequence. In addition, the CCAGCCATG sequence in this region of the cDNA is similar to the consensus eukaryotic initiation signal proposed by Kozak (22). This initiation site defines a 338 amino acid polypeptide which spans the open reading frame and thereby predicts a 24 amino acid stretch preceding the NH_2-terminal sequence of mature mMDH. Translation of the nucleotide sequence predicts leucine residues at positions 2, 5, and 13 in the 24

amino acid transit peptide. This prediction agrees with the partial sequence data obtained by Edman degradation of radioactive porcine pre-mMDH (Fig. 2). Therefore, precursors to rat and procine mMDH have 24 amino acid transit peptides which show some sequence conservation between species. The molecular mass of pre-mMDH calculated from the sequence is 35,764 daltons including the 2604 dalton pre-piece. This is consistent with data from *in vitro* synthesized precursor (Fig. 1).

DISCUSSION

In this paper we present the first nucleotide sequence encoding the precursor form of rat mMDH, a mitochondrial matrix enzyme. From the derived amino acid sequence, we conclude that mMDH is initially synthesized in a precursor form which bears a 24 amino acid NH_2-terminal extension. This conclusion is supported by determinations of molecular weight, partial sequence analysis of porcine pre-mMDH generated *in vitro*, and overlap with the mature rat sequence beginning with the 25th amino acid in the coding sequence.

The length of the amino acid NH_2-terminal extension predicted by nucleotide sequence is further supported by partial sequence analysis of porcine pre-mMDH. The transit peptide length and positions of leucine residues in the porcine pre-mMDH sequence correspond directly to predictions from the rat cDNA sequence. Homology between transit peptides in different species has been observed in pre-ornithine carbamoyltransferase (23). These rat and human enzymes share 69% identity in amino acid sequence of transit peptides while the mature portions of the enzymes share higher homology (93%). The extent and nature of sequence conservation among transit peptides may be of functional and evolutionary significance.

In vitro generated pre-mMDH is acetylated at the NH_2-terminus. NH_2-terminal acetylation is a common feature of cytoplasmic proteins (24). Cytosolic protein acetyltransferase activity is also present in cell-free wheat germ and reticulocyte lysate translation systems (12). In contrast to cytoplasmic proteins, secretory proteins are not acetylated *in vivo* or under *in vitro* conditions (11). Acetylation of pre-mMDH *in vitro* demonstrates its accessibility to cytoplasmic acyltransferases. It is likely that pre-mMDH would be subject to similar modification *in vivo*. This observation suggests a fundamental difference in accessibility of mitochondrial and secretory protein nascent chains to acetyltransferases.

According to the pre-mMDH sequence, the mature NH_2-terminus is generated by cleavage after an asparagine residue. This is an unusual site of proteo-

lytic processing which differs from those found in signal peptides of secretory proteins, pro proteins, zymogens, and complement or coagulation factors. This cleavage site was compared with those of several mitochondrial precursors. Processing after glutamine, a similar residue, has been reported (23). Other transit peptide cleavage sites are more heterogeneous. This diversity might suggest the involvement of multiple processing steps involving proteases with different specificities. Alternatively, proteolytic specificity might reflect regional conformation or secondary structure rather than specific amino acid sequences.

Alignment of the rat mMDH transit peptide sequence with seven other mammalian sequences shows striking homology among three rat sequences (Fig. 7). All three sequences are targeted to the mitochondrial matrix. The mMDH precursor sequence demonstrates identity of 12 residues (50% of the transit peptide) with the corresponding residues of rat ornithine aminotransferase. Rat ornithine transcarbamylase also shares several of these identities as does the human form of this enzyme. This homology does not continue into the mature regions of these enzymes (not shown). Recent experiments show that this homology has functional significance. A synthetic peptide corresponding to amino acids 1-27 of rat liver ornithine carbamyltransferase competes with pre-mMDH and other mitochondrial precursor proteins for mitochondrial import (25). This implies that common translocation apparatus is shared by homologous precursor proteins. In the proteins examined, sequence conservation is greatest in the first 20 residues, perhaps indicating that these regions are most important in targeting. Recent experiments have been designed to determine which segments of transit peptides have critical roles in targeting. Gene fusion experiments have shown that the first 12 amino acids of the

