The expression of *Escherichia coli* threonine synthase and the production of threonine from homoserine in mouse 3T3 cells

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We have subcloned the coding sequence for the *Escherichia coli* threonine synthase gene into a eukaryotic expression vector based on the simian-virus-40 early promoter. When mouse 3T3 cells which already expressed homoserine kinase were transfected with the new plasmid, the cells were able to incorporate radioactivity from [^{14}C]homoserine into their cell proteins. Stable cell lines were established by co-transfecting 3T3 cells with the plasmid coding for threonine synthase and another coding for homoserine kinase and G-418 (Geneticin) resistance. Cells were selected for G-418 resistance and then screened for an ability to synthesize threonine from homoserine and incorporate it into the cell protein. A cell line which expressed both the homoserine kinase and threonine synthase genes was capable of growth in a threonine-deficient medium containing homoserine.

INTRODUCTION

The essential amino acids are required in the diet of higher animals which lack the enzymes for their biosynthesis. In the nutrition of non-ruminants the utilization of diets based on cereal proteins can be significantly improved by feeding rations supplemented with synthetic amino acids, particularly lysine and threonine. The introduction of pathways for the biosynthesis of these limiting amino acids into animal cells would eliminate the need for supplementation, and we have previously discussed the possibility of producing transgenic animals capable of these biosyntheses (Rees et al., 1990). Our metabolic analysis showed that the favoured route for the production of both lysine and threonine in animal cells would be through the bacterial pathway, which uses aspartic acid as the precursor.

The biosynthesis of threonine in *Escherichia coli* (Figure 1) proceeds through the non-protein amino acid homoserine, which is produced from aspartic acid by three enzyme steps. Aspartokinase I/homoserine dehydrogenase I is a bifunctional enzyme encoded by the *thrA* gene in *E. coli*. One of its two functions phosphorylates aspartic acid to produce aspartyl phosphate, which is converted into aspartate β-semialdehyde by aspartate-semialdehyde dehydrogenase, encoded by the *asd* gene. The second *thrA* activity, homoserine dehydrogenase I, then converts the semialdehyde into the non-protein amino acid homoserine. Homoserine is phosphorylated by the enzyme homoserine kinase (*thrB*) to produce homoserine *O*-phosphate, which is converted into threonine by the enzyme threonine synthase (*thrC*). In *E. coli*, *thrA*, *thrB* and *thrC* are located in a single operon (Theze et al., 1974; Parsot et al., 1983), in which the threonine synthase gene, *thrC*, is the last gene in the operon. The 1.3 kbp coding region produces an enzyme with a molecular mass of 47 kDa. Threonine synthase requires pyridoxal phosphate as a cofactor and cleaves homoserine *O*-phosphate to produce threonine and P_i (Flavin and Slaughter, 1960).

The expression of a bacterial gene in animal cells has been achieved by the production of chimeric genes in which the coding region for the bacterial gene is combined with the promoter from an animal virus (Mulligan and Berg, 1980). Using a simian-virus-40 (SV40)-based expression vector system we have produced a chimeric gene using the *thrB* coding region, which we have placed between an SV40 late promoter and an SV40 poly-adenylation site (Rees et al., 1992). When this chimeric gene was transferred to mouse 3T3 cells in tissue culture, the new gene enabled the cells to produce homoserine *O*-phosphate, and a pool of this intermediate accumulated in the cells. As the next step in introducing the entire threonine-biosynthetic pathway, we have now prepared a chimeric gene which expresses threonine synthase and in this paper show that mouse cells expressing both activities can produce threonine from homoserine.

METHODS

General

The plasmid pIP3 (Parsot et al., 1983) was generously given by Dr. I. Saint-Girons, Institut Pasteur, Paris, France. The plasmid pSVpoly was generously given by Dr. A. Stacey, Colorado State University, Fort Collins, CO, U.S.A. The plasmid pSVL was from Pharmacia. Enzymes were supplied by Pharmacia, Boehringer or Northumbria Biologicals. Recombinant DNA methods were as described by Maniatis et al. (1982). L-[U-^{14}C]-Threonine (226 mCi/mmol, L-[U-^{14}C]homoserine (40 mCi/mmol) and [γ-^{32}P]dCTP were supplied by Amersham International. L-[U-^{14}C]Homoserine was further purified by ion-exchange chromatography on Dowex-50 (H^+ form) to remove a trace contaminant which was not retained by this column. Unlabelled amino acids were from Sigma.

Tissue culture

For this, 10 × Dulbecco's modified Eagles medium (DMEM) was obtained from Gibco. Threonine-free 10 × DMEM was prepared according to the same formulation, except that threonine was omitted. Newborn-calf serum was from Flow. Dialysed newborn-calf serum was prepared by dialysis for 48 h against 20 vol. of Hanks balanced salts buffer at 4 °C. The buffer was changed three times in this period. After dialysis the serum was

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline (10 mM sodium phosphate/0.15 M NaCl, pH 7.4); SV40, simian virus 40; G-418 Geneticin, *O*-2-amino-2,7-dideoxy-D-glycero-α-D-glucoheptopyranosyl(1–4)-*O*-3-deoxy-C^4-methyl-3-(methylamino)-β-L-arabinopyranosyl-D-streptamine.

