Expression of Amplified DNA Sequences
for Ornithine Transcarbamylase in HeLa Cells:
Arginine Residues May Be Required for
Mitochondrial Import of Enzyme Precursor

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ABSTRACT Expression of ornithine transcarbamylase (OTC), a nuclear-coded mitochondrial
enzyme, was programmed in HeLa cells by the use of a strategy of gene co-amplification.
HeLa cells, ordinarily devoid of OTC activity, were transfected with a plasmid containing viral
regulatory elements joined with two cDNA sequences, one encoding the human OTC
precursor and a second encoding a mutant mouse dihydrofolate reductase. After transfection
and selection in increasing concentrations of methotrexate, several hundred copies per cell
of the sequence encoding OTC were detected by blot analysis. Immunoprecipitation of
extracts of radiolabeled cells with anti-OTC antiserum revealed newly synthesized mature
OTC subunits. Furthermore, OTC enzymatic activity in cell extracts was comparable to that
of control human liver, and mitochondrial localization of OTC was demonstrated by immu-
nofluorescence. When we incubated transfected HeLa cells with dinitrophenol, a known
inhibitor of mitochondrial import, the only form of newly synthesized OTC detected was the
precursor. We estimated the rate of import of precursor by performing an inhibitor-free chase;
precursor was converted to mature subunit with a half-life of less than two minutes. When a
HeLa transformant was incubated with the arginine analogue canavanine, the major form of
newly synthesized OTC detected was a species migrating slightly more slowly than the normal
precursor; little mature-sized subunit was recovered. This indicates that substitution of the
analogue for arginine in the OTC precursor interferes with mitochondrial import and process-
ingar. Thus, arginine residues in the OTC precursor—most likely the four residues contained in
its NH₂-terminal leader sequence—probably play an important role in mitochondrial import
and/or processing.

The vast majority of mammalian mitochondrial proteins are
encoded in the nucleus, translated as larger precursors on free
cytoplasmic polyribosomes, and posttranslationally imported
by mitochondria. Import requires specific recognition by the
mitochondria, translocation across one or both mitochondrial
membranes, and proteolytic removal of NH₂-terminal leader
sequences to produce the mature protein. A number of details
concerning these steps have been elucidated during the past
few years: recognition involves outer membrane receptors;
translocation across the inner membrane requires an intact
electrochemical gradient; and proteolytic cleavage is catalyzed
by a divalent cation-dependent matrix protease (for review
see reference 1). However, a large number of biochemical and
cell physiological questions remain unanswered. Precursors
incubated with isolated mitochondria are imported, whereas
the corresponding mature proteins fail either to be taken up
or to interfere with uptake of the precursors (1). This indicates
that leader sequences are required for recognition. How do
these sequences function? Do they contain specific “address”
information?
One approach to answering these questions is to analyze the structure of the leader sequences. Recently we reported the complete amino acid sequence of the precursor of a human hepatic mitochondrial matrix enzyme, ornithine transcarbamylase (OTC) (2). We compared the leader sequence of OTC with the sequences of those leaders analyzed to date from precursors of mitochondrial enzymes from yeast (3–5), Neurospora (6), and rat (7), and failed to find any shared sequence. However, several features of amino acid composition are shared: the leaders do not contain stretches of hydrophobic residues; they are devoid of acidic residues; and they contain an average number of basic residues when compared with eukaryotic proteins. In particular, the OTC leader contains four arginine residues in a peptide of 32 residues. Because the overall amino acid composition of the leaders is basic, they are predicted to be positively charged (2).

To define further the characteristics of mitochondrial import, we sought to establish a cultured cell system expressing OTC. Such a system had not been available because the intact liver is usually the only site of major OTC expression; cell lines derived from either hepatic tumors or normal liver are essentially devoid of enzyme activity. We previously achieved low levels of OTC activity in HeLa cells after transfection with a plasmid programming expression of the cloned cDNA (2). Here we describe high-level expression of OTC in HeLa cells, with enzyme activity similar to that of intact liver. To achieve this, we used a strategy of gene amplification proposed by Simonsen et al. (8), in which sequences programming expression of the OTC product were co-amplified with sequences programming expression of a dominant, selectable marker, a mutant dihydrofolate reductase (DHFR).

