Threonine synthesis from homoserine as a selectable marker in mammalian cells

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The plasmid pSVthrBC expresses the *Escherichia coli thrB* (homoserine kinase) and *thrC* (threonine synthase) genes in mouse cells and enables them to synthesize threonine from homoserine. After transfection with pSVthrBC and culture in medium containing homoserine, only cells that have incorporated pSVthrBC survive. Homoserine at concentrations greater than 1 mM is toxic to mammalian cells. Mouse cells selected from medium containing 5 mM homoserine had incorporated 20–100 copies of the plasmid per cell and had homoserine kinase activities of 0.001–0.012 nmol/min per mg of protein per copy. Cells selected from medium containing 10 mM homoserine had incorporated one or two copies of the plasmid per cell and had homoserine kinase activities of 0.06–0.39 nmol/min per mg of protein per copy. By using high concentrations of homoserine, it is possible to use pSVthrBC to select and isolate cell lines that have one or two copies of the plasmid incorporated into an active region of chromatin. CHO and HeLa cells have also been successfully transfected with pSVthrBC. COS-7 cells are naturally resistant to homoserine as they are able to metabolize homoserine.

INTRODUCTION

Altering the nutritional requirements of an animal cell has provided a number of useful dominant-acting selectable markers for gene-transfer studies. New metabolic functions produced by the acquisition and expression of a foreign gene allows cell lines with the transformed phenotype to grow in a suitable medium. In animal cells, a number of genes have been shown to be suitable, including those coding for dihydrofolate reductase (Murray et al., 1983; Israel et al., 1987), aspartate transcarbamylase (de Saint Vincent et al., 1981; Ruiz and Wahl, 1986), asparagine synthetase (Cartier et al., 1987), histidinol decarboxylase, tryptophan synthase (Hartman and Mulligan, 1988) and glutamine synthetase (Cockett et al., 1990). In all of these cases, the enzyme encoded by the novel gene either provides a supply of an essential nutrient or converts a toxic component of the medium into a harmless product. In some cases, such as histidinol decarboxylase (Hartman and Mulligan, 1988), the new gene can carry out both of these functions. The precursor of histidine, histidinol, which is toxic to animal cells is converted into the essential amino acid histidine which can be used for growth.

Genes that code for enzymes that remove or modify toxic compounds are widely used as amplifiable markers. As the concentration of the toxic component is increased, only cells with higher enzyme activities capable of removing more of the toxin are able to survive. This has the effect of selecting for those cells that have incorporated the highest number of gene copies. Cell lines with up to 2000 copies of the foreign gene per cell can be isolated by the use of suitable selection systems and this provides a means of achieving high-level gene expression in mammalian cells.

The *Escherichia coli* genes that code for the enzymes homoserine kinase (*thrB*) and threonine synthase (*thrC*) could be suitable candidates for a selectable gene system. These enzymes synthesize threonine, which is not normally synthesized by animal cells, from its non-protein amino acid precursor homoserine. Homoserine is an analogue of threonine that is toxic to animal cells when it is present in the medium at high concentrations. In *E. coli*, homoserine is converted into threonine in a two-step reaction. The first enzyme, homoserine kinase, phosphorylates homoserine using ATP as the phosphate donor to produce homoserine *O*-phosphate. The second enzyme, threonine synthase, can then utilize homoserine *O*-phosphate to produce threonine. The *E. coli* genes for homoserine kinase and threonine synthase can be expressed in mouse 3T3 cells by placing the *E. coli* coding sequence under the control of the simian virus 40 (SV40) promoter and polyadenylation sequences (Rees et al., 1992; Rees and Hay, 1993). This allows the cells to utilize homoserine for the synthesis of threonine and permits growth in a threonine-free medium.

In the present paper, we report the combination of homoserine kinase and threonine synthase genes into a single construct which has been used to transfect animal cells. The transfected cells can survive and multiply without the essential amino acid threonine when grown in media containing homoserine. Unexpectedly we found that, unlike other selectable genes, the number of copies incorporated decreases as the homoserine concentration used for selection is increased.

MATERIALS AND METHODS

General

The plasmid pSVpoly was a generous gift from Dr. A. Stacey, Colorado State University, Fort Collins, CO, U.S.A., and pMC1neoPA was from Stratagene. Enzymes were supplied by Pharmacia, Boehringer or Northumbria Biologicals. Recombinant DNA methods were as described by Sambrook et al. (1989). L-[U-^{14}C]threonine (226 mCi/mmol) and L-[U-^{14}C]homoserine were supplied by Amersham International.

Abbreviations used: 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.

