Selection markers and expression enhancing elements: novel strategies to create mammalian production cell lines
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GENES INVOLVED IN THE SYNTHESIS OF THE AMINO ACID L-THREONINE CAN SERVE AS ‘MAINTENANCE’ MARKER TO CONVEY LONG-TERM STABILITY OF PROTEIN EXPRESSION IN CHO-K1 CELLS

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ABSTRACT

Keeping high expression levels of therapeutic proteins in mammalian cells stable over prolonged periods of time is difficult and often the continuous presence of toxic selection agents in the culture medium is needed. Here we tested the characteristics of the essential amino acid L-threonine for use as selection principle in mammalian cells. We employed co-transfection of the thrB and thrC genes, which are both involved in the synthesis of L-threonine. We found that removal of L-threonine from the culture medium resulted in a very low killing rate of wild type CHO-K1 cells. This characteristic excluded the use of the thrB/thrC genes for direct selection of colonies on L-threonine minus medium. Stable colonies were therefore first selected by means of another selection agent, such as Zeocin or Neomycin. When cells were subsequently switched to L-threonine minus culture medium, high protein expression levels remained stable over prolonged periods of time. During that ‘maintenance’ period, no Zeocin or Neomycin was needed for stability of protein expression. The results show that the thrB/thrC genes can be used ‘maintenance’ marker to achieve a high level of stability of protein expression in CHO-K1 cells.

INTRODUCTION

Novel genes that are introduced to an organism or cell lines are important biotechnological means to produce for instance monoclonal antibodies against various forms of human cancers. A problem is that these so-called transgenes are prone to gene silencing and that their ability to produce high protein expression levels is very limited. Gene silencing is thought to involve chromatin-associated repressor complexes, such as the Polycomb group proteins and heterochromatin-related proteins. Previously, we identified STAR elements, stretches of DNA that block the gene repressing abilities of Polycomb-group proteins and heterochromatin-related proteins1. When these STAR elements are utilized to flank gene expression cassettes, gene expression levels were strongly elevated. Also, they induce the formation of many more colonies than plasmids without STAR elements. A population of colonies consists of an even distribution of low to high protein expressing colonies. Within a larger population, more colonies have to be isolated to identify a high-protein expressing colony. To circumvent this, one would like to reduce in particular the low-expressing colonies. To achieve this, we developed a very stringent selection system to create relatively few, but highly productive mammalian cell lines2. This selection system involves the use of startcodons that confer attenuated translation initiation frequency. These codons are used for translation of the selection marker that is placed upstream of a gene of interest that has a startcodon with optimal translation initiation properties. The modified startcodon can be a GTG or TTG instead of an ATG, resulting in selection markers such as GTG Zeocin (GTG Zeo), TTG Zeocin (TTG Zeo) or TTG Neomycin (TTG Neo). The selection marker is translated at a low frequency from the transcribed bicistronic mRNA due to the attenuated translation initiation codon. In order to produce enough selection marker protein for the cell to survive, high amounts of bicistronic mRNA levels have to be expressed. Since the bicistronic mRNA also encompasses the mRNA of the gene of interest that has an optimal translation initiation codon, concomitant high levels of the protein of interest will be produced. In order to achieve these high mRNA levels, STAR elements have to be incorporated in such constructs. This procedure results in the formation of clones that express proteins at high levels. We called this selection system STAR-Select2,3.
Once cell clones are obtained that are characterized by high protein expression levels, keeping these protein expression levels high over prolonged periods of time remains a problem. STAR elements do not convey long-term stability of protein expression when used in the context of the STAR-Select system. The simplest means to maintain high protein expression levels is to keep selection agents such as Zeocin or Neomycin constantly present in the culture medium. However, this procedure is not favored since such agents are both toxic and expensive.

Another approach is to couple the gene of interest to an enzyme that metabolizes one or more essential steps in a metabolic pathway. Essential signifies that the cell is not able to synthesize the specific metabolic building blocks itself. For the cell to survive, the essential metabolic building blocks need to be present in the culture medium. The enzyme involved in the synthesis of this building block can be used as selection marker. Therefore, besides for instance the TTG Zeo gene, an expression cassette can be equipped with a gene encoding a specific, essential metabolic building block. In culture medium devoid of the essential metabolic building block, this may keep the cells under constant selection pressure, once stable clones have been established through TTG Zeo or TTG Neo. The advantage is that initial high protein expression can be achieved through the TTG Zeo or TTG Neo selection markers, and that these high expression levels can be maintained, without the need to keep Zeocin or Neomycin in the culture medium.

