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 immunofluorescence. 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 processing. Thus, arginine residues in the OTC precursor—most likely the four residues contained in its NH_2-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 NH_2-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)[1] (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. Ferstenberg of Lederle Laboratories Div., American Cyanamid Co., Wayne, NJ) (8). After initial selection, six transfectants 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 washes 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 [^{35}S]methionine, and incubation was continued for 1 h and followed by harvest of the cells. L-Ethionine and *p*-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).

Immunofluorescence: Cells were plated on round glass coverslips, cultured for 24–48 h in complete medium, and fixed by the use of 3% paraformaldehyde 0.05% glutaraldehyde in PBS (15 min, 22°C). Fixed cells were permeabilized in cold methanol (−20°C, 30 s), quenched in PBS containing 50 mM NH_4Cl, and incubated in a 1:100 dilution of either anti-OTC or control rabbit serum (30 min, 22°C). After being extensively washed in PBS, coverslips were incubated in a 1:40 dilution of a fluorescein-conjugated affinity purified $F(ab')_2$ goat anti-rabbit IgG (Tago Inc., Burlingame, CA) (30 min, 22°C). After coverslips were washed, they were mounted in Moviol and examined under a Zeiss microscope equipped with an epifluorescence illuminator. All antibody containing preparations were diluted in PBS containing 0.2% gelatin just before use, and centrifuged (2 min, 12,000 *g*).

For staining mitochondria with R6G (14), cells were incubated for 30 min at 37°C in medium containing R6G at a concentration of 1 μg/ml. After they were washed in PBS, coverslips were examined immediately (without fixation) under a Zeiss 40X water immersion lens.

Enzyme Purification and Assay: We prepared extracts of cultured cells by sonication in 0.5% Triton X-100, 10 mM HEPES pH 7.5, and 1 mM 2-mercaptoethanol. Extracts of human liver were prepared by homogenization in the same buffer. After 30 min of incubation at 0°C, the extracts were centrifuged for 10 min at 10,000 *g* and supernatants were assayed for OTC enzymatic activity as described previously (15).

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 K_i that does 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_f and pOD_b, contained head-to-tail and head-to-head orientations of the expression segments, respectively.

[1] *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; DNP, dinitrophenol; kb, kilobase; MEME, minimum essential medium with Earle's salts; OTC, ornithine transcarbamylase; R6G, rhodamine 6G.