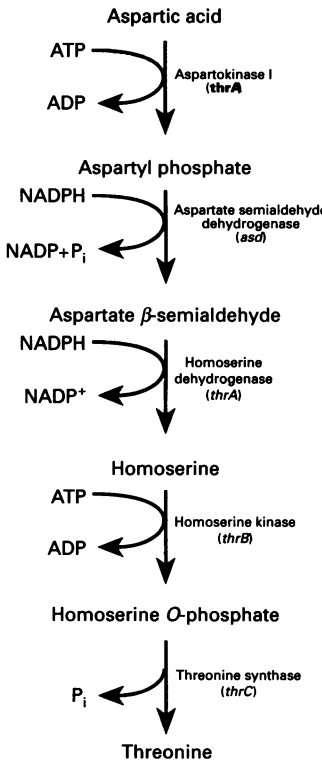

Figure 1 The pathway for threonine biosynthesis in *E. coli*

sterilized by filtration through an 0.2 μm-pore filter and stored frozen.

Routine culture of mouse 3T3 cells used DMEM containing 12% newborn-calf serum. Threonine-deficient medium was prepared by using the threonine-free DMEM supplemented with 12% newborn-calf serum. Threonine-free medium was prepared using the threonine-free DMEM supplemented with 5% dialysed newborn-calf serum, insulin, vitamins and transferrin/selenium (Sigma) and epidermal growth factor (Sigma; 10 ng/ml) (Hartman and Mulligan, 1988). The free threonine content of the different media was analysed by precipitating the serum proteins with $HClO_4$ and analysing the free amino acids remaining in the supernatant on an amino acid analyser. Complete medium contained 664 nmol/ml, deficient medium 16.7 nmol/ml and the threonine-free medium contained no detectable free threonine.

Transfections

The cells were transfected with DNA by the method of Parker and Stark (1979), using a calcium phosphate precipitate. First, 5×10^5 3T3 cells were plated in a 6 cm Petri dish and cultured overnight. The transfection mixture was prepared by mixing the DNA in 1.0 ml of 38 mM Hepes/0.15 M NaCl, pH 7.10, with 0.16 ml of 70 mM sodium phosphate and 88 μl of 2 M $CaCl_2$. Salmon sperm DNA (Sigma) was added when necessary to give a final DNA concentration of 25 μg/0.5 ml. The medium was removed, and 0.5 ml of the transfection mixture was added to the cells and incubated for 1 h with regular agitation. Then 2 ml of medium was added and the incubation was continued for a further 4 h. The cells were then subjected to osmotic shock by addition of sterile 10 mM Tris/0.15 M NaCl, pH 7.4) containing 10% glycerol. The same transfection procedure was used for the establishment of stable cell lines, except that 1×10^5 cells were transfected, and after 12 h incubation in complete 12% medium 400 μg/ml G-418 (Geneticin; Gibco) was added to the medium. The control cell line 3T3 neo was produced by transfection with pMC1neoPA. The line 3T3thrBneo 6/1 was produced by transfection with pSVthrBneo, and selection for G-418 resistance, and identified by screening for homoserine kinase activity (Rees et al., 1992).

Incorporation of radioactivity into cell protein

Cells were cultured in media containing [^{14}C]homoserine or [^{14}C]threonine, and the incorporation into $HClO_4$-insoluble protein was assessed. Cells were plated at 2×10^4 cells/35 mm dish in complete medium. After 24 h the medium was changed for threonine-deficient medium which contained either [^{14}C]homoserine or [^{14}C]threonine. Unlabelled threonine or homoserine was added to give a final concentration in the medium of 10 μM and a final specific radioactivity of 1.25 mCi/mmol. The cells were then cultured for up to 4 days. To estimate the radioactivity incorporated into the cell protein, the medium was removed and the dish was washed five times with ice-cold PBS. The cell monolayer was scraped off the dish in 0.5 ml of 0.3 M NaOH and the dish was washed with a further 0.5 ml of NaOH. The protein in the extract was precipitated by addition of 0.3 ml of 20% $HClO_4$ and collected by centrifugation at 10000 *g* for 10 min. The supernatant was removed and the pellet resuspended in 1 ml of 0.3 M NaOH. The precipitation process was repeated and the sample taken up in 0.1 ml of 0.3 M NaOH. Samples were then taken for measurement of radioactivity and protein (Lowry et al., 1951).

Hydrolysis of cell proteins

Purified cell protein (1–5 mg) was placed in a screw-cap tube together with 0.1 ml of 6 M HCl, and the tube was sealed and heated to 110 °C overnight. After cooling, the HCl was removed under vacuum and the product resuspended in 0.1 ml of water, dried, and then resuspended once more in 0.1 ml of distilled water.

Ion-exchange chromatography

A 50 μl portion of the hydrolysate was loaded on the column (30 cm × 3 mm) of an amino acid analyser (Chromaspek, Rank–Hilger) and eluted with a lithium/citrate/borate buffer system (flow rate 0.2 ml/min). The effluent was collected in a fraction collector with 60 fractions covering a pH gradient of 1.9–11.4. Samples of each fraction were counted for radioactivity.

T.l.c.

Samples (1 μl) of the hydrolysate were separated on cellulose thin-layer plates (Merck) together with an authentic threonine standard. The separation was performed with butan-1-ol/ethanol/water (12:3:5, by vol.) as solvent. After development the plates were dried and cut in half. One half was sprayed with EnHance (New England Nuclear) and the distribution of radioactivity was made visible by fluorography. The other half was dipped in a 0.01% solution of ninhydrin in acetone and heated briefly to reveal the location of the amino acid standards.

Growth curves

Cells were plated at 1×10^4 cells/6 cm Petri dish and cultured in threonine-free medium containing either 2 mM L-threonine or 2 mM L-homoserine. The medium was changed daily. At the

required time intervals, replicate sets of the dishes were taken and washed three times with PBS to remove the serum protein. The cells were dissolved in 1.0 ml of 0.3 M NaOH and samples were assayed for protein by the method of Lowry et al. (1951).