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Tissue culture

Mouse 3T3 cells, CHO, HeLa and COS-7 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wilts., U.K.), and the cells were grown in the media described previously (Rees and Hay, 1993). The cells were transfected with DNA by the method of Parker and Stark (1979) using a calcium phosphate precipitate. Because of the differences in the molecular sizes of the different plasmids, the cells were transfected with the equivalent of 10 μg of pSVthrC2 per 6 cm dish, i.e. 11.4 μg of pSVthrB1 per dish, 7.6 μg of pSVthrB2 per dish and 12.5 μg of pSVthrBC per dish, so as to preserve the molar ratio of plasmid copies. Briefly, 5×10^5 cells (for transient-expression experiments) were plated in a 6 cm Petri dish and cultured overnight. The following day the cells were transfected with a mixture of DNA in Hepes-buffered saline (pH 7.10) containing sodium phosphate and $CaCl_2$. The cells were incubated with the transfection mixture for 1 h, and then 2 ml of medium was added and the incubation continued for a further 4 h. The cells were then subjected to osmotic shock by the addition of sterile Tris-buffered saline (2.7 mM KCl/1.5 mM KH_2PO_4/137 mM NaCl/10 mM Tris, pH 7.5) containing 10% glycerol. The same transfection procedure was used to establish stable cells lines except that the cells were at a lower density of 1×10^5 cells/6 cm dish. 3T3 cells were co-transfected with 25 μg of the test plasmid and, if required, 2.5 μg per dish of the plasmid pMC1neoPA. Then 24 h after transfection, the cells were transferred to medium containing G-418 (400 μg/ml) or homoserine-supplemented threonine-free medium as required. Threonine-free medium was prepared by supplementing threonine-free Dulbecco's modified Eagles medium with 5% (v/v) dialysed newborn calf serum, insulin, vitamins, transferin/selenium (Sigma) and epidermal growth factor (Sigma; 10 ng/ml) (Rees and Hay, 1993).

Preparation of [^{3}H]homoserine

DL-[^{3}H]Homoserine was prepared by isotope exchange with tritium gas (Amersham TR-2) of L-homoserine. The crude reaction mixture was purified by paper chromatography using Whatman 3MM paper and a butanol/acetic acid/water (12:3:5, by vol.) solvent system. The radioactive product co-migrated with an authentic homoserine standard when analysed by t.l.c. on cellulose t.l.c. plates (Merck). Analysis with D- and L-amino acid oxidases revealed that approximately 60% of the product was D-[^{3}H]homoserine.

DL-[^{3}H]Homoserine was resolved by oxidation of the D-isomer with D-amino acid oxidase. DL-[^{3}H]Homoserine was diluted to give a specific radioactivity of 100 mCi/mmol by the addition of unlabelled L-homoserine (Sigma), and 10 mCi was dissolved in 2 ml of 20 mM sodium phosphate buffer (pH 7.2) containing 5 mg/ml D-amino acid oxidase (Sigma). The mixture was gassed thoroughly with oxygen and incubated at 37 °C for 24 h. The product was applied to a column (1.2×5.0 cm) of Amberlite GC-120 (Na^+) which had been equilibrated with 0.2 M sodium citrate buffer, pH 3.1. Buffer (50 ml) was passed through the column and 10 ml fractions were collected. The radioactivity was distributed between two peaks, the second of which, containing approximately 50% of the total activity, was collected and applied to a column (1.2 cm × 5.0 cm) of Dowex 50 (H^+), washed with 20 ml of 0.01 M HCl and eluted with 10 ml of 2 M NH_4OH. The NH_4OH fractions were concentrated using a rotary evaporator, with repeated additions of distilled water; the concentrated eluate was not allowed to dry completely. More than 95% of the radioactivity co-chromatographed with an authentic homoserine standard. Oxidation with D- and L-amino acid oxidases suggested that the final product was still contaminated with approx. 10% D-[^{3}H]homoserine.

Enzyme assays

The assay of homoserine kinase has been described previously (Rees et al., 1992). The assay of homoserine-derived threonine incorporated into protein used the same method as described previously (Rees and Hay, 1993) except that in the experiments described here L-[^{3}H]homoserine tracer was used. The threonine synthase reaction results in the loss of hydrogen atom from both C-2 and C-3 of the homoserine molecule (Flavin and Slaughter, 1960), and this produces differences in the amount of label incorporated from L-[^{3}H]homoserine and L-[^{14}C]homoserine.

Some 79% of the L-[^{3}H]homoserine was phosphorylated when it was treated with a semipurified preparation of *E. coli* homoserine kinase and ATP. When L-[^{3}H]homoserine *O*-phosphate was converted into threonine with an *E. coli* enzyme extract, 9.1% of the radioactivity was retained in the threonine produced. When the ^{3}H radioactivity incorporated into [^{3}H]threonine in cells expressing homoserine kinase and threonine synthase was corrected for the loss of ^{3}H to water, the L-[^{3}H]homoserine and L-[^{14}C]homoserine tracers gave identical rates for the synthesis of threonine. Hydrolysis of the cell proteins from 3T3 cells expressing homoserine kinase and threonine synthase cultured in L-[^{3}H]homoserine showed only a single labelled product which corresponded to an authentic threonine standard.

Amino acid uptake

The uptake of homoserine and threonine was studied in 24-well plates using the protocol of Gazzola et al. (1981). 3T3 cells were plated at 1×10^5 cells per well in 1 ml of medium. When the cells were confluent, the medium was removed and replaced with Hanks balanced salts medium containing 25 mM D-glucose. After 30 min, this medium was removed and replaced with 0.2 ml of the same medium containing L-[^{3}H]homoserine (0.5 μCi/well). The uptake was stopped by the addition of 2 ml of ice-cold PBS containing 1 mM $HgCl_2$. The cell monolayer was washed with a further 2 ml of the same solution. The cells were lysed by the addition of 0.22 ml 5% (w/v) trichloroacetic acid. After 15 min incubation at room temperature, 0.2 ml of the supernatant was removed and counted for radioactivity. The cell protein that adhered to the bottom of the well was dissolved in 0.3 M NaOH, and samples were taken for protein determination. The progress curve for the uptake of 10 μM homoserine remained linear for approx. 90 s.