MATERIALS AND METHODS

Recombinant DNAs, DNA-mediated Gene Transfer, and DNA Hybridization Analysis: We prepared recombinant DNAs using previously described methods of restriction endonuclease digestion, purification of DNA fragments, joining of DNA fragments, bacterial transformation, and colony screening (2). Plasmid DNA was transferred into cultured cells by calcium-phosphate co-precipitation (9, 10). Preparation of high molecular weight DNA, agarose gel electrophoresis, transfer of DNA, and hybridization with a nick-translated plasmid were performed as described elsewhere (11).

Transfection and Selection: HeLa cells were grown in minimal essential medium with Earle's salts (MEME) containing 10% fetal calf serum. Two different transfection protocols were carried out, each using the calcium phosphate-mediated procedure of Wigler et al. (10). In one, HeLa cells were co-transfected with two plasmids, one encoding OTC and the other encoding the bacterial gene for neomycin resistance. Selection was carried out by addition of G418 (Geneticin, Gibco Laboratories, Grand Island, NY) to the medium at a concentration of 400 μg/ml (12). In the second experiment, cells were transfected with a single plasmid that encodes both OTC and a mutant form of DHFR. Selection was carried out by addition of 4 μg/ml folic acid and 0.2 μM methotrexate (added from a stock solution, 25 mg/ml, kindly supplied by Dr. K. Yen of Lederle Laboratories, Inc., Milwaukee, WI) (8). After initial selection, six transfecants were grown in progressively higher concentrations of methotrexate added to the same medium (see Results).

Inhibitors and Amino Acid Analogues: In pulse-chase experiments, 4 mM dinitrophenol (DNP) was added to the medium one-half hour before the labeling period and was present during radiolabeling and washing in nonradiolabeled medium, but not during the chase period (13). Rhodamine 6G (R6G) was used as previously described (2). Canavanine incorporation studies were performed as follows. Cells grown to 70% confluence in 60-mm dishes were rinsed twice with phosphate-buffered saline (PBS), then 2 ml of arginine and methionine-deficient MEME (Select-Amine Kit, Gibco Laboratories) was added. The cells were incubated at 37°C for 1 h, and canavanine (L-canavanine sulfate, Sigma Chemical Co., St. Louis, MO) was then added to the desired concentration. After 20 min, the medium was replaced with fresh arginine/methionine-deficient medium containing the same concentration of canavanine and 10 μCi/ml [3H]sulfate (0.2 μCi, 12,000 cpm) for 1 h, and followed by harvest of the transfected cells. L-Ethionine and 3-fluoro-phenylalanine (Sigma Chemical Co.), amino acid analogues of methionine and phenylalanine, respectively, were used similarly. Competition with the corresponding natural amino acid was verified by parallel incubations in which total incorporation of the labeled natural amino acid into TCA-precipitable products was measured in the presence and absence of the analogue. In all cases, incorporation in the presence of analogue was <5% of that in its absence.

Immunoprecipitation: Procedures for radiolabeling cultured cells and for harvesting, immunoprecipitation, and SDS PAGE have been described previously (13).