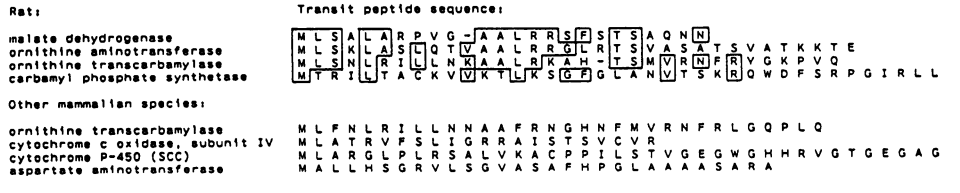

Figure 7. Alignment of mammalian transit peptides. Transit peptides from three different rat matrix enzymes (29,23,30) were aligned with that from the precursor of rat mMDH. Gaps were introduced to maximize homology. Identifies in the rat enzyme sequences from other mammalian species were included in the figure. These include those from human ornithine transcarbamylase, bovine cytochrome c oxidase (subunit IV), the bovine cytochrome P450 side chain cleavage enzyme, and porcine aspartate aminotransferase (31-34).

transit peptide from yeast cytochrome c oxidase subunit IV are sufficient to target a mouse cytosolic protein to yeast mitochondria (4). This result suggests that a limited sequence or structure is capable of conferring the full functional capability of the transit peptide. In efforts to define import regions of human ornithine carbamyltransferase more thoroughly, Horwich and coworkers have constructed various mutants containing deletions within the transit peptide segment (26). These studies have demonstrated that deletion of the first 12 residues does not abolish the targeting function, while deletion of an internal region from amino acids 8 through 22 results in the complete loss of import. It is clear that homologous sequences in functionally important regions may represent important structural determinants for mitochondrial import.

The homology noted among rat mitochondrial transit peptides might be interpreted to suggest a species-specific recognition signal. However, the sequence of rat carbamyl phosphate synthase, also a matrix enzyme, is not homologous to the other rat sequences. Some evidence indicates that multiple receptors or uptake mechanisms may be involved with translocation of different mitochondrial proteins in the same tissue (27). It is possible that enzymes with homologous transit peptides share the same receptor, while others utilize different receptors. However, it is unlikely that this specificity is highly restrictive as import of precursor proteins by mitochondria from different tissues or even different species has been widely reported (26).

There is no significant homology with the other mammalian transit peptides shown. Comparison of predicted secondary structures and hydropathy profiles based on transit peptide sequences shows no unifying features. For comparison, compositions of mitochondrial precursors were tabulated (not shown) and compared with average composition values (28). In almost all mitochondrial transit peptide sequences reported to date, there is an abundance of charged, basic residues and a paucity of acidic residues. Arginine represents 11.1% of the overall composition of transit peptides. In contrast, the average percent composition of arginine in proteins is 4.5. Arginine comprises 12.5% of the pre-mMDH transit peptide. These basic residues are dispersed throughout the transit peptide, thereby precluding existence of a long hydrophobic stretch. Like most mitochondrial presequences, the mMDH transit peptide is devoid of charged, acidic residues. In fact, acidic residues constitute less than 0.5% of the overall composition of mitochondrial presequences. In contrast, acidic residues comprise >11% of proteins.

Serine residues are also of increased abundance in transit peptides of pre-mMDH and several other mitochondrial enzymes. In general, the composition of transit peptides is basic and polar, while those of the mature portions of mitochondrial proteins do not deviate appreciably from average values. These similarities may indicate a similar mechanism of mitochondrial import among eukaryotes.

In summary, we have isolated the first cDNA clone encoding the precursor of mMDH. Pre-mMDH bears a 24 amino acid NH_2-terminal transit peptide which is absent from the mature form. This peptide is blocked by acetylation and removed upon mitochondrial import. Significant homology is apparent upon comparison with transit peptides from two other enzymes targeted to the rat mitochondrial matrix. These homologous segments may constitute part of a tissue or species-specific binding site which is recognized by receptors on the mitochondrial surface. Creation of site-specific mutations through recombinant DNA technology will be a useful tool for detailed analysis of the functional implications of specific amino acid residues found in this and other mammalian transit peptides. In this way, structures required for mitochondrial import may be defined.

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