RESULTS

Construction of pSVthrB2 and pSVthrBC

Our previous studies had suggested that the SV40 early-promoter vector pSVpoly (Stacey and Schnieke, 1989) improved the expression of *E. coli* threonine-synthesis genes in mouse 3T3 cells compared with the SV40 late-expression vector pSVL. Homoserine kinase (*thrB*) was expressed in mouse cells from the plasmid pSVthrB1 which was based on the SV40 late promoter (Rees et al., 1992). To compare the SV40 early and late promoters directly, a second construct with the *thrB*-coding region in the vector pSVpoly was prepared. The *thrB* coding region was excised from pSVthrB1 by digestion with *Bam*HI and *Xba*I, and ligated into the same sites in the multiple cloning site of pSVpoly. A recombinant plasmid pSVthrB2 with the expected restriction map was isolated from *E. coli* DH5α (Figure 1).

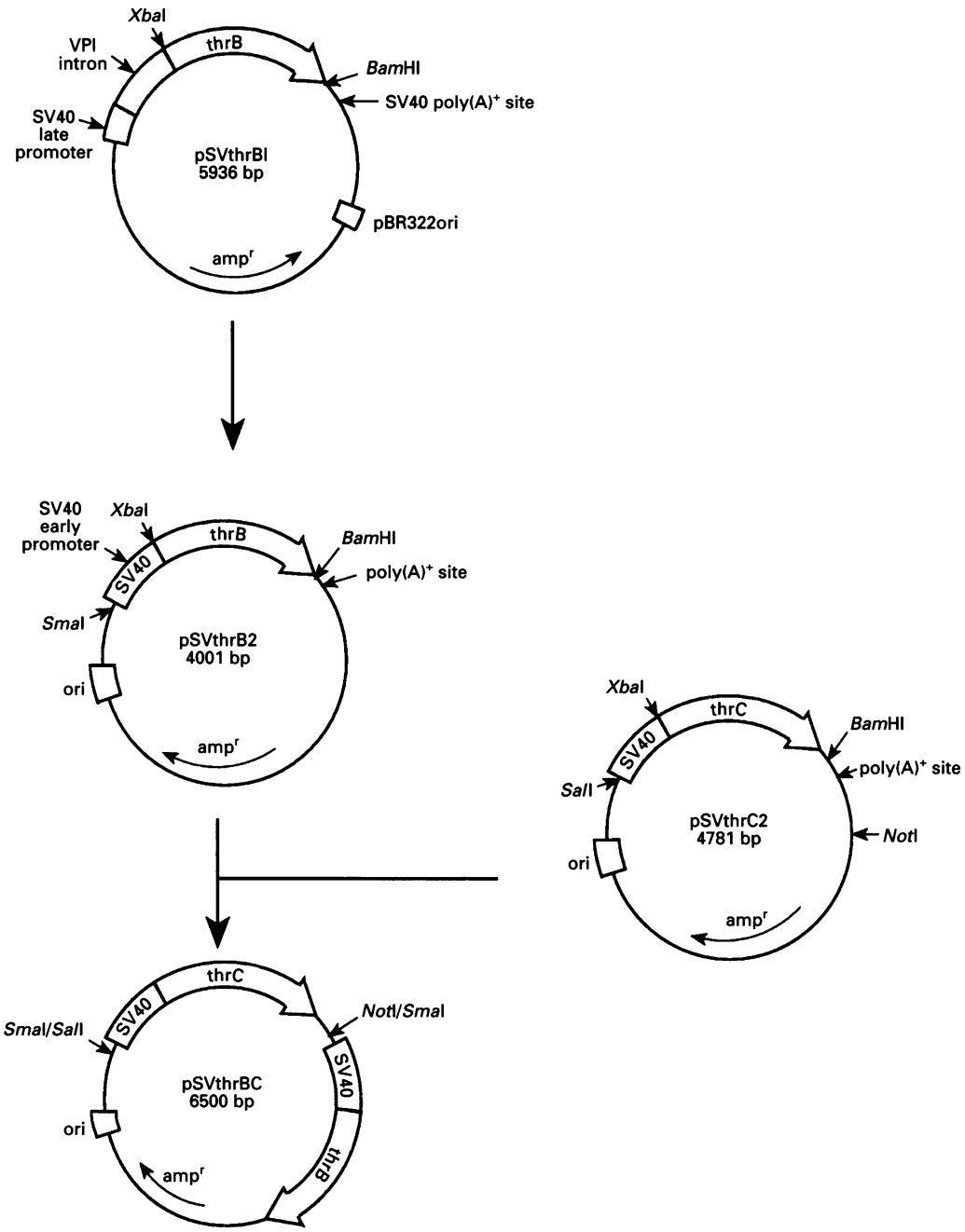

Figure 1 Construction of pSVthrB2 and pSVthrBC

A 0.9 kb *XbaI*–*Bam*HI fragment of pSVthrB1 which contained the *E. coli thrB*-coding region was cloned into the multiple cloning site of the vector pSVpoly to produce pSVthrB2. A 2.6 kbp *SalI*/*NotI* fragment of pSVthrC2 which contained the SV40/*thrC*-expression cassette was cloned into a unique *SmaI* site in pSVthrB2 to produce pSVthrBC.