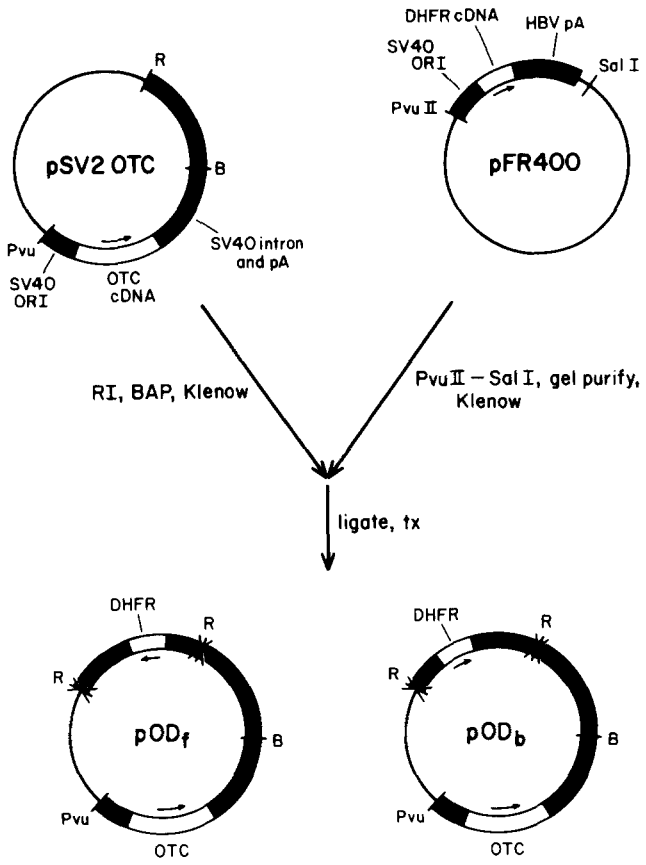

FIGURE 1 Construction of plasmids containing cDNA sequences encoding both the OTC precursor and a mutant DHFR joined with viral regulatory sequences. The plasmid pFR400 (8) was kindly supplied by Christian Simonsen. The DNA fragment containing cDNA coding for a mutant DHFR is shown by the open bar, the 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 Polymerase 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 designated pOD$_f$ (OTC-DHFR head-to-tail) and pOD$_b$ (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 chosen as the recipient because SV40 regulatory elements, used for expression of both DHFR and OTC cDNA sequences, have been shown to function efficiently in this cell line. 48 h after transfection, selection was started by the addition of methotrexate to the culture medium at a concentration of 200 nM. After 12 d of growth in selective medium, transformant clones were identified in equal numbers for the two plasmids. 20 were analyzed for OTC enzymatic activity. Of these, 18 contained detectable activity; no activity was measured in untransformed HeLa cells. The six transformants (three pOD$_f$, three pOD$_b$) 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 methotrexate and were maintained in this concentration of methotrexate. The lines were designated OD cell lines because they contain both OTC and DHFR sequences. The lines were analyzed for the presence of immunoprecipitable OTC, for OTC enzymatic activity, and for copy number and arrangement of OTC DNA sequences. The results of immunoprecipitation of extracts of radiolabeled OD cell lines with anti-OTC antiserum are shown in Fig. 2. All five lines contained precipitable 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 co-transformants.) The OD lines also contained a much less intensely labeled immunoprecipitable product that co-migrated 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 cotransformant, 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 OD5 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

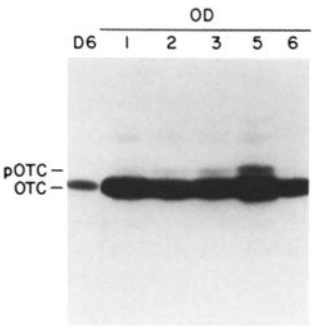

FIGURE 2 OTC expression in stable HeLa cell transformants. Stable HeLa cell transformants, derived either from transfection with pSV2OTC and pSV2Neo (*D6*) or from transfection with pOD$_f$ and pOD$_b$ (*OD 1, 2, 3, 5, 6*), were grown to near confluence in 60-mm dishes in MEME + G418 (line D6) or MEME + 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 [^{35}S]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.

TABLE I

OTC Enzymatic Activity in HeLa Transformants and Human Liver

	U*/mg protein
HeLa	<0.2
Neo co-transformants	
A1	0.8
D6	1.4
Mtx^R 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.

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, pHO731, containing a nearly full-length OTC cDNA joined with pBR322. Two Bam HI sites are present in the input plasmids pOD_f and pOD_b—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 hybridizing 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 presence 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 OD5 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 hybridizing bands in the OD lines is unclear, although the single additional band observed for the OD5 line corresponds precisely 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 enzymatic 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.

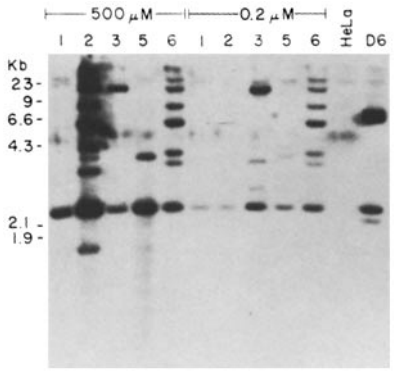

FIGURE 3 Hybridization analysis of DNA from HeLa cell transformants. High molecular weight DNA was isolated from the OTC-DHFR transformants (OD *1, 2, 3, 5, 6*), both after initial selection in 0.2 μM methotrexate and after subsequent amplification in 500 μM methotrexate. DNA was also prepared from untransfected HeLa cells and from the OTC-Neo cotransformant D6. DNA was digested with BamHI, separated by electrophoresis in a 0.8% agarose gel, and blotted to nitrocellulose. The filter was hybridized with a nick-translated plasmid, pHO731, which contains a nearly full-length human OTC cDNA. The 2.2-kb hybridizing fragments correspond in size to a Bam fragment containing almost all of the OTC cDNA sequence present in plasmids pOD_f and pOD_b, and in pSV2OTC. See Results for details concerning hybridization pattern.