Well-known examples of essential metabolic building blocks are the essential amino acids. These cannot be synthesized by a mammalian cell and need to be present in the culture medium to allow the cell to survive. One such example is L-threonine. Which mammalian cells are unable to synthesize. Therefore L-threonine is an essential amino acid for mammals and they need the amino acid as food supplement. Likewise, cultures of mammalian cells require L-threonine in the culture medium in order to survive. Two bacterial enzymes (E.coli) catalyze the synthesis of L-threonine from a precursor, L-homoserine. These enzymes are homoserine kinase (designated thrB), which catalyzes the conversion of L-homoserine to O-phospho-L-homoserine, and this product is converted to L-threonine by threonine synthase (designated thrC).\(^4\) Previously, Rees et al. showed that thrB can be expressed in an animal cell and that it can successfully phosphorylate L-homoserine. A cell line which expressed both the thrB and thrC genes was capable of growth in L-threonine-deficient medium containing L-homoserine and thrB and thrC were used to select for transfected cells. In these experiments, the constructs only harbored the thrB and thrC genes to enable production of L-threonine from L-homoserine. Our aim, however, is to use the thrB and thrC genes for high-level expression of a reporter gene of interest.

In principle, the thrC gene alone could be used as selection marker when incorporated into the expression plasmid, provided that the end-product L-threonine is absent and the precursor O-phospho-L-homoserine is present in the culture medium. Unfortunately, the usefulness of O-phospho-L-homoserine in combination with transfection of the thrC gene is not easy to assess, since O-phospho-L-homoserine is not commercially available. However, the thrB gene can produce O-phospho-L-homoserine, when the readily available precursor L-homoserine is present in the culture medium. Therefore, with both thrB and thrC genes placed on the expression plasmid, this combination should work as selection marker in the absence of L-threonine and the presence of L-homoserine. Here, we tested this possibility, in combination with our very stringent STAR-select expression system, to enable high expression levels of reporter genes of interest.
RESULTS

thrB and thrC genes convey highly stable protein expression in stably transfected clones

Like all mammalian cells, Chinese Hamster Ovary (CHO) -K1 cells lack the thrB and thrC genes and these cells therefore need L-threonine in the culture medium to survive. We first tested how long wild type CHO-K1 cells survive in the absence of L-threonine. Tryphan blue staining was used to monitor the viability of cells. Figure 1B shows that after one week approximately 25% of the CHO-K1 cells had died and after two weeks only ~40% cells had died. In contrast, addition of 150 μg/ml Zeocin already lead to 50% cell death after three days and >90% cell death after one week (Figure 1B). Apparently, Zeocin is a more effective means to kill wild type CHO-K1 than removal of the essential amino acid L-threonine. Any selection methodology depends on the killing rate of untransfected cells in a transfected culture. When the killing rate is slow, stably transfected colonies will emerge only slowly from the background of untransfected cells. This hampers the easy identification of stably transfected colonies. The rather slow killing rate of cells in the absence of L-threonine (Figure 1B) indicates that the employment of removing L-threonine as selection principle is not likely to provide a very stringent and efficient selection system.

In order to test the usefulness of the thrB and thrC genes as selection marker we made two plasmids that harbored the thrB and thrC genes. Both plasmids harbored the d2EGFP gene as reporter gene. The thrB and thrC genes were coupled to the d2EGFP gene by an IRES (Internal Ribosome Entry Site) sequence. We placed two different selection markers upstream of the d2EGFP reporter genes. A modified TTG Zeocin (TTG Zeo) selection marker was