RESULTS

High-level Expression of OTC in HeLa Cells

We wished to achieve high-level expression of the precursor of OTC through co-amplification of a sequence programming its expression with that of a mutant methotrexate-resistant DHFR (8). The mutant DHFR has a greater Ki for the normal enzyme for the folate antagonist, methotrexate, and therefore serves as a dominant selectable marker, permitting growth of transformants that express this sequence in concentrations of methotrexate that kill nontransformed cells. Exposure of methotrexate-resistant transformants to increasingly higher concentrations of methotrexate permits selection of transformants that may contain several hundred copies of the sequences expressing the mutant DHFR (8). To increase the likelihood that OTC sequences would be included in the amplified unit, the sequences programming OTC expression were physically joined with those programming expression of the mutant DHFR as shown in Fig. 1. Plasmid pFR 400 (kindly provided by C. Simonsen, Genentech Inc., San Francisco, CA) contains a cDNA sequence encoding the mutant DHFR, joined upstream with the SV40 early promoter and enhancer sequence and downstream with hepatitis B virus sequences encoding a polyadenylation signal. The sequences that program expression of the mutant DHFR were inserted at the unique Eco RI site of pSV2OTC, a plasmid that programs expression of cDNA for the OTC precursor from SV40 regulatory elements (2). The two derived plasmids, pOD and pODa, contained head-to-tail and head-to-head orientations of the expression segments, respectively.
these, 18 contained detectable activity; no activity was mea-

plasmids. 20 were analyzed for OTC enzymatic activity. Of

mant clones were identified in equal numbers for the two

after transfection, selection was started by the addition of

methotrexate to the culture medium at a concentration of

for expression of both DHFR and OTC eDNA sequences,

viral regulatory sequences. The plasmid pFR400 (8) was kindly

hGURE 1 Construction of plasmids containing cDNA sequences

encoding both the OTC precursor and a mutant DHFR joined with

viral regulatory sequences. The plasmid pFR400 (8) was kindly

supplied by Christian Simonsen. The DNA fragment containing
cDNA coding for a mutant DHFR is shown by the open bar, the
direction of transcription by an arrow. The DHFR sequence is joined
at its upstream terminus with SV40 sequences and at its downstream
terminus with regulatory sequences from hepatitis B virus, shown by
dark bars. The Pvu II-Sal I fragment containing the DHFR cDNA
and regulatory sequences was purified and treated with Klenow
fragment of Polymerase I to produce flush termini. The fragment
was joined with plasmid pSV2OTC, containing a human OTC cDNA
sequence, shown by the open bar, and adjoining SV40 regulatory
elements, shown by dark bars. pSV2OTC was prepared for the
joining reaction by digestion with Eco RI, incubation with bacterial
alkaline phosphatase, and treatment with Klenow fragment of Poly-
merase I. Blunt-ended joining allows two possible orientations of
the cDNA sequences relative to each other. Recombinant plasmids
with both orientations were identified and the plasmids were desig-
nated pOD. (OTC-DHFR head-to-tail) and pODb (OTC-DHFR
head-to-head). Loss of Eco RI recognition sequences at the sites of
joining is indicated by X marks. ORI, SV40 origin of replication; RI,
Eco RI; BAP, bacterial alkaline phosphatase.

The OTC-DHFR plasmids were introduced into HeLa cells
by calcium phosphate co-precipitation. HeLa cells were cho-

sured in untransformed HeLa cells. The six transformants
(three pOD, three pODb) with the greatest OTC activity were
subjected to stepwise increases of methotrexate concentration,
first to 10 then to 500 μM.

Five transformants survived selection in 500 μM metho-
trexate and were maintained in this concentration of metho-
trexate. The lines were designated OD cell lines because they
contain both OTC and DHFR sequences. The lines were ana-
alyzed for the presence of immunoprecipitable OTC, for
OTC enzymatic activity, and for copy number and arrange-
ment of OTC DNA sequences. The results of immunoprecip-
itation of extracts of radiolabeled OD cell lines with anti-OTC
antisera are shown in Fig. 2. All five lines contained precipi-
table product that co-migrated in SDS PAGE with the mature
rat OTC subunit. The intensity of this band was significantly
greater than that found upon analysis of a similar extract
from the cell line D6, a stable transformant derived after
cotransfection of HeLa cells with two separate plasmids,
pSV2Neo and pSV2OTC. (The D6 line contained the greatest
amount of OTC enzymatic activity of 30 stable G418-resistant
cotransformants.) The OD lines also contained a much less
intensely labeled immunoprecipitable product that co-mi-
grated with the rat OTC precursor (Fig. 2). The latter is
presumed to be newly synthesized precursor that has not been
processed to the mature form.

