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EXPRESSION IN ESCHERICHIA COLI OF FUNCTIONAL PRECURSOR TO THE RAT LIVER MITOCHONDRIAL ENZYME, ORNITHINE CARBAMYL TRANSFERASE. PRECURSOR IMPORT AND PROCESSING IN VITRO

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cDNA encoding the full-length cytosolic precursor of rat liver ornithine carbamyl transferase, a mitochondrial matrix enzyme, was inserted into pKK223-3 and expressed under the control of the tac promoter. Following transformation of E. coli strain JM105 and induction by isopropylthiogalactoside, a polypeptide was synthesized which reacted with antibody against ornithine carbamyl transferase and co-migrated in SDS-polyacrylamide gels with authentic enzyme precursor (pOCT, M_r = 40,000 kDa); it constituted ~ 0.1% of total E. coli protein. pOCT was synthesized in vitro by coupled transcription-translation of the recombinant plasmid in an E. coli S30 extract; upon subsequent addition of purified rat liver or heart mitochondria, the precursor was imported and processed to mature form. Synthesis of pOCT in a bacterial system, therefore, results in production of a functional precursor polypeptide which does not require additional cytosolic factors from eukaryotic cells to support its uptake and processing by mitochondria in vitro.

Ornithine carbamyl transferase (E.C. 2.1.3.3) catalyzes the formation of citrulline from L-ornithine and carbamyl phosphate. It is found in hepatocytes and epithelial cells of the intestinal mucosa (1,2) where it is located exclusively in the mitochondrial matrix compartment (2,3). Like most mitochondrial proteins, the enzyme is encoded by a single-copy nuclear gene, synthesized as a cytosolic precursor polypeptide (pOCT[1], M_r = 40,000), and rapidly translocated into mitochondria where it is processed to mature enzyme (monomer M_r = 36,000) within the matrix compartment (4-6). The amino acid sequence of pOCT has recently been derived in several laboratories from cloned full-length cDNA (7-11); its N-terminal signal sequence is comprised of 32 amino

[1]Abbreviations: pOCT, precursor of ornithine carbamyl transferase; IPTG, isopropylthiogalactoside; SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRP, signal recognition particle.

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acids and, like other precursors, is characterized by basic residues, 5 in human (7) and 8 in rat (8-11), with no acidic amino acids.

Further analysis of pOCT structure and function would clearly benefit from having large quantities of the purified precursor polypeptide. To date, however, this has proven difficult to achieve because of the rapid turnover of pOCT *in situ*. Expression of the precursor molecule in *E. coli* offers an attractive alternative, but it must first be demonstrated that the bacterial precursor is functionally active. This is particularly important in view of recent reports showing that precursor polypeptides, including pOCT, require the presence of cytosolic factors, either from reticulocytes or yeast, to support precursor import and processing by mitochondria *in vitro* (12-16). In the present study, we have expressed rat liver pOCT cDNA using a bacterial expression vector and find that subsequent import and processing of pOCT by isolated rat mitochondria in vitro can occur efficiently in the complete absence of cytosolic components from eukaryotic cells.

MATERIALS AND METHODS

General. The procedures followed for routine methodology have been described elsewhere. These include isolation of rat liver mRNA (18), *in vitro* translation in a messenger-dependent (19) rabbit reticulocyte cell-free system containing [^{35}S]methionine (18), immunoprecipitation of radioactive proteins synthesized *in vivo* and *in vitro* (20), SDS-PAGE and fluorography of dried gels (20), and bacterial transformation and manipulation of recombinant DNA plasmids (21). Further details are provided in the Figure Legends.

Synthetic Oligonucleotides. Complementary oligonucleotides were employed to construct DNA adaptors. They were synthesized by an automated phosphotriester procedure, labeled at their 5' ends with polynucleotide kinase and [^{32}P]γATP, purified by preparative 20% polyacrylamide gel electrophoresis, eluted into H_2O, and annealed under the conditions described in ref. 21.

Plasmids. pMN152 was constructed from pBR322; it contains the Okayama-Berg SV40 tailing linker and a rat liver cDNA insert encoding the entire precursor polypeptide of ornithine carbamyl transferase. Details are given in refs. 2 and 11. The bacterial expression vector, pKK223-3, is described in ref. 23; it was purchased from Pharmacia.