Threonine synthase assay

Mouse 3T3 cells were grown to confluence in 90 mm Petri dishes. The cells were washed with PBS, scraped into 0.5 ml of PBS with a rubber policeman and harvested by centrifugation at 10000 *g* for 1 min. The PBS was removed and the cells was resuspended in assay buffer (100 mM KCl, 20 mM Tes, pH 7.4, 0.1 mM EDTA). The cells were lysed by sonication for 15 s at 60 W with a 0.1 cm probe. The extract was then centrifuged at 100000 *g* for 15 min and the supernatant was used for the assay.

Homoserine kinase was assayed as described previously (Rees et al., 1992). The protein content of the extract was determined by the method of Lowry et al. (1951).

Threonine synthase activity has been assayed previously by measuring the release of P_i from homoserine *O*-phosphate (Flavin and Slaughter, 1960). This method was not sufficiently sensitive to assay the small amounts of material available from cultured cells, and we have developed a radio-enzyme assay to monitor the hydrolysis of [^{14}C]homoserine *O*-phosphate. At the end of the reaction, unchanged [^{14}C]homoserine *O*-phosphate is precipitated as its barium salt, leaving the [^{14}C]threonine produced in the supernatant.

[^{14}C]Homoserine *O*-phosphate was prepared by phosphorylating homoserine with a partially purified preparation of *E. coli* homoserine kinase prepared by the method described by Szczesiul and Wampler (1976). Briefly, *E. coli* (DH5α) was grown in minimal medium; the cells were then harvested and resuspended in a buffer containing 100 mM KCl, 20 mM Tes, pH 7.4, and 1 mM EDTA. The cells were broken by sonication and the debris was removed by centrifugation. Nucleic acids were precipitated with 3.0 g/l streptomycin sulphate. A crude protein extract was obtained by the addition of 388 g/l $(NH_4)_2SO_4$. The pellet was dialysed against buffer before being applied to a DEAE-Sephadex column (Pharmacia A-50) and eluted with a linear gradient of KCl (0.1–0.6 M). The fractions collected were analysed for homoserine kinase and threonine synthase activities. The homoserine kinase, which emerged at 0.2 M KCl, was precipitated with $(NH_4)_2SO_4$ and stored as a slurry. The specific activity of the enzyme was 3 μmol/min per mg of protein. Incubation of the enzyme with [^{14}C]homoserine *O*-phosphate showed that the preparation was free of threonine synthase, which emerged from the column at 0.4 M KCl, or any other activities which degraded homoserine *O*-phosphate.

[^{14}C]Homoserine (0.02 μCi) was incubated with 10 mM ATP and 1 mM unlabelled homoserine together with 0.01 unit of the *E. coli* homoserine kinase preparation in a 150 μl volume. After incubation for 3 h the [^{14}C]homoserine *O*-phosphate produced was used for the assay. A 10 μl portion (5×10^4 c.p.m.; sp. radioactivity 1.1 mCi/mmol) of this product was incubated with 20 μl of 3T3-cell extract at 37 °C. After a suitable time the [^{14}C]homoserine *O*-phosphate was precipitated by addition of 100 μl of 0.15 M $Ba(OH)_2$ and 100 μl of 0.15 M Na_2CO_3. The mixture was centrifuged for 1 min at 10000 *g* in a microfuge, and a 100 μl sample of the supernatant was taken for measurement of radioactivity. Blank values were determined by incubating homoserine *O*-phosphate with boiled cell extracts.

Under these conditions, with extracts of cell line 10-7, the progress curve remained linear for up to 4 h. When extracts of cell line 10-7 were incubated overnight with the homoserine *O*-phosphate tracer, 80% of the barium-precipitable radioactivity was converted into a non-precipitable form. In transient expression experiments, there was a correlation between the hydrolysis of homoserine *O*-phosphate and the amount of plasmid used in the transfection (see the Results section). It should be noted that this assay does not directly identify the product, and it is impossible to differentiate between threonine synthase and non-specific phosphatase activity. High-speed centrifugation removes a large part of the phosphatase activity present in these 3T3-cell extracts (Rees et al., 1992), but the activity remaining can still result in significant hydrolysis of homoserine *O*-phosphate. The degree of phosphatase contamination is variable, and in the absence of an effective threonine synthase inhibitor it is not possible to correct the results. It is therefore important to ensure that extracts of non-transfected cells prepared in parallel possess a low phosphatase activity.

RESULTS

Plasmid preparation

The *E. coli* threonine synthase gene is part of the *E. coli* threonine operon which has been isolated in the plasmid pIP3 (Cosart et al., 1981). A convenient *Sca*I site is located 4 bp upstream of the start codon for *thrC*, and we were able to isolate this 1.727 kbp *Sca*I/*Ava*II fragment of the plasmid pIP3 as shown in Figure 2. In initial experiments we attempted to insert this fragment into the polylinker site of the vector pSVL, which is based on the SV40 late promoter and the SV40 polyadenylation sequence. The plasmids isolated from the *E. coli* host DH5α showed an unexpected restriction pattern when the insert was in the correct (sense) direction relative to the SV40 promoter. A portion of the 3′ end of the coding region appeared to have become inverted, reversing the orientation of two restriction sites, one in the insert and the other in the polyadenylation sequence of the vector. Inserts in the opposite (anti-sense) orientation were apparently unaffected. A single plasmid with the expected restriction map for an insert in the sense direction was isolated, but this did not show any activity in the transient expression assay (results not shown). The reasons for these changes are not clear, but may involve rearrangements due to the presence of part of the ribosomal terminator sequence from the *E. coli* threonine operon, which is present at the 3′ end of the insert.