The results of assay of the OD cell lines for OTC enzymatic
activity are shown in Table I along with the results obtained
for two OTC-Neo co-transformants, untransformed HeLa
cells, and control human liver. As mentioned above, HeLa
cells contain no detectable activity. The OTC-Neo cotrans-
formant, D6, contains ~5% of the activity found in liver. The
OD lines (OD1, OD2, OD5) have activities an order of
magnitude greater than that of D6 and comparable to the
activity found in human liver. The line designated ODS
contained the greatest amount of activity, at the upper limit
of the range of activity of human liver.

OTC Sequences in HeLa Cell Transformants

To analyze the DNA sequences encoding OTC in the HeLa
transformants, high molecular weight DNA was prepared from the
lines initially during growth in 0.2 μM methotrexate

FIGURE 2 OTC expression in stable HeLa cell transform-
ants. Stable HeLa cell transform-
ants, derived either from
cotransfection with pSV2OTC and pSV2Neo (D6) or from
cotransfection with pOD and pODb (OD 1, 2, 3, 5, 6), were
grown to near confluence in 60-mm dishes in MEME +
G418 (line D6) or MEME +
folate + 500 μM methotrexate
(OD transformants 1, 2, 3, 5,
6). The growth medium was replaced by labeling medium and the
cells were incubated for 1 h with [35S]methionine. The monolayers
were washed and harvested by detergent lysis, and the extracts
were immunoprecipitated with anti-OTC antiserum. The products
were electrophoresed through an SDS-polyacrylamide gel, and
the gel was fluorographed. Portions of the same fluorogram are shown;
exposure times were the same for all lanes. pOTC, OTC precursor;
OTC, mature OTC subunit.
and again after selection in 500 µM methotrexate. DNA was
digested with Bam HI, electrophoresed through a 0.8% agarose
gel, transferred to nitrocellulose by the method of Southern (16),
and probed with a nick-translated plasmid, pH0731,
containing a nearly full-length OTC cDNA joined with
pBR322. Two Bam HI sites are present in the input plasmids
pODf and pODb—one in an SV40 regulatory segment (see
Fig. 1) and the other in the OTC leader coding sequence (site
not shown in Fig. 1). Cleavage of the plasmid DNA at these
sites produces a fragment of 2.2 kilobases (kb), containing
nearly all of the OTC sequences, and a fragment of 4.0 kb,
containing the DHFR sequences, pBR322 sequences, and the
5' terminal OTC sequences (40 base pairs). Analysis of all
five OD cell lines, shown in Fig. 3, revealed a 2.2-kb hybrid-
zizing fragment that probably corresponds to the OTC-bearing
fragment of the input plasmids. The intensity of this band in
tracks containing DNA from cells grown in 0.2 µM methotrexate
is greater than that of the two high molecular weight bands in
the nontransfected HeLa track characteristic of the normal
X chromosome OTC locus (11), indicating the pres-
ence of multiple OTC sequences even at the initial methotrexate
concentration used for selection. After selection in 500
µM methotrexate, the intensity of hybridization of the 2.2-kb
fragment from OD1, OD2, and OD6 is about two orders of
magnitude greater than observed at 0.2 µM methotrexate,
which suggests a corresponding increase in copy number of
OTC sequences in these lines. The source of additional hy-
broidizing bands in the OD lines is unclear, although the single
additional band observed for the OD5 line corresponds pre-
cisely to the 4.0-kb Bam HI fragment of the pOD plasmids
and suggests that, in this line, the multiple copies of plasmid
dNA have assumed a simple head-to-tail configuration.