Homoserine kinase and threonine synthase (*thrC*) activities were combined into a single plasmid as shown in the schematic diagram in Figure 1. The SV40/*thrC*-expression cassette was removed from pSVthrC2 (Rees and Hay, 1993) by digestion with *Sal*I and *Not*I. The overhanging ends of the fragment were end-filled using deoxynucleotide triphosphates and the Klenow fragment of DNA polymerase I. pSVthrB2 was digested at a unique *Sma*I site 5′ to the SV40 promoter outside the SV40/*thrB*-expression cassette. A 2:1 ratio of the SV40/*thrC* fragment and pSVthrB2 digested with *Sma*I and treated with alkaline phosphatase was then used in the subsequent ligation. The mixture was used to transform *E. coli* DH5α. In two separate syntheses and transformations, only plasmids that had both genes in the same orientation (shown in Figure 1) were obtained. Although it was possible for the SV40/*thrC* gene to be ligated into pSVthrB2 in either orientation, there were no transformants in which the two promoters were close together.

Threonine synthesis and enzyme activity in mouse 3T3 cells

The homoserine kinase activity of the constructs was measured in transient-expression experiments, and the results are shown in

Table 1 Homoserine kinase activity and threonine synthesis in transient expression

For the estimation of homoserine kinase, 3T3 cells were transfected with the equivalent of 10 μg of pSVthrB2 per 6 cm dish. After 48 h, the homoserine kinase activity of cell-free extracts was measured, and the data ($\pm$S.D. $n = 3$) given are averages of three experiments. Threonine synthesis was determined in cells transfected with either pSVthrB1 or pSVthrB2 and pSCthrC2 or with pSVthrBC alone. Three dishes of cells were used for each treatment and the results from two experiments are shown.

Plasmid	Homoserine kinase activity (nmol/min per mg of protein)	Homoserine-derived threonine incorporated (nmol/mg of protein)	
		Experiment 1	Experiment 2
pSVthrB1	0.0080 $\pm$ 0.0025	0.29	0.29
pSVthrB2	0.2003 $\pm$ 0.0727	0.42	0.44
pSVthrBC	0.0711 $\pm$ 0.0430	0.60	0.49

Table 1. Assuming that the transfection efficiency was similar for both plasmids, then cells transfected with pSVthrB2 express at least twice as much enzyme activity as those transfected with a similar number of copies of pSVthrB1.

We also examined the activity of the constructs by following the synthesis of threonine from homoserine (Table 1). After being co-transfected with either pSVthrB1 or pSVthrB2 and an equivalent amount of pSVthrC2 which encodes threonine synthase, the cells were grown in medium containing [^{3}H]homoserine so that the threonine produced was incorporated into the cell protein. The increased homoserine kinase activity of the pSVthrB2 construct is reflected by increased threonine production in cells transfected with this plasmid compared with cells transfected with pSVthrB1. In the cells that had been transfected with pSVthrBC alone, threonine synthesis was further improved with this plasmid recording the highest incorporation. This may in part be the result of all cells receiving an equal dose of both genes on transfection. Alternatively the close proximity of the two transcriptional units in the plasmid may lead to interactions that enhance expression of one or both genes.

Analysis of mRNA

Interactions between the threonine synthase and homoserine kinase genes when they are placed close together in pSVthrBC might be expected to appear as changes in the size or quantity of the mRNAs produced. A stable cell line expressing pSVthrB2 was produced by co-transfecting the plasmid together with a second plasmid pMC1neoPA coding for G-418 resistance. Of the six representative cell lines isolated from cells transfected with pSVthrB2 and selected for G-418 resistance, four expressed homoserine kinase activity. The best, 3T3 B2-3, produced 0.34 $\pm$ 0.05 nmol of homoserine *O*-phosphate/min per mg of protein. A cell line transfected with pSVthrBC was obtained by selecting for growth on medium containing 5 mM homoserine (see below), and single colonies were isolated by plating the cells at a low density. One of these colonies was used to isolate the cell line 3T3 BC1 which was used in these experiments.

Total RNA was isolated from the different cell lines, and Northern blots were probed with ^{32}P-labelled probes prepared from either the *Xba*I–*Bam*HI fragment of pSVthrB2 or a 1.4 kbp *Eco*RI fragment of pSVthrC2. Cell lines expressing homoserine kinase showed a single RNA species of approx. 1.5 kb which hybridized with the *thrB* probe (Figure 2a). In the blot probed for