We were able to insert the 1.7 kbp fragment into an SV40 early expression vector system, pSVpoly (Stacey and Schnieke, 1989). The *Sca*I/*Ava*II fragment of the plasmid pIP3 was isolated and the 3′ overhang was end-filled with Klenow enzyme and dNTPs. This fragment was then ligated into a unique *Eco*RV site in the polylinker of the plasmid pSVpoly, and this recombinant DNA was used to transform *E. coli* strain DH5α. Ampicillin-resistant colonies were isolated and screened for the presence of recombinant plasmids. These were isolated and characterized by restriction mapping. Approximately equal numbers were found with inserts in the sense and antisense orientation, and there was no evidence of the rearrangements seen with the vector pSVL. A schematic diagram of the construction of pSVthrC1 is shown in Figure 2. A representative plasmid which showed the expected restriction pattern was taken and tested for activity in mouse cells.

A line of 3T3 cells, Bneo6/1, which expresses the *E. coli* gene for homoserine kinase (Rees et al., 1992) was transfected with pSVthrC1 (10 μg/6 cm dish) and cultured in threonine-deficient medium which contained tracer L-[U-^{14}C]homoserine. Between 0.7 and 1.1% of the added radioactivity was incorporated into the cellular protein of cells transfected with pSVthrC1 to give specific radioactivities of 2500–3500 c.p.m./mg of protein. In contrast, control 3T3 cells or a clone expressing homoserine

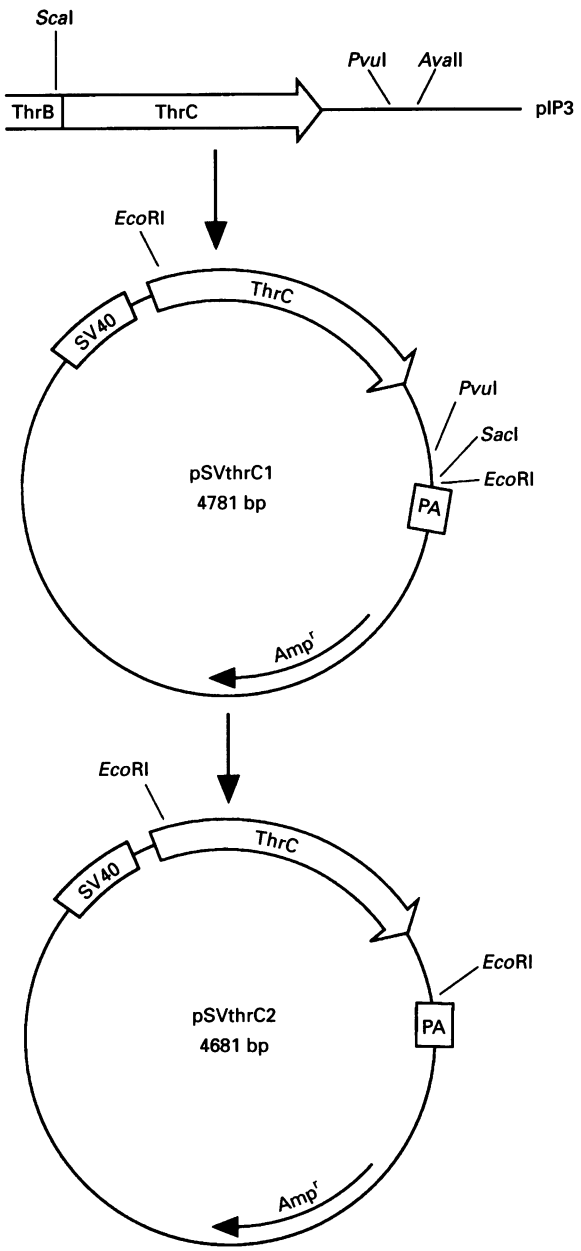

Figure 2 Schematic diagram of the preparation of the plasmids pSVthrC1 and pSVthrC2

Abbreviations: SV40, SV40 early promoter; ThrC, *E. coli* threonine synthase coding region; PA, SV40 polyadenylation site; Amp^r, ampicillin-resistance gene. The diagram is not drawn to scale.

kinase alone (3T3 Bneo 6/1) incorporated between 0.03 and 0.05% of the added radioactivity into $HClO_4$-precipitable proteins to give specific radioactivities of 200–300 c.p.m./mg of protein. The results indicated that homoserine *O*-phosphate produced by the homoserine kinase activity was converted into threonine, which was incorporated into the cell protein.

If this incorporation was due to the expression of threonine synthase, we expected to be able to demonstrate the increased hydrolysis of homoserine *O*-phosphate in control 3T3 cells transfected with pSVthrC1. We used [^{14}C]homoserine *O*-phosphate with a high specific radioactivity (40 mCi/mmol), which was prepared by incubating [^{14}C]homoserine with *E. coli* homoserine kinase and ATP. 3T3 cells were transfected with 5, 10 or 25 μg of pSVthrC1/dish, and after 2 days the rate of homoserine *O*-phosphate hydrolysis by cell extracts was measured. The hydrolysis was dependent on the amount of pSVthrC1 used for the transfection. In a 2 h incubation the total hydrolysis increased from 23–25% with 5 μg/dish to 65–75% of the tracer with 25 μg/dish. Mock-transfected cells hydrolysed 13–18%, showing that there was an increase in the rate of homoserine *O*-phosphate hydrolysis which was dependent on the amount of pSVthrC1 used for the transfection.

In the hope that the partial removal of the *E. coli thr* operon ribosomal terminators would enhance threonine synthase expression, 100 bp of DNA was deleted from pSVthrC1 to give pSVthrC2 by using conveniently sited *Pvu*I and *Sac*I sites (Figure 2). The activity of threonine synthase in transient expression assays was not in fact enhanced (0.13 compared with 0.12 nmol/min per mg of protein), but pSVthrC2 was used in subsequent experiments to generate stable cell lines expressing threonine synthase.