Analysis of DNA from the OTC-Neo transformant D6 also
reveals a 2.2-kb hybridizing fragment, probably corresponding
to the OTC-bearing Bam HI fragment present in the input
plasmid pSV2OTC (see Fig. 1). The intensity of hybridization
of this fragment is well above single copy (compare HeLa
lane in Fig. 3) and is similar to that of OD lines 3 and 6
selected in 500 µM methotrexate, yet, curiously, OTC enzym-
atic activity in D6 is an order of magnitude less. Both the
D6 and OD5 lines were subjected to karyotype analysis, which
detected a hypotetraploid chromosome complement but
failed to detect either a homogeneously staining region or
double minute chromosomes.

**Localization of OTC in OD5 Cells**

To demonstrate directly the presence of OTC in the mito-
chondria of a transformant, cells from the OD5 line were
fixed, permeabilized, and incubated with monospecific rabbit
anti-OTC antiserum, then fluorescein isothiocyanate-conju-
gated goat anti-rabbit IgG antiserum was added. Fig. 4 shows
the results of this study. A threadlike pattern of fluorescence
is visible, characteristic of the morphologic pattern of mito-
chondria in HeLa cells (see below). Virtually all of the cells
in the population were positive for OTC expression by this
assay. No fluorescent pattern was detected when the procedure
was carried out either with nontransformed HeLa cells or
with normal rabbit serum instead of anti-OTC antiserum (not
shown). However, when either HeLa cells or OD5 cells were
incubated with R6G, a fluorescent dye that binds selectively
to mitochondrial membranes (14), we observed a fluorescent
pattern identical to that obtained for OD5 cells incubated
with anti-OTC antiserum (not shown).

**Rate of Import of OTC by Mitochondria**

The establishment of cultured cell lines that express OTC
affords the possibility of estimating the rate of import by
mitochondria of cytoplasmically synthesized OTC precursor.
Such an analysis relies on proteolytic cleavage of the precursor
polypeptide by one or more proteases localized to the matrix
space (17) as an indication of entry of the OTC subunit. To
permit this analysis of import, we synthesized radiolabeled
precursor by metabolically labeling the cells with [35S]methi-
onine in the presence of DNP, an uncoupler of oxidative
phosphorylation, which has been demonstrated to inhibit the
import of the OTC precursor (17). After labeling was done in
the presence of DNP, [35S]methionine and DNP were washed
from the cells and incubation was carried out in nonlabeled

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Table I

| OTC Enzymatic Activity in HeLa Transformants and Human Liver | U*/mg protein |
|-------------------------------------------------------------|--------------|
| HeLa                                                        | <0.2         |
| Neo co-transformants                                        |              |
| A1                                                          | 0.6          |
| D6                                                          | 1.4          |
| Mtx4 DHFR co-transformants                                 |              |
| OD-1                                                       | 29.7         |
| OD-2                                                       | 25.3         |
| OD-3                                                       | 12.3         |
| OD-5                                                       | 38.3         |
| OD-6                                                       | 9.2          |
| Human liver                                                | 30 (20–40 range) |

Cell-free extracts of HeLa cell transformants (one or two confluent 10-cm dishes) and liver tissue were prepared as in Materials and Methods and assayed for OTC enzymatic activity by the method of Kalousek et al. (15). Activity is expressed as micromoles of citrulline produced per hour per milligram extract protein.

* U, micromoles citrulline per hour.
FIGURE 4 Localization of OTC in OD5 cells by indirect immunofluorescence. OD5 cells were grown on glass coverslips, fixed, permeabilized in methanol, and stained for OTC by the use of a rabbit anti-rat OTC antiserum. In some cases, the fluorescent organelles correspond to structures seen by phase-contrast microscopy (right). See text for details. × 360.

medium for various times. The results of immunoprecipitation of the extracts of the harvested cells are shown in Fig. 5. In the absence of DNP, only the mature OTC subunit is detected, whereas in its presence only the precursor is detected. Within 2 min of release from DNP inhibition, more than half of the radiolabeled precursor has been converted to the mature subunit. By 6 min nearly all precipitable material corresponds to the mature subunit. Thus, the \( t_{1/2} \) for import and cleavage in this system is estimated to be <2 min.