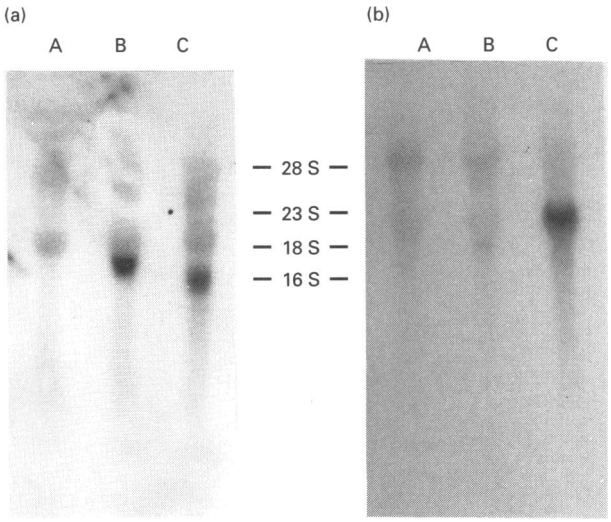

Figure 2 Total cell RNA (20 μg/lane) from lines 3T3 (lane A), 3T3 B2-3 (lane B) and 3T3 BC1 (lane C) probed for *thrB* (a) and for *thrC* (b)

The positions of the 18 and 28 S eukaryotic ribosomal RNAs and 16 and 23S prokaryotic ribosomal RNAs used as standards are indicated.

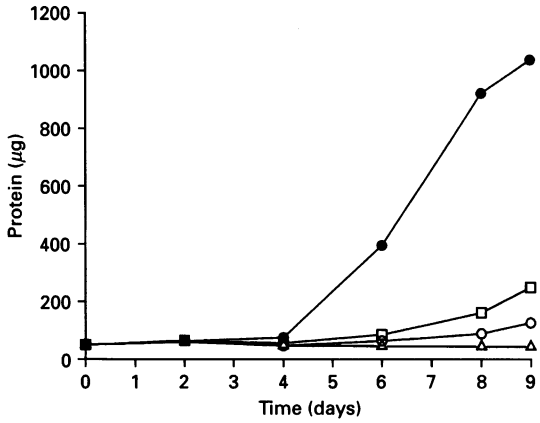

Figure 3 Growth of mouse 3T3 cells in homoserine-containing threonine-free medium

Cells were grown in threonine-free medium containing 2 mM threonine (●) or 0.5 mM (□), 1.0 mm (○) or 10 mM (△) homoserine. Each time point represents the average protein content of three dishes.

thrC (Figure 2b), only 3T3 BC1 expressed a single *thrC* mRNA with a size of approx. 1.7 kb, similar to the pSVthrC2 transcript reported previously (Rees and Hay, 1993). The *thrB* transcript produced by pSVthrBC is slightly smaller than the corresponding transcript from pSVthrB2, seen from its position relative to the non-specific labelling of the 18S ribosomal RNA. A similar sized transcript was seen in another cell line expressing pSVthrBC (results not shown), suggesting that the larger transcript is a feature of 3T3 B2-3. The SV40/*thrB*-expression cassette has not been modified in either the promoter or the region immediately 3′ to the polyadenylation site in either construct, and it is likely that the small change in mRNA length is due to components gained when pSVthrB2 integrated into the cell DNA in 3T3 B2-3. Despite the close proximity of the two transcriptional units in pSVthrBC, this would appear to be the only difference in the

Table 2 Characteristics of clones isolated in different homoserine concentrations

3T3 cells were transfected with pSVthrBC and selected for growth in medium containing 5, 10 and 25 mM homoserine. The cells from two separate transfections and selections (5-1 and 5-2 etc.) were analysed for the incorporation of homoserine-derived threonine and homoserine kinase activities. The results for homoserine kinase activity are from three experiments (±S.D., $n = 3$).

Homoserine concentration (mM)	Transfection number	Number of colonies per dish	Homoserine-derived threonine incorporated (nmol/mg of protein)	Homoserine kinase activity (nmol/min per mg of protein)
5	1	10	30.5	0.121 ± 0.041
5	2	9	28.5	0.240 ± 0.051
10	1	7	27.5	0.193 ± 0.083
10	2	11	31.0	0.145 ± 0.082
25	1	5	28.3	0.123 ± 0.053
25	2	6	33.3	0.395 ± 0.057