Stable clones expressing both homoserine kinase and threonine synthase

To produce stable cells lines which integrated the genes for both homoserine kinase and threonine synthase, we transfected 3T3 cells simultaneously with pSVthrBneo and pSVthrC2 and selected for G-418 resistance. The plasmid pSVthrBneo codes both for G-418 resistance and for homoserine kinase activity. We have shown previously that a majority of the G-418-resistant lines also express homoserine kinase (Rees et al., 1992). When pSVthrC2 is co-transfected with pSVthrBneo, its incorporation into G-418-resistant cells depends on chance, as there is no positive selection for its presence and it is not linked to G-418 resistance. By using different ratios of the plasmids coding for homoserine kinase and threonine synthase, we hoped to isolate a series of cell lines with differing activities of the two enzymes. 3T3 cells were transfected with a mixture of 1 μg of pSVthrBneo and 5, 10 or 20 μg of pSVthrC2 per dish. The cells were selected with G-418 for 21–28 days, during which time 30–50 colonies per dish appeared. The number of colonies obtained in the co-transfection experiments was similar to the number obtained when cells were transfected with pSVthrBneo alone. Representative colonies were isolated by means of cloning rings, and the cell lines were screened for the ability to incorporate homoserine into the cell proteins.

Cells from each of the lines were established in standard medium and then transferred to threonine-deficient medium containing 10 μM L-[U-^{14}C]homoserine. The culture was allowed to continue for 5 days before the proteins were extracted. As a control, the incorporation of 10 μM L-[U-^{14}C]threonine was measured under identical conditions, except that the protein was harvested 65 h after the radioisotope was added. With all the cell lines, approx. 80% of the threonine added was incorporated into protein regardless of the ability of the cell line to incorporate homoserine. At the end of the experiment, the cell proteins had incorporated an average of 43.61 ± 6.8 nmol of threonine/mg of protein.

With labelled homoserine we found that less than 0.01% of the radioactivity was taken up by the control (non-transfected by pSVthrBneo-transfected) cells. In several of the cell lines transfected with pSVthrBneo and pSVthrC2 there was significant incorporation of labelled amino acid into the cell proteins, as shown in Figure 3. The prefix to the clone number indicates the ratio of pSVthrC2 to pSVthrBneo used for transfection. Generally there was a progressive increase in the number of active cell lines as the ratio of the two plasmids was increased. This is consistent with a greater chance of pSVthrC2 becoming incorporated with pSVthrBneo. There was also a progressive

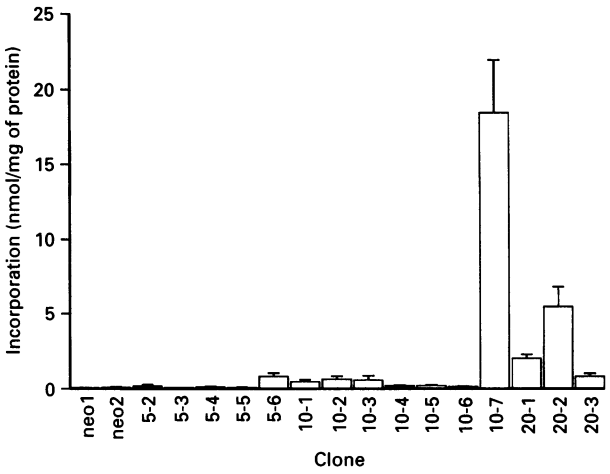

Figure 3 Incorporation of 10 mM L-[^{14}C]homoserine into 3T3 clones transfected with homoserine kinase and threonine synthase

neo1 and 2 are control cell lines which only express the neomycin-resistance gene. Cell lines prefixed 5, 10 and 20 were respectively transfected with a 5:1, 10:1 and 20:1 ratio of pSVthrC2:pSVthrBneo. The results are means ± S.E.M. of four experiments with duplicate observations in each case.

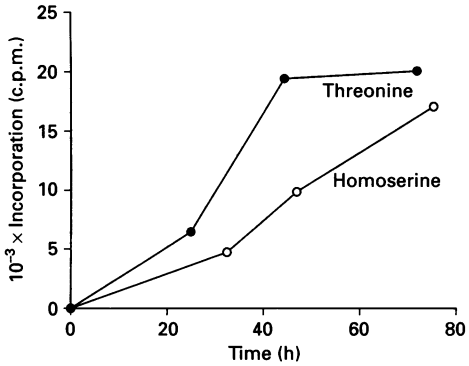

Figure 4 Time course for the incorporation of [^{14}C]threonine (●) and [^{14}C]homoserine (○) into the clone 3T3 10-7

Both amino acids were at a final concentration of 10 μM.

increase in the amount of radioactive amino acid incorporated; however, clone 10-7 stands out as having a significantly higher activity than the other lines. At the end of the experiment, the cell protein from this line had incorporated 18.4 ± 3.0 nmol of threonine/mg of protein (average of four experiments with duplicate observations in each experiment).

The time course for the incorporation of the labelled amino acids into the cell proteins of the cell line 10-7 is shown in Figure 4. The cells were cultured under conditions identical with those in the experiments shown in Figure 3 with both amino acids at a final concentration of 10 μM. The incorporation of radioactivity was not linear, but had a lag period corresponding to the lag phase in cell growth after subculture or a change of medium. The specific radioactivity of the cell protein, and hence the content of labelled amino acid, remained constant after this lag period of 12 h was over and only began to decline slowly after 40–80 h, when the free radioisotope in the medium was exhausted. In the experiment shown in Figure 4, 66.7 ± 7.4 nmol of threonine/mg of protein had been derived from free threonine present in the medium when the cells were grown in threonine-containing medium, and 46.7 ± 3.7 nmol of threonine/mg of protein had been derived from homoserine when the cells had been grown in homoserine-containing medium (in both cases the averages of six determinations between 12 and 72 h of culture). The final yields of protein were 141 μg and 112 μg respectively. There is less protein-bound threonine derived from the medium by cells grown with homoserine than by cells grown with threonine. When growing in the medium lacking threonine but containing homoserine, we estimate that the cell line 3T3 10-7 is producing threonine at a rate sufficient to satisfy approx. 60 % of its requirement.