Coupled Transcription/Translation in vitro. A prokaryotic DNA-directed translation system, containing *E. coli* S30 and supplemented with [^{35}S]methionine, was purchased from Amersham and employed according to the manufacturer's instructions. When programmed with pOX1, the system routinely yielded ~ 4 x 10^4 cpm/μl incorporated into trichloracetic acid insoluble product.

Import and Processing of pOCT by Rat Heart Mitochondria in vitro. [^{35}S]-labeled translation products, present either in a rat liver mRNA-directed reticulocyte lysate system (40 μl) or in a pOX1-directed *E. coli* S30 system (20 μl), were incubated at 30° for 60 min in a total volume of 120 μl containing rat heart mitochondria (60 μg protein, purified according to ref. 12), 0.25M sucrose, 40 mM KCl, 1.0 mM Mg acetate, 0.5 mM ATP, 10 μg/ml cycloheximide, 10 mM Hepes, pH 7.5, 0.25 mM dithiothreitol, 2.5 mM Na succinate, 0.04 mM ADP, and 1.0 mM K_2HPO_4, pH 7.5 (12,14). Radioactive products were precipitated with

antibody against ornithine carbamyl transferase (6,12) and analyzed by SDS-PAGE and fluorography. The import system employed in this study is a heterologous one, in which the source of purified mitochondria was heart tissue. Heart does not express ornithine carbamyl transferase. In all cases, identical results were obtained using purified rat liver mitochondria.

RESULTS

Construction of a Recombinant pKK223-3 Vector Expressing Rat Liver pOCT.

pMN152 carries a 1.6 kb cDNA insert which spans the entire rat liver pOCT coding region plus 110 bp of 5' and 360 bp of 3' non-translated sequences, and a polydA stretch of undetermined length (11). As outlined in Fig. 1, pMN152 was modified to render its pOCT cDNA insert suitable for cloning into pKK223-3; an oligonucleotide adaptor molecule was employed to replace the pOCT 5'-untranslated