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**Figure 1.** The metabolic pathway in which homoserine kinase and threonine synthase are involved and the effects of culturing in the absence of L-threonine on CHO-K1 wild type cells.  
A. The metabolic pathway of L-threonine synthesis. Two enzymes are required to catalyze the synthesis of L-threonine from L-homoserine. The homoserine kinase (thrB) converts L-homoserine to O-phospho-L-homoserine, and this product is converted to L-threonine by threonine synthase (thrC).  
B. Killing curve of wild type CHO-K1 cells by Zeocin and in culture medium devoid of L-threonine. Tryphan blue staining monitored cell viability for 14 days. The percentage of viable cells is indicated.
placed upstream of d2EGFP-IRES-thrC and the modified TTG Neomycin (TTG Neo) selection marker was placed upstream of d2EGFP-IRES-thrB (Figure 2). The use of these two different selection markers warranted that after co-transfection of the two plasmids, the presence of both Zeocin and Neomycin in the culture medium would force expression of both the thrB and thrC encompassing cassettes. The expression cassettes were flanked by STAR elements, specifically, a combination of STAR 7 and STAR 67 upstream of the CMV promoter and STAR 7 downstream of the expression cassette (Figure 2).

The slow killing rate of CHO-K1 cells in L-threonine minus medium suggested that direct selection of transfected cells in such culture medium would be inefficient (Figure 1B). Still we attempted to directly select colonies that were transfected with the thrB/thrC genes simply by omitting L-threonine from the culture medium and adding 1 mM L-homoserine as precursor to the culture medium. However, as expected, no colonies formed; instead a monolayer of cells emerged, mostly cells that did not express d2EGFP, which was monitored under a fluorescence microscope (result not shown). This result is in agreement with the slow killing rate of wild type CHO-K1 in the absence of L-threonine in the culture medium (Figure 1B).

![Figure 2](image-url)

**Figure 2.** Co-transfection of plasmids harboring the thrB/thrC genes.
The components of the plasmids, such as promoter and selection markers are indicated. CHO-K1 cells were co-transfected with two plasmids that contain the thrB or thrC gene, coupled to the d2EGFP reporter gene through an IRES sequence. Stably transfected CHO-K1 clones were isolated and propagated before d2EGFP values were measured. First measurements were performed after selection with Zeocin and Neomycin, and in the presence of L-threonine in the culture medium (day 1). After this first measurement cells were split and cultured for 65 days under different selection conditions, as indicated. After 65 days d2EGFP values were determined. The mean d2EGFP values per colony are shown. The horizontal bars signify the average d2EGFP values of the colonies that are cultured under the indicated experimental conditions.
The thrB/thrC genes are placed downstream of the d2EGFP reporter gene, separated by two IRES sequences, as indicated. CHO-K1 cells were transfected with this plasmid and selection was performed with Zeocin alone. Stably transfected CHO-K1 clones were isolated and propagated before d2EGFP values were measured. The same protocol of first measurements, subsequent culturing under different selection conditions and the representations of the results are as described in Figure legend 2.

Therefore we tested the following scenario in which colonies are first selected by another, more effective selection agent and in which the thrB/thrC genes are subsequently used for keeping protein expression levels stable over prolonged periods of time. We used Zeocin and Neomycin as initial selection agents to establish stably transfected colonies. The above-described plasmids were transfected to CHO-K1 cells and we isolated up to 24 stably transfected clones (100 μg/ml Zeocin and 250 μg/ml Neomycin). Approximately two weeks after isolation and propagation of the clones we quantified d2EGFP expression by flow cytometry (FACS). As shown in Figure 2, the average d2EGFP value in these TTG Zeo IRES thrC/ TTG Neo IRES thrB colonies was 395 (day 1), when measured in the presence of 100 μg/ml Zeocin and 250 μg/ml Neomycin. To assess the influence of the presence or absence of L-threonine minus selection pressure on the stability of protein expression, we split the cells and they were further cultured under three conditions:  
- Zeocin/ Neomycin selection (100 μg/ml Zeocin and 250 μg/ml Neomycin);  
- No selection (no Zeocin or Neomycin);  
- Threonine selection (no Zeocin or Neomycin, without L-threonine, but with 1 mM of the precursor L-homoserine in the medium).

After 65 days we again measured the d2EGFP values. As shown in Figure 2, continuous selection with Zeocin and Neomycin resulted in stable d2EGFP expression (380), but with absence of Zeocin/Neomycin selection, the average d2EGFP values in the clones dropped to 75. However, d2EGFP values remained also remarkable stable under the threonine selection
conditions (397). This result indicates that \textit{thrB} and \textit{thrC} genes, in combination with removal of L-threonine from the culture medium may be useful to maintain high protein expression levels in the absence of selection agents such as Zeocin and Neomycin.