Fate of homoserine in line 10-7

To verify that the new product produced from homoserine was indeed threonine, we investigated the nature of the radioactivity associated with the component amino acids of the cell proteins from 3T3 10-7 by separating them by ion-exchange chromatography or on thin-layer chromatograms. The cells were cultured in threonine-deficient medium containing either [^{14}C]threonine or [^{14}C]homoserine (both 40 mCi/mmol), and after 5 days the proteins were isolated and hydrolysed. The hydrolysate contained 58–60 % of the radioactive threonine and 25–27 % of the radioactive homoserine which had been added.

One portion of the protein hydrolysate was analysed by ion-exchange chromatography using an amino acid analyser. The radioactivity was eluted in the region where threonine and homoserine would be expected, and the distribution is shown in Figure 5(a). The major peak accounts for 59–76 % of the activity which was applied to the column, and both threonine and homoserine tracers gave similar recoveries. When authentic threonine standards were run, the ninhydrin-reactive material was eluted in fraction 19, whereas a homoserine standard was eluted in fraction 17. The separation between homoserine and threonine is poor, since these amino acids are isomers of one another. There is also a small peak, containing less than 10 % of the total activity in fraction 16, which is present in both threonine- and homoserine-fed cells. We have been unable to identify this product, but since it is present in both samples we believe it to be a breakdown product from threonine produced during the hydrolysis procedure. None of the other fractions contained significant amounts of radioactivity, and there was no evidence for any of the non-essential amino acids becoming labelled.

Portions of the protein hydrolysates were also analysed by t.l.c., and the distribution of radioactivity is shown in Figure 5(b). Lanes A and B are the products from cells cultured in medium containing [^{14}C]threonine, and lanes C and D are the products from cells grown in medium containing [^{14}C]homoserine. The location of the radioactivity is identical in both threonine- and homoserine-fed cells and corresponded to the position of an authentic threonine standard.

Growth in homoserine-containing medium

As the line 3T3 10-7 was able to produce threonine from homoserine, we were interested to determine what proportion of its requirement could be supported by the new pathway. The control 3T3 cell line 3T3neo was able to grow in threonine-deficient DMEM supplemented with 12 % calf serum, although addition of homoserine at concentrations above 1 mM inhibited growth (results not shown). An analysis of the amino acid content of 3T3 cells showed that the cell protein extracted from a confluent 6 cm dish of cells contained 483 nmol of threonine (166 nmol of threonine/mg of protein or 124.5 nmol of threonine/

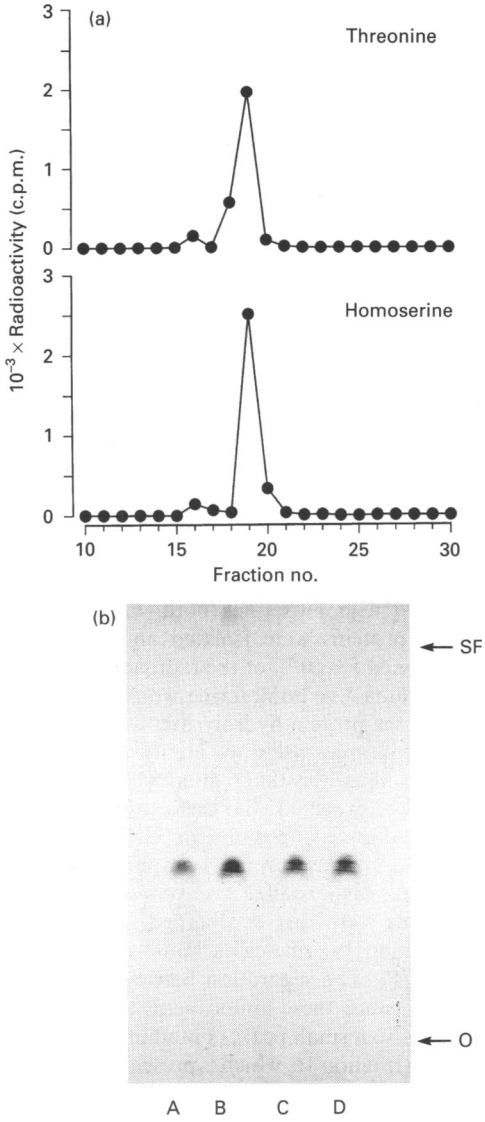

Figure 5 Amino acid analysis of the hydrolysate of cell proteins from cell line 3T3 10-7 cultured in the presence of L-[^{14}C]threonine or L-[^{14}C]homoserine

Cell proteins were harvested and hydrolysed, and the component amino acids were analysed by either anion-exchange chromatography or t.l.c. (a) Distribution of radioactivity in a part of the effluent when the hydrolysate was separated on a Chromaspek amino acid analyser. An authentic threonine standard emerged in fractions 19–20 and a homoserine standard in fractions 17. The cells were labelled with [^{14}C]threonine (upper panel) and [^{14}C]homoserine (lower panel). The experiment was repeated twice with identical results in each case. (b) Fluorograph of the protein hydrolysate separated on a cellulose thin-layer plate. Lanes A and B are separate cell cultures grown in the presence of [^{14}C]threonine and lanes C and D from cells grown with [^{14}C]homoserine. Key: O, origin; SF, solvent front.