Localization of OTC in OD5 Cells

To demonstrate directly the presence of OTC in the mitochondria of a transformant, cells from the OD5 line were fixed, permeabilized, and incubated with monospecific rabbit anti-OTC antiserum, then fluorescein isothiocyanate–conjugated 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 mitochondria 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 [^{35}S]methionine 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, [^{35}S]methionine and DNP were washed from the cells and incubation was carried out in nonlabeled

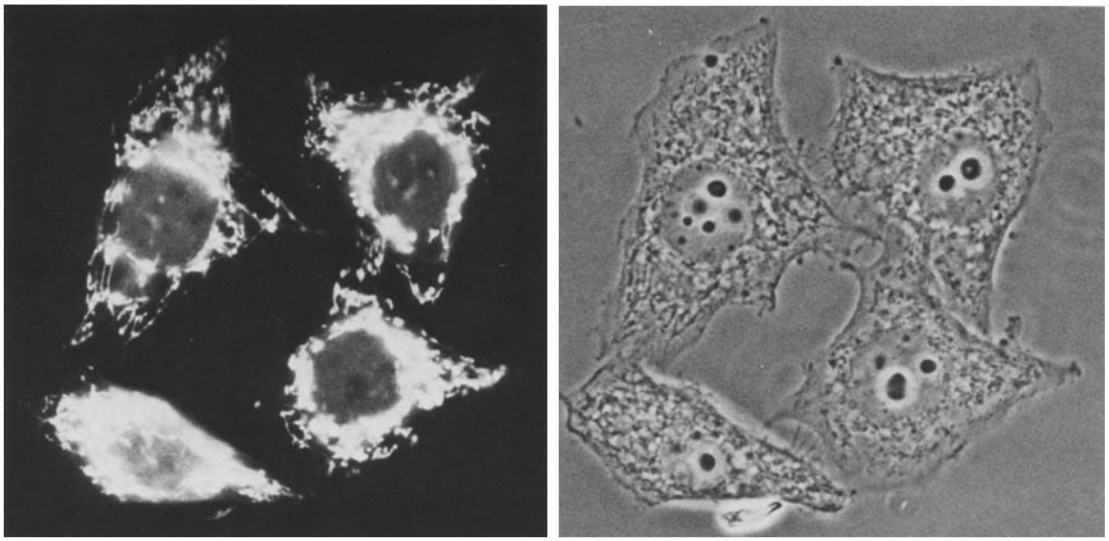

FIGURE 4 Localization of OTC in OD5 cells by indirect immunofluorescence. OD5 cells were grown on glass coverslips, fixed, permeabilized in methanol, and stained for OTC by the use of a rabbit anti-rat OTC antiserum. In some cases, the fluorescent organelles correspond to structures seen by phase-contrast 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.

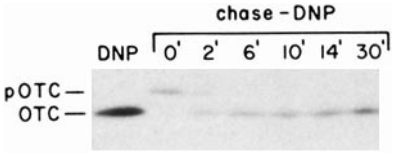

FIGURE 5 Time course of mitochondrial import and processing of OTC. Cultures of the stable transformant (D6) were grown to confluence in 60-mm dishes in MEME. The monolayers were washed, incubated with 4 mM DNP, and labeled with [^{35}S]methionine for 1 h. Then the labeling medium was removed and replaced 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.

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. 6*A*, lane *1*). When the mito-

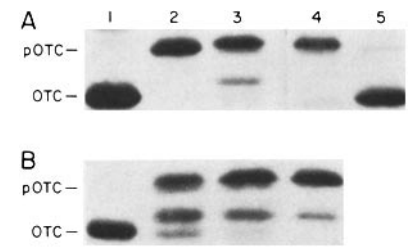

FIGURE 6 Effect of canavanine on mitochondrial import and processing of OTC. The OTC-DHFR cell transformant OD5 was grown to near confluence in 60-mm dishes in MEME + folate + 500 μM methotrexate. Cells were radiolabeled with [^{35}S]methionine in the absence or presence of canavanine and/or R6G. Monolayers 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 L-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*).

chondrial import inhibitor, R6G, was added to the control culture, only the OTC precursor was observed (Fig. 6*A*, lane *2*). In the presence of 2 mM canavanine, two precipitable products were detected (Fig. 6*A*, 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. 6*A*, 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. 6*A*, 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. 6*B*, 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 normal-sized precursor and mature products are reduced at 2 mM canavanine concentration and are totally absent at 10 mM concentration (Fig. 6*B*, 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 $t_{1/2}$ for import was estimated to be <2 min. Similar determinations have been carried out for three other mammalian mitochondrial precursors, and $t_{1/2}$ 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 $t_{1/2}$ 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 OD5 Cells by the Arginine Analogue Canavanine

The substitution of the amino acid analogue, canavanine, for arginine in the medium of OD5 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, perhaps 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 pk_a of the canavanine side-chain is 6.6 as compared with 9.0 for arginine (21), 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 their 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.

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