\textbf{thrB and thrC genes can be used in a tricistronic gene configuration}

Co-transfection of two plasmids to produce one protein of interest (in our case d2EGFP) is not very elegant and requires two different selection markers for initial selection of colonies (in our case Zeo and Neo). We therefore placed both \textit{thrB} and \textit{thrC} genes behind the d2EGFP gene, separated by two IRES sequences. We thus created the following expression cassette: STAR7/67-CMV promoter-TTG Zeo-d2EGFP-IRES-\textit{thrC}-IRES-\textit{thrB}-STAR7. We transfected this plasmid to CHO-K1 cells and we isolated stably transfected clones (150 μg/ml Zeocin). Approximately two weeks after isolation and propagation of the clones we quantified d2EGFP expression. As shown in Figure 3, the average d2EGFP value in these colonies was 381 (day 1), when measured in the presence of 150 μg/ml Zeocin. To assess the influence of the presence or absence of selection pressure on the stability of protein expression, we split the cells and they were further cultured under three conditions:

- Zeocin selection (150 μg/ml Zeocin);
- No selection (no Zeocin);
- Threonine selection (no Zeocin and without L-threonine, but with 1 mM of the precursor L-homoserine in the medium).

After 65 days we again measured the d2EGFP values. As shown in Figure 3, without Zeocin selection, the average d2EGFP value in the clones dropped to 45, whereas these d2EGFP values remained rather stable (357) in the continuous presence of Zeocin. d2EGFP values remained also stable in the absence of Zeocin and L-threonine (365). This result indicates that \textit{thrB} and \textit{thrC} genes can be placed in a tricistronic gene configuration in order to achieve a high degree of long-term stability of protein expression.

\textbf{L-homoserine itself can be used as selection agent with thrB/thrC as maintenance marker}

In the course of performing control experiments we observed that when only the \textit{thrB} gene was transfected with the TTG Zeo-d2EGFP-IRES-\textit{thrB} plasmid, cells very rapidly died when 1 mM L-homoserine was present in the culture medium that contained L-threonine (Figure 4). Within one day, more than 90% of the cells had died. In contrast, when 1 mM L-homoserine was present with cells that were transfected with only the TTG Neo-d2EGFP-IRES-\textit{thrC}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Toxicity of L-homoserine in thrB transfected cell. CHO-K1 cells were transfected with the plasmids as shown in Figure 2, containing either the \textit{thrB} or the \textit{thrC} gene. Stably transfected colonies were selected with either Zeocin (\textit{thrC}) or Neomycin (\textit{thrB}). In either case cells were cultured in medium containing L-threonine. 1 mM L-homoserine was added to an exponentially growing cell population. Tryphan blue staining monitored cell viability for two days. Approximately every 4-8 hours a sample was monitored. The percentage of viable cells is indicated.}
\end{figure}
plasmid, no cell death was observed (Figure 4). In comparison, addition of 1 mM L-homoserine to wild type CHO-K1 cells had no apparent effects on the cells and cell growth remained normal. Only when the concentration of L-homoserine was raised to 5 mM we observed a reduced growth rate of the cells, but still this concentration was not toxic (data not shown). The result that 1 mM L-homoserine was highly toxic for cells transfected with the thrB gene alone, suggested that the product formed by the thrB enzyme, O-phospo-L-homoserine, might be very toxic itself. If this were the case, this could be a useful property. We raised the concentration of L-homoserine in the culture medium to modulate the levels of the intermediate product O-phospo-L-homoserine. Since this can only be tested in cells that harbor functional thrB enzyme, we tested the idea on clones that were established with the STAR7/67-CMV promoter-TTG Zeo-d2EGFP-IRES-thrC-IRES-thrB-STAR7 plasmid.