**Effect of Canavanine on Import of OTC in OD5 Cells**

The establishment of cultured cells that express and import the OTC precursor permits a direct analysis of the amino acid residues required for import by substituting amino acid analogues in the OTC precursor for the corresponding natural amino acids. We have previously noted that the leader sequences of those mitochondrial precursors analyzed to date share an overall basic composition. In the OTC leader sequence below, all four basic residues are arginines (2):

met leu phe asn leu arg ile leu leu asn asn ala ala phe arg asn gly his asn phe met val arg asn phe arg cys gly gln pro leu gln ↓.

Given the possibility that these arginine residues play an essential role in mitochondrial import, the effect of substitution of these residues, as well as of eight arginine residues in the mature portion of the precursor, by the analogue canavanine, was examined.

The results of such an experiment are shown in Fig. 6. OD5 cells were incubated for 20 min with arginine-free medium supplemented with 2 mM canavanine. The medium was replaced with fresh canavanine-supplemented, arginine-free medium containing \(^{35}\)S methionine, and incubation was carried out for 1 h and followed by harvest and immunoprecipitation with anti-OTC antiserum. A control radiolabeled culture incubated in arginine-containing medium contained only the mature OTC subunit (Fig. 6A, lane 1). When the mito-

FIGURE 5 Time course of mitochondrial import and processing of OTC. Cultures of the stable transformant (D6) were grown to confluence in 60-mm dishes in MEME. The monolayers were washed, incubated with 4 mM DNP, and labeled with \(^{35}\)S methionine for 1 h. Then the labeling medium was removed and replaced with MEME without DNP. At each indicated time, one plate of cells was harvested by detergent lysis, and the labeled OTC products were recovered by immunoprecipitation. The products of immunoprecipitation were separated by polyacrylamide gel electrophoresis and fluorographed. The first lane (−DNP) shows the product obtained when both the labeling and the chase were performed in the absence of DNP.

FIGURE 6 Effect of canavanine on mitochondrial import and processing of OTC. The OTC-DHFR cell transformant OD5 was grown to near confluence in 60-mm dishes in MEME + folate + 500 μM methotrexate. Cells were radiolabeled with \(^{35}\)S methionine in the absence or presence of canavanine and/or R6G. Monolayers were washed, harvested by detergent lysis, the OTC products immunoprecipitated and electrophoresed, and the gel fluorographed. Portions of the same fluorogram are shown. (A) Extract of cells radiolabeled under control conditions (lane 1); in the presence of 1 μg/ml R6G (lane 2); in 2 mM 1-canavanine (lane 3); in 1 μg/ml R6G and 2 mM canavanine (lane 4); in 2 mM canavanine and 0.6 mM arginine (lane 5). (B) Extracts of cells radiolabeled in the presence of 0 mM (lane 1), 0.2 mM (lane 2), 2 mM (lane 3), and 10 mM canavanine (lane 4).
The mitochondrial import inhibitor, R6G, was added to the control culture, only the OTC precursor was observed (Fig. 6A, lane 2). In the presence of 2 mM canavanine, two precipitable products were detected (Fig. 6A, lane 3), a major one migrating slightly more slowly than the normal precursor, and a minor one migrating slightly more slowly than the mature subunit from untreated cells. The altered mobilities of these OTC products show that canavanine has been incorporated into them. The major product appears to be an altered form of the OTC precursor. This is supported by the observation that when R6G is added to the canavanine-containing medium only this OTC product is detected (Fig. 6A, lane 4). The minor OTC product, which disappears in the presence of R6G, is a product of proteolytic processing by the mitochondria. Whether this cleavage product is an altered mature form of OTC or an intermediate form resembling that previously reported by our group and others cannot be distinguished.