10^6 cells). As the 4 ml of threonine-deficient medium in the dish contained only 33.4 nmol of free threonine, this is clearly insufficient for growth. However, there was a further 16.3 μmol of threonine/ml present in the serum proteins, and hydrolysis of even a small part of this protein could provide the source of additional threonine. We found that the growth rates in the threonine-deficient medium were variable, and to achieve more consistent growth curves we found it necessary to use a threonine-free medium. This used dialysed calf serum and was based on the medium described by Hartman and Mulligan (1988). It contained no detectable free threonine, although the significant amount of

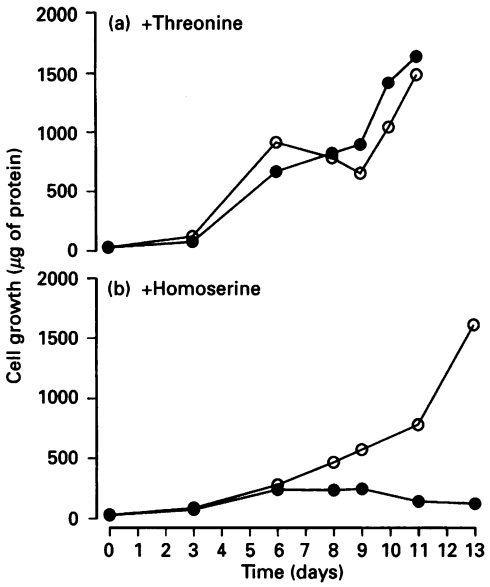

Figure 6 Growth curves for the cell lines 3T3 Bneo6/1 (●) and 3T3 BneoC 10-7 (○) in threonine-free medium supplemented with (a) 2 mM threonine and (b) 2 mM homoserine

threonine in the serum proteins was probably unchanged by dialysis.

We have measured the growth rates of cells from the lines 6/1 (homoserine kinase alone) and 10-7 (homoserine kinase and threonine synthase) by measuring the accretion of protein when the cells are grown in threonine-free medium containing either threonine or homoserine. It was necessary to change the medium daily at the later stages, as an unidentified component rapidly became exhausted and the growth rate slowed. When threonine was present, the two cell lines gave identical growth rates and had accumulated a similar amount of protein (1.60 mg of protein for 3T3neo and 1.47 mg of protein for 3T3 10-7) (Figure 6a). The growth and final protein accretion was similar to that seen in the standard medium (complete DMEM + 12% calf serum). When homoserine replaced threonine (Figure 6b) the cell line expressing homoserine kinase alone was unable to grow and there was only a slight accretion of protein, which declined after 2 days as the cells began to become detached from the dish. The cell line expressing both homoserine kinase and threonine synthase grew normally and gave a final accretion of 1.59 mg of protein, which was not dissimilar to that achieved when these cells were cultured in threonine-containing medium. From these results we would estimate that the line 10-7 grows in homoserine containing medium at about half the rate at which it can grow in the threonine-containing medium. This is consistent with the data shown in Figure 4, for the incorporation of radioactively labelled homoserine into the cell proteins, from which we deduced that the new pathway could supply about 60% of the cells' threonine requirement.

Enzyme activity

The activity of threonine synthase in the stable cell lines was measured by a method similar to that used for the transient expression experiments, except that the final concentration of homoserine *O*-phosphate in the assay mixture was increased to 0.3 mM. Homoserine kinase was assayed by the ability to incorporate label from [^{14}C]homoserine into homoserine *O*-phosphate, which can then be precipitated as its barium salt. The

Table 1 Enzyme activities of some 3T3 cell lines which are able to synthesize threonine from homoserine

Results are the means (±S.E.M.) of six separate experiments with duplicate estimations in each experiment.

Cell line	Homoserine kinase activity (nmol/min per mg of protein)	Threonine synthase activity (nmol/min per mg of protein)
3T3 neo	0.005±0.005	0.020±0.025
3T3 6/1	0.112±0.052	0.001±0.039
3T3 5-6	0.014±0.016	0.331±0.085
3T3 10-7	0.051±0.031	0.127±0.091
3T3 20-2	0.028±0.027	0.089±0.121

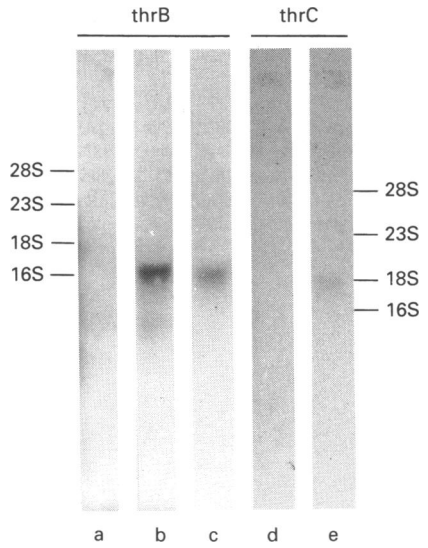

Figure 7 Northern blots (25 μg/lane) of total cell RNA from the lines 3T3neo (lane a), 6/1 (lane b) and 10-7 (lane c) probed for *thrB* (homoserine kinase), and lines 6/1 (lane d) and 10-7 (lane e) probed for *thrC* (threonine synthase)

The arrows indicate the positions of the 18S and 28S eukaryotic RNA and the 16S and 23S prokaryotic ribosomal RNAs.

results of both enzyme assays are shown in Table 1. We estimated the rate of threonine synthase activity in the cell line 10-7 to be 0.127±0.092 nmol/min per mg of protein (average of six experiments) which is in the same order as the activity of homoserine kinase, which gave a rate of 0.051±0.030 nmol/min per mg in the same cell line. Also shown in Table 1 are the enzyme activities found in the cell lines 5-6 and 20-2, both of which were also identified as producing significant amounts of threonine from homoserine. Despite a high level of threonine synthase, the total synthetic activity of line 5-6 would seem to be limited by the lower activity of homoserine kinase in this cell line. The activities of both enzymes in line 20-2 would appear to be lower than in 10-7, and these results are consistent with the total activity observed in the screening experiment (Figure 4).