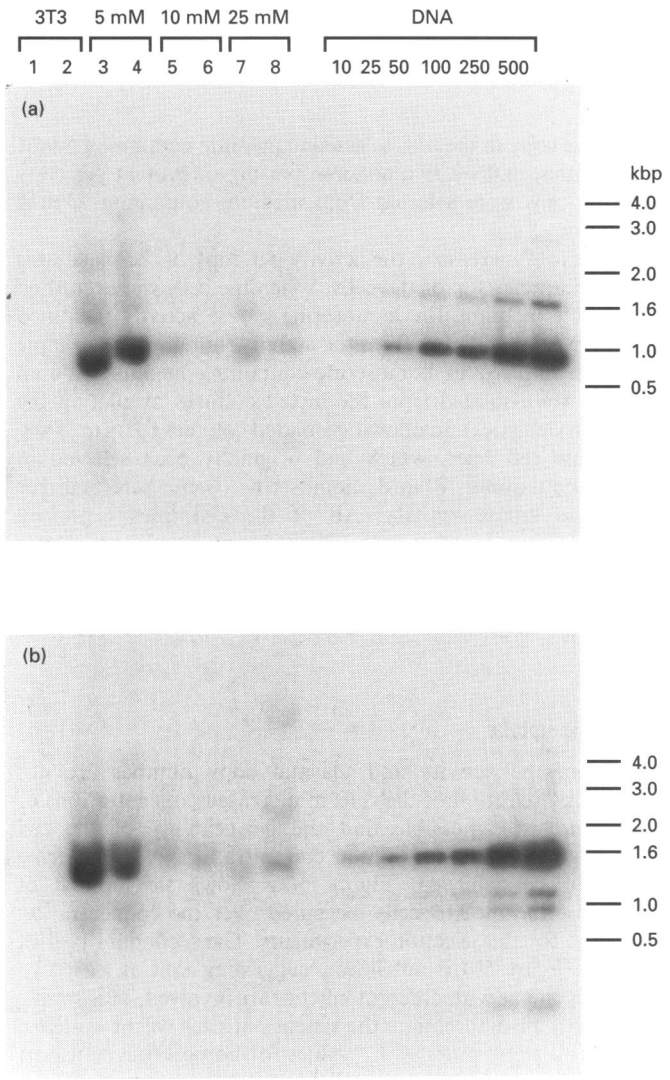

Figure 4 Southern blot of genomic DNA from cells transfected with the plasmid pSVthrBC

Genomic DNA (10 μg) was digested with *Hin*dIII and *Bam*HI and separated on a 0.6% agarose gel. The samples are from two separate transfections of 3T3 cells selected for growth in medium containing 5 mM homoserine (5-1 lane 3 and 5-2 in lane 4), 10 mM homoserine (10-1 in lane 5 and 10-2 in lane 6) and 25 mM homoserine (25-1 in lane 7 and 25-2 in lane 8). Lanes 1 and 2 contained DNA from non-transfected 3T3 cells. The lanes on the right-hand side contained a 10–500 pg serial dilution of the plasmid DNA digested with *Hin*dIII and *Bam*HI. (**a**) Blot probed for *thrB* and (**b**) blot probed for *thrC*.

messages produced. The proportion of the *thrC* message relative to *thrB* seemed to be similar, and there was no evidence for interactions between the two promoters.

Selection for growth on homoserine-containing medium

To determine the minimum concentrations of homoserine required to produce an effect on the growth and survival of 3T3 cells, the cells were cultured in threonine-deficient medium containing increasing concentrations of homoserine. The amount of protein synthesized is shown in Figure 3. When the homoserine concentrations were less than 1 mM, growth was inhibited but the cells were not killed. However, 10 mM homoserine caused the total amount of protein to decrease, and, under the microscope, cell death could be observed. A concentration of 2.5 mM was found to be the minimum at which this toxic effect could be observed (results not shown).

Homoserine-resistant 3T3 cells were produced by transfecting with 10 μg of pSVthrBC per dish. After 24 h, the medium was changed to a threonine-free medium containing 5, 10, 25 or 50 mM homoserine. When the cell culture was continued for 21–28 days, the vast majority of cells died, leaving a number of isolated viable colonies (Table 2), except for the cells in medium containing 50 mM homoserine where all of the cells were killed and no colonies were observed. In a similar experiment, transfections with pMC1neoPA yielded 20–30 G-418-resistant colonies. The larger size of pSVthrBC compared with pMC1neoPA may be responsible for reducing its transfection efficiency but the need for both the *thrB* and *thrC* genes to be functional may also influence the chances of a successful integration event.

The homoserine-resistant cells were harvested as mixed cell lines, and their capacity for threonine synthesis, homoserine kinase activity and gene copy numbers were determined. All of the homoserine-resistant cells were able to synthesize threonine and showed homoserine kinase activity (Table 2). Regardless of the homoserine concentration used for selection, the homoserine kinase activity and threonine production rates were similar and there was little or no amplification of the enzyme activity.

The number of plasmid copies incorporated into the genomic DNA in each of the cell lines was determined by Southern blotting. Figure 4 shows blots of chromosomal DNA digested with *Hin*dIII and *Bam*HI hybridized to ^{32}P-labelled probes for either *thrB* or *thrC*. *Hin*dIII and *Bam*HI digestion produces a fragment of 1050 bp from the *thrB* portion of pSVthrBC and a 1700 bp fragment from *thrC*. The copy number can be determined by comparing the extent of probe hybridization to the genomic DNA with that of a serial dilution of the digested plasmid shown on the right hand side of the blot. The haploid DNA content of

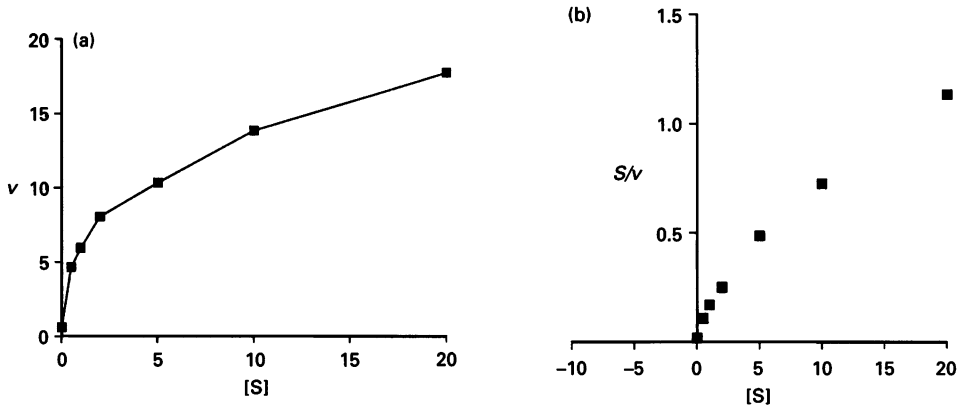

Figure 5 Uptake of homoserine by mouse 3T3 cells

The cells were mixed with L-[^{3}H]homoserine and incubated for 37 °C for 45 s before the uptake was stopped by the addition of ice-cold stopping solution. (**a**) Rate of homoserine uptake at different external homoserine concentrations. (**b**) The same data plotted as S/v versus S. The data are averages of three experiments.