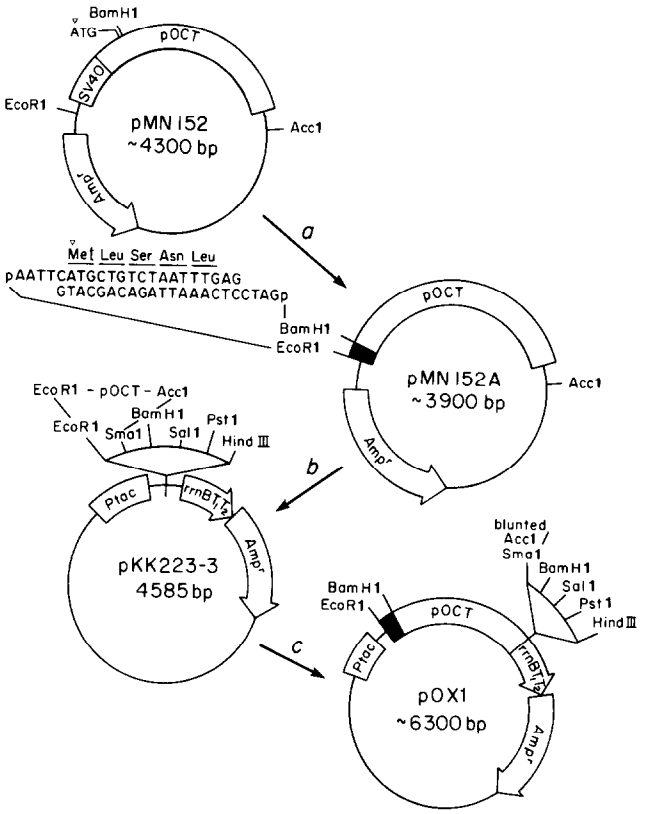


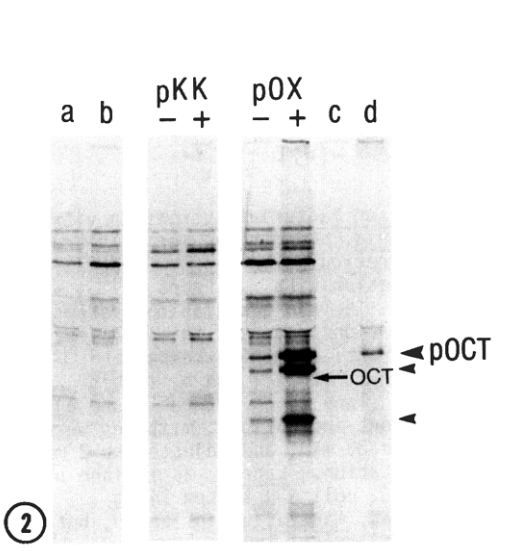
Figure 1. Construction of pOX1. Step a. Following digestion of pMN152 at unique EcoR1 and BamH1 sites, the larger fragment was purified electrophoretically and ligated to a synthetic 21-mer oligonucleotide adaptor, resulting in formation of a unique EcoR1 site followed by an in-frame reconstruction of codons specifying the amino terminus of pOCT. Step b. pMN152A was linearized with Acc1, blunt-ended using the Klenow fragment of DNA polymerase I in the presence of deoxynucleoside triphosphates, and further digested with EcoR1. Step c, the 1.8 kb (5')EcoR1/blunted Acc1(3') fragment generated in step b was purified and inserted between the EcoR1 and Sma1 sites in the polylinker of pKK223-3. The positons of the tac promoter (Ptac) and sequences specifying transcriptional termination ($rrnBT_1T_2$) are indicated.

sequence with an EcoRI sticky-end placed immediately in front of the pOCT translational start codon. The modified pOCT cDNA insert was recovered and inserted between the EcoR1 and Sma1 sites in the polylinker of pKK223-3, thereby bringing the pOCT translational start codon 10 nucleotides downstream of the hybrid promoter, tac (22,23).

The recombinant pKK223-3 plasmid was designated pOX1. To confirm that pOX1 was capable of expressing pOCT, the plasmid was employed to transform E. coli strain JM105. When transformed cells were incubated in the presence of IPTG and [^{35}S]methionine, synthesis was induced of a radioactive polypeptide which reacted with antibody against rat liver ornithine carbamyl transferase and comigrated in SDS-polyacrylamide gels with authentic rat liver pOCT (M_r = 40,000) (Fig. 2); the polypeptide was absent in JM105 cells transformed with pKK223-3 and was reduced by 90% in non-induced (-IPTG) pOX1- transformed cells (Fig. 2).

In addition to full-size pOCT, JM105 cells transformed with pOX1 and induced with IPTG also contained relatively high levels of two smaller products (37 kDa and 28 kDa) that reacted with anti-ornithine carbamyl transferase (Fig. 2); we assume that these presumptive pOCT polypeptide fragments arose either from premature translational termination or from proteolysis. The leader sequence of rat liver pOCT is particularly sensitive to trypsin-like digestion (unpublished), presumably because of its high content of lys and arg residues; this could explain the origin of the 37 kDa product. Importantly, however, pOX1-transformed JM105 cells did not produce a polypeptide corresponding to mature enzyme (Fig. 2).

Coupled Transcription/Translation of pOX1 In Vitro. Transport of precursor proteins into mitochondria, and subsequent removal of the mitochondrial signal peptide, are most widely studied by reconstitution in vitro, in which eukaryotic mRNA translation systems (usually reticulocyte lysate) are mixed with purified mitochondria following completion of protein synthesis. Because E. coli would not be expected to contain a compartment equivalent to either the outer or inner membrane of mitochondria, tests for functional expression of pOCT by bacteria must necessarily entail tests for the ability of the precursor to be delivered into mitochondria in vitro. In order to compare import of bacterial pOCT with