To demonstrate further that canavanine is incorporated into the OTC precursor, we performed two additional experiments. In one, arginine was added to the culture medium at the same time as canavanine (Fig. 6A, lane 5). Under these conditions only the mature form of OTC was detected, which indicates successful competition by arginine for incorporation into the OTC precursor. In the second, the effect of adding varying concentrations of canavanine was determined. At a canavanine concentration of 0.2 mM (Fig. 6B, lane 2), four products were detected. We propose that, at 0.2 mM, canavanine competes only partially with endogenous arginine for incorporation into the precursor. As a result, the precursor is canavanine substituted to a variable extent. The least substituted precursor is converted to mature OTC, whereas the most completely substituted appears as an altered precursor. The nature of the two forms that are intermediate in mobility is unclear. In agreement with this interpretation, the normalized precursor and mature products are reduced at 2 mM canavanine concentration and are totally absent at 10 mM concentration (Fig. 6B, lanes 3 and 4).

The effect of canavanine upon import of the OTC precursor was not reversed by the subsequent removal of the analogue from the culture medium and replacement with arginine. When cells were radiolabeled in the presence of canavanine, and a cold chase in arginine-containing medium was then performed, no new products were observed and the half-life of the observed products was estimated to be 40 min. In another experiment designed to test whether the mitochondrial import system is functional after incubation in canavanine, cells were first incubated with canavanine and were then radiolabeled in medium in which canavanine was replaced by arginine. At all points examined, mature OTC was the only product detected (data not shown).

DISCUSSION

High-level Expression of the OTC Precursor in HeLa Cells

The results presented above have described the high-level expression of a mammalian mitochondrial precursor in cultured HeLa cells. This was achieved through co-amplification of a sequence that programmed expression of the precursor with a sequence that programmed a dominant selectable marker. Physical linkage of the two sequences in the input plasmid probably contributed to the uniformly observed amplification of the nonselected sequences. The derived cell lines contained several hundred copies of these sequences, which had been joined in the input plasmid with a promoter and enhancer sequence that function efficiently in the HeLa cell recipient. Both the high template copy number and an efficient promoter led to high-level expression of OTC products in cells that do not normally express OTC.

The OTC precursor synthesized in the derived OD cell lines is efficiently and faithfully imported by mitochondria, as supported by three lines of experimental evidence: immunoprecipitation of cell extracts reveals almost exclusively the mature form of the OTC subunit; cell extracts contain OTC enzymatic activity, which has been observed only with the assembled trimer of mature subunits; and indirect immunofluorescent analysis of the fixed cells with OTC antiserum reveals a pattern of mitochondrial fluorescence. As a reflection of the efficiency of import, only relatively small amounts of OTC precursor were detected in immunoprecipitates, and no fluorescence was detected in the cytoplasm, the site of synthesis of the precursor.

The level of synthesis of precursor could be roughly estimated by assay of the processed product, the OTC enzyme. The amount of OTC enzymatic activity detected in the OD cells is comparable to that found in normal liver, the usual site of expression; this enzymatic activity is about two orders of magnitude greater than that obtained with most OTC-Neo co-transformants previously analyzed. From analysis of total radiolabeled proteins from the OD5 cell line, we estimate OTC enzyme to comprise ~0.1% of total soluble protein (data not shown). This estimate agrees well with the figure for normal liver and also corresponds to the detected level of enzyme activity. Apparently, expression of OTC in HeLa cells at a level similar to that of liver does not saturate the pathway of mitochondrial import of these cells, because there was no observable accumulation of OTC precursor. It is interesting that the amount of DHFR in the cells is estimated at several percent of total soluble protein (data not shown). We cannot at this time explain why this product should be present at a level an order of magnitude greater than OTC.