Table 3 Incorporation of radioactivity from L-[^{3}H]homoserine into the cell proteins of mammalian cell lines

CHO, HeLa and COS cells were transfected with pSVthrBC and selected for growth in a threonine-deficient medium containing 10 mM homoserine. CHO and HeLa cells resistant to homoserine from two separate transfections (pSVthrBC-1 and pSVthrBC-2) were harvested as mixed cell lines. These cells together with non-transfected controls were cultured in threonine-deficient medium containing 10 μM L-[^{3}H]homoserine, and the incorporation of radioactivity into the cell proteins was measured. The data are averages of four separate determinations.

Cell line	Radioactivity incorporated (c.p.m./μg of protein)
CHO control	0.34
CHO + pSVthrBC-1	2.45
CHO + pSVthrBC-2	7.89
HeLa control	0.56
HeLa + pSVthrBC-1	19.29
HeLa + pSVthrBC-2	14.29
COS-7 control	4.30

mammalian cells is approximately 3×10^9 bp (Sambrook et al., 1989), but the 3T3 cell is almost tetraploid (Pollock et al., 1970a,b) giving the 3T3 cell a genome size of approximately 12×10^9 bp, compared with the pSVthrBC plasmid which is 6500 bp. Therefore a cell line with one copy per cell would be expected to contain 5.4 pg of plasmid DNA per 10 μg of chromosomal DNA. In the blots shown in Figure 4, the expected fragments are present in all cell lines except the non-transfected controls. The cells selected in 5 mM homoserine appear to have integrated 20–100 copies of the plasmid in each cell. As the probe hybridizes to the expected restriction fragments, both the *thrB* and *thrC* coding regions are evidently intact in a large number of the plasmids that have become inserted into the cellular DNA. As the homoserine concentration is increased, the number of copies integrated is reduced, and the hybridization shows that the cells selected in 10 and 25 mM homoserine have only one or two copies of the plasmid per cell. Despite this reduced copy number, there is no corresponding decrease in the enzyme activity which remains the same in the cells with one or two copies as it does in the cells with 20 or more copies. The homoserine kinase activity per copy rises from 0.001–0.012 nmol/min per mg of protein per copy in the cells selected in medium containing 5 mM homoserine to 0.06–0.39 nmol/min per mg of protein per copy when the cells were selected from medium containing 25 mM homoserine.

This tenfold increase in the activity per copy of the plasmid is a stable characteristic of the cells. When the cells were returned to complete medium, the homoserine kinase activity remained high and was no different from the activity found when the same cells were cultured in homoserine-containing medium. Clonal cell lines were isolated from the mixed cultures by plating the cells at low densities and allowing isolated colonies to form. Then 15 of these cell lines, which had originally been selected in medium containing 10 mM homoserine, were screened for homoserine kinase activity. All of the cell lines expressed homoserine kinase activity with a range from 0.6 to 0.1 nmol/min per mg of protein. The addition of 10 mM homoserine to the culture medium 24 h before the enzyme assay had no significant effect on the homoserine kinase activity of any of the cell lines tested.

Homoserine uptake

As the enzyme activity and plasmid copy number are not amplified by selection of the cells in increased concentrations of homoserine, it is possible that the permeability of the cell membrane limits the intracellular concentrations of homoserine which can be achieved. Figure 5(a) shows the uptake of [^{3}H]homoserine by 3T3 cells measured over the concentration range used for the selection experiments. The secondary plot of the data (Figure 5b) is not linear, suggesting that at least two transport systems with different kinetics are involved. This simple analysis cannot differentiate the kinetics of each of the systems, but the data show that the K_m values for homoserine transport range from about 10 μM to 5 mM. Homoserine is very similar in charge and structure to threonine, and the time course of the uptake of 10 μM homoserine by 3T3 cells is almost identical with that of threonine (results not shown). Threonine has been shown to be a substrate for both Na^+-dependent and Na^+-independent transporters (Gazzola et al., 1981), and it is likely that both amino acids share the same transport systems.