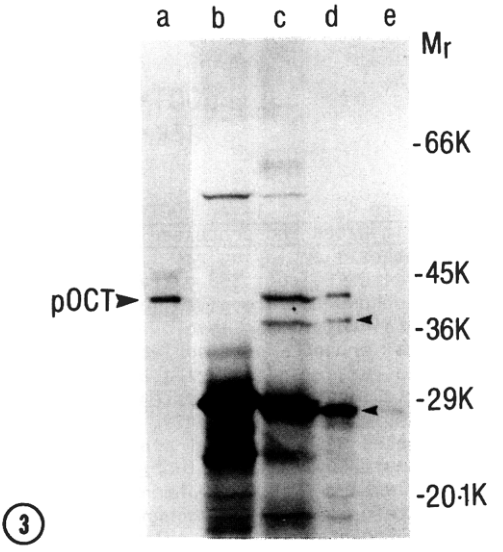


Figure 2. Expression of pOCT in pOX1-transformed E. coli JM105 cells. One-ml cultures of JM105 were transformed with pKK223-3 or pOX1 (21), grown to 0.6 A_{550} at 37° in M9CA medium containing 50 μg/ml ampicillin, maintained overnight in the presence of chloramphenicol (68 μg/ml), recovered by centrifugation, washed twice in M9 salts to remove chloramphenicol, and resuspended in M9 salts containing 0.5X Difco methionine assay mix, 50 μg/ml ampicillin, and 25 μCi/ml [^{35}S]methionine (>1000 Ci/mmol, NEN Corp.), either with (+) or without (-) IPTG (5 mM), and incubated for 2 hours. Untransformed JM105 cells were treated in the same way, but in the absence of ampicillin and lacking the chloramphenicol treatment. Cells were recovered by centrifugation, dissolved in 50 μl boiling 2% (w/v) SDS, diluted to 1 ml with ice-cold medium containing 1% (w/v) Triton X-100, 1 mM phenylmethylsulfonylfluoride, 10 mM ethylenediaminetetraacetate, 20 mM methionine, and phosphate buffered saline, and adjusted to 1.0 M NaCl from a 4.0 M stock. Immunoprecipitation was carried out with anti-ornithine carbamyl transferase IgG and precipitates were subjected to SDS-PAGE and fluorography, exactly as described (12). Incorporation of [^{35}S]methionine into polypeptide products was similar for each culture (2 x 10^6 cpm). Lane a, untransformed cells, minus IPTG; lane b, untransformed cells, plus IPTG; lanes pKK, cells transformed with pKK223-3, minus (-) or plus (+) IPTG induction; lanes pOX, cells transformed with pOX1, minus (-) or plus (+) IPTG induction; lanes c and d, rat liver mRNA-directed rabbit reticulocyte lysate translation products precipitated with pre-immune (lane c) or anti-ornithine carbamyl transferase (lane d). The positions of pOCT (40 kDa) and mature monomeric enzyme (36 kDa) are indicated. Unlabelled arrowheads denote two immunoreactive polypeptides, 37 kDa and 28 kDa in size, whose synthesis was induced by IPTG. Numerous polypeptide products, whose synthesis was unaffected by IPTG, reacted with anti-ornithine carbamyl transferase; they arise because antibody production in rabbits had been potentiated with attenuated bacteria. OCT, ornithine carbamyl transferase; pOCT, precursor to OCT.

Figure 3. Coupled transcription/translation of pOX1 in vitro. Plasmid DNA was purified by CsCl density gradient centrifugation and transcribed and translated in vitro in the presence of [^{35}S]methionine. Radioactive products were analyzed by SDS-PAGE and fluorography. Lane b, total products made from pKK223-3 DNA; lane c, total products made from pOX1 DNA; lane d, approximately half the products shown in lane c were subjected to immunoprecipitation with anti-ornithine carbamyl transferase IgG; lane e, as in lane d, but using pre-immune IgG. Lane a, shows rat liver pOCT synthesized in a rabbit reticulocyte lysate system. Small arrowheads denote 37 kDa and 28 kDa products precipitated by anti-ornithine carbamyl transferase. The positions of standard molecular weight markers are indicated.

standard animal systems, coupled transcription-translation was carried out, employing an E. coli S30 extract programmed with pOX1. Fig. 3 shows that full-