In addition to assaying for enzyme activity, we also examined RNA from these cells for transcripts corresponding to the *E. coli thrB* and *thrC* coding regions. Figure 7 shows Northern blots of total RNA extracted from the cell lines 3T3neo, 6/1 and 10-7. The RNA was hybridized with ^{32}P-labelled probes prepared either from the *Xba*I/*Bam*HI fragment of pSVthrB or from the 1.4 kbp *Eco*RI fragment of pSVthrC2. In blots probed for *thrB* it can be seen that there is a signal consistent with a message of approx. 1.5 kb in both 6/1 and 10-7. In the blot probed for *thrC* there is a signal corresponding to a size of approx. 1.7 kb only in the cell line 10-7. Analysis of the mRNA from transient expression experiments showed the presence of two large transcripts larger than 4.2 kb, similar to those found previously for the plasmid pSVThrB (results not shown). Since the small 1.7 kb transcript is only present in the cell line 10-7, which can incorporate homoserine into the cell protein, this would suggest that this is the translationally active mRNA species. Standard plasmid dilutions, run as controls with the Northern-blot analysis shown in Figure 7, suggested that the *thrB* and *thrC* probes had a similar specific radioactivity and gave similar signals with equal quantities of their respective plasmids. In cell line 10-7 the levels of the mRNAs coding for *thrB* and *thrC* are therefore roughly equal.

The levels of *thrB* mRNA are in approximate agreement with the levels of activity recorded by the enzyme assay. The signal given by mRNA from cell line 10-7 is about half as intense as that from cell line 6/1, and this corresponds to the measured enzyme activities. This is important, since the assay of homoserine kinase activity is complicated by the presence of threonine synthase. This may convert some of the [^{14}C]homoserine *O*-phosphate produced into threonine, which is not precipitated, and this could lead us to underestimate the homoserine kinase activity. We have not corrected the rates of homoserine *O*-phosphate production for the effect of threonine synthase, but the result from the Northern blot suggests that the presence of threonine synthase in the crude extracts has not markedly affected the result of the enzyme assay.

DISCUSSION

When the *E. coli* threonine synthase and homoserine kinase genes were expressed in mouse 3T3 cells, the cells were able to convert homoserine into threonine, which they subsequently incorporated into cell proteins. Threonine synthase was expressed at a similar level to other *E. coli* genes expressed in mammalian cells, although we have not been able to measure the kinetic parameters of the enzyme in 3T3 cells. There is no change in the K_m *in vitro* for homoserine kinase expressed in 3T3 cells (Rees et al., 1992), and we can see no reason to assume that this is not also the case for threonine synthase. As both enzymes are present in the supernatant after high-speed centrifugation there is no evidence for their being associated with any subcellular structures.

We can compare our values for enzyme activity in 3T3 cells with those given by Szczesiul and Wampler (1976) for a wild-type *E. coli* strain CU-1. In *E. coli* the activity of homoserine kinase was found to be 19 units/ml of cell water and that of threonine synthase 9 units/ml cell water, which gives a ratio of kinase to synthase of 2.1, compared with our values for 3T3 10-7 cells, which give a ratio of 0.4. In the other cell lines which we examined, we found ratios of 0.04 for line 5-6 and 0.3 for line 20-2 (Table 1). The simplest explanation is that more copies of pSVthrC2 have been incorporated. However, the mRNA levels would appear to be roughly equal and therefore similar to the 1:1 ratio in the polycistronic *E. coli* message. It would seem reasonable to assume that the lower activity of homoserine kinase is a function of our expression system rather than a decreased catalytic activity when the enzyme is expressed in a mouse cell. The two genes use different expression vector systems, using different promoters, and the pSVthrB gene also

incorporates an intron which has to be removed before the message can be translated. It is most likely that these factors influence the translation of the message, and this is in turn reflected in the enzyme activities.

The structure of the pSVthrC2 plasmid may also not be optimal for maximum expression of threonine synthase activity. The pSVpoly expression vector has an out-of-sequence ATG codon in its polylinker, and this is located 4 bp upstream of the authentic start site in pSVthrC2. The construct also contains a 328 bp portion of non-coding DNA at the 3′ end of the *thrC* coding sequence between the stop codon and the polyadenylation site. This DNA contains the *E. coli* ribosomal terminator sequences: it is not clear what effect the presence of these palindromic sequences may have on the translation and stability of the chimeric mRNA.

Although only one of the cell lines that we have isolated shows a high rate of conversion of homoserine into threonine, it should be noted that this is a passive selection for incorporation of both *thrB* and *thrC*, and that the cells are selected for G-418 resistance rather than threonine biosynthesis. When control cell lines were cultured in homoserine-containing media there was a significant inhibition of cell growth, suggesting that homoserine is an inhibitor of one or more cellular processes. The presence of both homoserine kinase and threonine synthase would detoxify homoserine by converting it into threonine, which is harmless and can be used for growth. The differences in growth observed in threonine-free homoserine-containing medium suggest that a combination of pSVthrB and pSVthrC may provide a selectable gene system, allowing cells to be selected on the basis of their growth in medium in which threonine has been replaced by homoserine.

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