When the homoserine concentration in the medium rises above 5 mM, its uptake begins to approach its maximum rate. The enzyme activity of transfected cells isolated in 5 mM homoserine

should therefore be sufficient to render them resistant to 25 mM homoserine. When cells from lines isolated from medium containing 5 mM homoserine were cultured in medium containing 25 mM homoserine, they grew normally and their growth rate was no different from that of a control group cultured in medium containing 5 mM homoserine (results not shown). Hence a homoserine kinase activity of about 0.2 nmol/min per mg of protein is sufficient to render cells resistant to homoserine concentrations greater than 5 mM.

pSVthrBC as a selectable marker in other cell types

To assess pSVthrBC as a selectable marker in cells from other species, some commonly used cell lines were transfected with pSVthrBC and cultured in homoserine-containing media. CHO, HeLa and COS-7 cells were transfected with pSVthrBC, and, after 24 h, were transferred to threonine-deficient medium containing homoserine. The experiments with 3T3 cells had suggested that it was not essential to eliminate free threonine from the medium, so for these experiments a threonine-deficient medium supplemented with complete newborn calf serum and homoserine was used. With both CHO and HeLa cells, viable colonies began to appear after 21–28 days when the majority of cells had died and become detached from the dish. The surviving cells were harvested as mixed cell lines. The COS-7 cells did not appear to be sensitive to 10 mM homoserine and continued to grow normally so that we were unable to isolate any transfected cells.

The cells were then cultured in threonine-deficient medium containing L-[^{3}H]homoserine. The homoserine-resistant CHO and HeLa cells transfected with pSVthrBC incorporated significantly more radioactivity from homoserine into their cell proteins than the non-transfected parent lines (Table 3). Assuming that this radioactivity is all associated with threonine, the CHO cells had incorporated 8.6–27.8 nmol of homoserine-derived threonine/mg of protein, and the HeLa cells had incorporated 48.6–65.6 nmol of homoserine-derived threonine/mg of protein. These values are very similar to those obtained with 3T3 cells transfected with pSVthrBC (30 nmol of homoserine-derived threonine/mg of protein), and strongly suggest that both CHO and HeLa cells resistant to homoserine can convert it into threonine at a very similar rate to 3T3 cells.

Non-transfected COS-7 cells also incorporate significantly more radioactivity from homoserine into the cell proteins than the control 3T3, CHO or HeLa cells. As COS-7 cells are derived from a kidney epithelium, this could well be due to the presence of the enzyme cystathionine γ-lyase which is expressed in this tissue. This enzyme converts homoserine into 2-oxobutyrate which is oxidized through the tricarboxylic acid cycle and would allow radioactivity from homoserine to be used for the synthesis of non-essential amino acids. An ability to oxidize homoserine explains why COS-7 cells are not subject to the toxic effects of homoserine as it is cleared from the cell by metabolism.

DISCUSSION

By combining the homoserine kinase and threonine synthase activities into a single construct, we have produced a plasmid that allows animal cells to grow in a threonine-free medium and overcome the toxic effects of high concentrations of homoserine. In cells that are unable to metabolize homoserine, it is possible to select cell lines that have incorporated the pSVthrBC into their genome by selecting for cells resistant to homoserine. It is likely that a large part of the selective advantage is due to the detoxification of homoserine rather than the provision of a supply of threonine, as this selection can be carried out in media that contain some free threonine. There is some evidence that even a small supply of free threonine can protect cells against homoserine toxicity, and higher concentrations of homoserine are required for complete cell killing in threonine-containing media.

Because the selection requires relatively high concentrations of homoserine, close to those that saturate homoserine entry into the cell, pSVthrBC behaves differently from other selectable plasmids. Provided that the rate of homoserine removal by its conversion into threonine is greater than the rate of uptake, the homoserine concentration will remain below toxic levels. Cells selected in 5 mM homoserine express sufficient activity to grow in 25 mM homoserine, and there is no amplification of the plasmid copy numbers as seen with other genes such as glutamine synthase (Cockett et al., 1990) or asparagine synthase (Cartier et al., 1987).

Indeed the unusual feature of this plasmid is that when the selection uses a high concentration of homoserine in the medium, the total number of plasmid copies integrated is reduced and there is a compensatory increase in the activity per gene copy. This enhancement is stable and the enzyme activity remains high when the cells are cultured in complete medium. This suggests that, rather than an effect on enzyme or mRNA stability, the increase is due to the transcriptional activity of the inserted gene. It is well known that the activity of foreign genes inserted into the chromosome varies depending on the nature of the integration site. Integration near to an endogenous enhancer can markedly increase the expression compared with a similar insertion in another site. It would appear that this is the case with pSVthrBC, as high concentrations of homoserine are apparently selecting for cells where the plasmid has integrated into these more active regions.

A possible explanation for this phenomenon is that homoserine is a good substrate for amino acid transport, and, at very high concentrations, would strongly inhibit the uptake of the other amino acids that share the same transporters. This would have the effect of starving the cells of the other essential amino acids that are taken up through the same route and placing the cells under additional stress. The selection pressure for cells in 25 mM homoserine is not just the provision of threonine and the removal of toxic homoserine but also for efficient growth when starved of other amino acids. This extra pressure selects for cell lines in which the integration site is most active and against cells with multiple low-activity integrations.

Threonine synthesis from homoserine adds a new selectable marker to those available for mammalian cell transformations. The direction of pSVthrBC to transcriptionally active regions of the genome may be of use in the identification of enhancers and other chromosomal components that influence promoter activity. We are also interested in using this selection to introduce the remaining parts of the *E. coli* threonine pathway. The two genes that code for the enzymes to produce homoserine can now be inserted by co-transfecting with pSVthrBC and selecting for homoserine-resistant cells. The ability to modulate the copy number by means of the amount of homoserine used is also potentially valuable in allowing us to adjust the number of copies of the gene controlling the first step in the pathway.

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