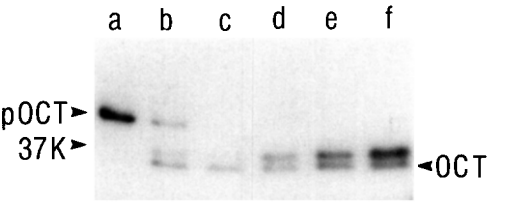


Figure 4. Rat liver pOCT synthesized in mammalian and bacterial systems in vitro is imported and processed by purified rat mitochondria. Conditions for in vitro synthesis employing rat liver mRNA-directed rabbit reticulocyte lysates or pOX1-directed E. coli S30 extracts, and subsequent incubation with rat heart mitochondria, are given in Experimental Procedures. The two translation systems contained similar levels of radioactive pOCT translation product. In all cases, total products were subjected to precipitation with anti-ornithine carbamyl transferase and analyzed by SDS-PAGE and fluorography. Lanes a and b, rabbit reticulocyte lysate system (25 mg protein/ml), with (lane b) or without (lane a) addition of mitochondria. Lanes c-f, following import incubations, mixtures were treated with 150 μg/ml proteinase K, kept on ice for 30 min, and adjusted to 2 mM phenylmethylsulfonylfluoride prior to immunoprecipitation. Lane c, as in lane b but with protease treatment; lane d, pOXI-directed E. coli S30 system (4 mg protein/ml), plus mitochondria and protease treatment; lane e, as in lane d, but supplemented with reticulocyte lysate (20 μl) during import; lane f, as in lane d, but import incubations were supplemented with 20 μl E. coli S30. Precursor (pOCT) and processed (OCT) products are indicated; 37K denotes a 37kDa breakdown product of pOCT (12).

size pOCT was made with relatively good efficiency; additionally, however, the two smaller products (37 kDa and 28 kDa) that had been detected in vivo (Fig. 2) also appeared in vitro (Fig. 3).

pOX1-programmed E. coli S30 Supports Import and Processing of pOCT by Purified Rat Heart Mitochondria. Following transcription/translation of pOX1, the E. coli S30 translation system was added to purified mitochondria from rat heart, employing conditions that closely approximate those that support in vitro import of pOCT in a rabbit reticulocyte lysate translational system (Fig. 4). Fig. 4 demonstrates that E. coli S30 programmed with pOX1 supports import and processing of pOCT in a manner very similar to pOCT made in a rat liver mRNA-directed reticulocyte lysate system. Import was monitored by conversion of pOCT from a protease-sensitive form (cf. lanes b and c) to a product which became insensitive to added protease and which comigrated in SDS gels with mature enzyme (lane d). Addition of extra reticulocyte lysate (lane e) or E. coli S30 (lane f) resulted in a modest stimulation of pOCT import and processing.

In both bacterial and reticulocyte lysate import systems, variable amounts of a ~ 37 kDa pOCT conversion product were observed in addition to bona fide processed polypeptide. However, like the ~ 37 kDa pOX1-translation product

observed both *in vivo* (Fig. 2) and *in vitro* (Fig. 3), it may have arisen by non-specific events (see also ref. 12). The level of 37 kDa product relative to OCT varied from experiment to experiment (not shown).

DISCUSSION

We have described the construction of a recombinant plasmid, pOX1, which expresses rat liver pOCT under the control of the *tac* promoter to produce a functionally active precursor polypeptide, as demonstrated by coupled transcription/translation of pOX1 in an *E. coli* S30 extract followed by uptake and processing of the primary pOCT translation product upon addition of purified rat mitochondria. Of particular interest, however, was the finding that a bacterial system supported import and processing of pOX1-produced pOCT. Earlier reports had demonstrated that import of precursor proteins, including pOCT, by mitochondria *in vitro* shows an absolute dependence on the presence of cytosolic fractions from reticulocyte (12-16) or yeast (16) cells, the suggestion being that cytosolic import factors may play a defined and specific role in targeting proteins to the organelle, perhaps analogous to the role of SRP in delivering nascent proteins to the endoplasmic reticulum (17). The fact that *E. coli* S30 extracts also support *in vitro* import of pOCT, however, raises doubts concerning such a conclusion. The trivial explanation that cytosolic import factors were being introduced adventitiously into the bacterial import system upon addition of rat heart mitochondria is exceedingly unlikely since previous studies (14) showed that purified mitochondria are free of import factors and, moreover, do not themselves bind factors from exogenous sources (e.g., reticulocyte lysate). The present results, therefore, indicate that cytosolic import factors, or their functional equivalents, are present in bacterial as well as eukaryotic cells. The fact that a bacterial system can be engineered to produce functionally-active mitochondrial precursor protein should prove useful for a variety of studies, including those involving the use of pOCT as an affinity matrix to bind and purify components of the mitochondrial import apparatus.

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