Rate of Import of OTC in HeLa Cells

Accumulation of the OTC precursor could be observed in the presence of an inhibitor of import, DNP. Removal of the inhibitor permitted subsequent observation of formation of mature subunit as a measure of the rate of import. The t1/2 for import was estimated to be <2 min. Similar determinations have been carried out for three other mammalian mitochondrial precursors, and t1/2 values were obtained that range from 1 to 10 minutes (13, 18, 19). Differences of these measurements may reflect experimental error and do not indicate whether different rates and pathways of import are followed by different precursors. Experiments involving cold chase in the presence of DNP indicate a half-life for the cytoplasmic OTC precursor of ~30 min, significantly greater than the t1/2 for its import (data not shown). The high efficiency of mitochondrial protein transport argues strongly for the involvement of receptors, one of which has been demonstrated in the outer membrane of the mitochondria of Neurospora (20). The receptors involved must not only rapidly bind precursors but must also be able to release them rapidly to the subsequent steps in the pathway.
Inhibition of Import of OTC in O5D Cells by the Arginine Analogue Canavanine

The substitution of the amino acid analogue, canavanine, for arginine in the medium of O5D cells had a striking effect on the forms of precipitable OTC detected in these cells. When canavanine was added at a concentration of 2 mM a form of OTC was detected that is presumed, based on nearly identical mobility to the normal product and upon detection in the presence of R6G, to be a canavanine-substituted OTC precursor. A small fraction of this product has apparently been imported by mitochondria and proteolytically processed because in the presence of 2 mM canavanine a minor product was also detected that was intermediate in mobility between that of normal precursor and normal mature subunit. It is clearly a mitochondrial proteolytic product because its formation is blocked by R6G. It is unclear whether this product has been cleaved at a site other than that producing the mature subunit, or whether it is a canavanine-substituted mature subunit. In either case, most of the canavanine-substituted precursor fails to negotiate one or more steps of import and/or processing.

We have no direct evidence for which step is involved but offer several hypotheses. One possibility is that intramitochondrial proteolytic processing cannot be carried out on the substituted precursor. The arginine residue nearest the site of cleavage of the mature subunit is at position -7. Based on studies of other proteolytic enzymes, it seems unlikely that this residue is directly involved with protease recognition. It formally remains possible, however, that altered folding of the canavanine-substituted precursor obscures the site of proteolytic cleavage. The much more likely possibility, in our view, is that the canavanine-substituted precursor is inefficiently recognized by mitochondria, maybe failing to bind to an outer membrane receptor. If the canavanine-substituted precursor can be synthesized in vitro, its ability to bind to isolated rat liver mitochondria can be compared with binding of an unsubstituted OTC precursor.

At the amino acid level, the major effect of substitution of canavanine for arginine is on the charge of the synthesized polypeptide. The pKₐ of the canavanine side-chain is 6.6 as compared with 9.0 for arginine (23), which suggests that at neutral pH, the substituted residues may not exhibit as great a net positive charge as is carried at this pH by arginine residues. Thus, a net loss of positive charge in the substituted precursor may explain its failure to be efficiently imported and/or processed. Consistent with a role for charged residues, import and processing was not affected by analogue substitution of two charge-neutral residues, methionine and phenylalanine, present in both the leader and mature portion of the OTC precursor.

Given the prediction that leader sequences carry an overall positive charge, we may consider that loss of charge, brought about by canavanine substitution of all four arginine residues in the OTC leader, is responsible for inhibition of import. Recent experiments suggest that the leader itself may carry sufficient information to direct mitochondrial import (Horwich, A. L., manuscript in preparation), supporting the idea that it is substitution of leader residues per se that interferes with import. However, there are eight arginine residues in the mature OTC subunit and we cannot, at this time, exclude the possibility that the effect of canavanine results from its substitution. Site-directed mutagenesis studies will permit direct evaluation of the hypothesis that the positive charge conferred by basic residues in the leader sequence is required for import and/or proteolytic processing.

We are grateful to Christian Simonsen of Genentech Inc. (San Francisco, CA) for providing the mutant DHFR plasmid, and for helpful discussions; to Lederle Laboratories for supplying methotrexate; to Mike Watson for karyotype analyses of transformants; to Tony Hunter for helpful comments on the manuscript; and to Connie Woznick for secretarial expertise.

This work was supported by grants from the National Institutes of Health (GM32156, GM20124, GM29765, and GM33904). Dr. Firgaira was supported by a Fulbright Fellowship.

Received for publication 4 December 1984, and in revised form 11 January 1985.

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