Cells were split (designated day 1) and cultured in the absence of Zeocin, but in the presence of either 1 or 5 mM L-homoserine and in the presence of 0.1 mM L-threonine. This experimental set-up was different from the previous experiments in which we used culture medium that was devoid of L-threonine. In that case a concentration of 1 mM L-homoserine was not toxic, possibly because the intermediate product, O-phospo-L-homoserine, could efficiently be processed by the thrC enzyme to produce L-threonine. That resulted in the maintenance of high protein expression levels (Figure 2 and 3). In the present situation the culture medium contains L-threonine, which therefore does not require high expression levels of the thrB/thrC combination to produce L-threonine. As shown in Figure 5, addition of 1 mM L-homoserine to the culture medium (containing L-threonine) did not prevent a sharp decline in d2EGFP values (from 405 to 57) in 65 days. Obviously, the presence of L-threonine in the

![Figure 5](image-url)
culture medium is not a condition that requires high expression levels of the \textit{thrB}/\textit{thrC} genes in order to produce L-threonine in the cell. As a consequence, low expression levels of the \textit{thrB} gene would not produce high concentrations of the toxic O-phospho-L-homoserine. However, when cells were cultured in the presence of 5 mM L-homoserine, d2EGFP values remained very stable after 65 days (average 438 versus 405). This result indicates that higher levels of L-homoserine precursor may indeed induce higher levels of the toxic intermediate product O-phospho-L-homoserine. Only the presence of elevated \textit{thrC} enzyme levels (concomitant with higher tricistronic mRNA levels and thus high d2EGFP expression) can rescue the cell from this toxic, intermediate product.

Importantly, this result was achieved in the absence of Zeocin, in the presence of L-threonine and with only a high concentration of L-homoserine. Since even high concentrations of L-homoserine are relatively harmless to wild type cells, this procedure cannot be employed for direct selection. The use of the \textit{thrB}/\textit{thrC} genes as ‘maintenance’ marker is, however, less complicated when only L-homoserine needs to be added to the medium. Culture medium without L-threonine is expensive since it has to be prepared by special order. Furthermore, the culture medium containing L-threonine needs to be removed carefully before the switch of culture media becomes effective. Obviously, addition of 5 mM L-homoserine to L-threonine-containing culture medium is much less complicated.

\textbf{thrB/thrC genes can be used as maintenance marker with a secreted therapeutic protein}

We next used the secreted therapeutic protein human erythropoietin (hEPO) to assess the potential of L-homoserine as selection agent. The construct STAR7/67-CMV promoter-TTG Zeo-hEPO-IRES-\textit{thrC}-IRES-\textit{thrB}-STAR7 was transfected to CHO-K1 and stable colonies

![Figure 6. Stability of hEPO expression with the use of the \textit{thrB}/\textit{thrC} genes in a tricistronic configuration and with L-homoserine as selection agent.](image)

A. The same plasmid as described in Figure 3 was used, but with hEPO as reporter gene instead of d2EGFP. CHO-K1 cells were transfected with this plasmid and initial selection was performed with Zeocin. Stably transfected CHO-K1 clones were isolated and propagated before hEPO values were determined by an ELISA. First measurements were performed after selection with Zeocin. Subsequent culturing under different selection conditions are indicated. Shown are the specific hEPO activities of the clones, expressed as pg/ cell/ day. B. The average doubling time (in hours) of the clones as described in A are shown.
were isolated by selection with Zeocin. After approximately two weeks production of hEPO into the culture medium was measured by using an ELISA. As shown in Figure 6A, the average specific hEPO productivity was 16 pg/cell/day. From here on, cells were split and cultured in the continuing presence of 150 μg/ml Zeocin in the medium, no selection agent at all, or 5 mM L-homoserine, but no Zeocin in the culture medium. We found that also with hEPO as reporter protein the average specific productivity dropped to 2.2 pg/cell/day, after 65 days without selection pressure. In contrast, the specific productivity remained stable in the continuing presence of 150 μg/ml Zeocin (Figure 6A). Importantly, the average specific hEPO productivity remained also stable at 17.2 pg/cell/day in the absence of Zeocin, but in the presence of 5 mM L-homoserine and L-threonine. Not only the specific hEPO productivity remained stable over these 65 days. As shown in Figure 6B, also the growth rates of the cells in the colonies stayed unaffected. Therefore, taken together with a higher specific productivity, the volumetric hEPO productivity remained stable over a prolonged time period in the absence of Zeocin, but in the presence of 5 mM L-homoserine. These results indicate that L-homoserine itself, in combination with transfection of the thrB and thrC genes is useful as selection agent.

**DISCUSSION**

The prevalent methodology to produce therapeutic proteins at an industrial scale involves the growth of stably transfected mammalian cell lines in large-scale reactors. Selection of such cell lines mostly involves the use of protein selection markers that neutralize toxic antibiotics. In principle there is no objection against this methodology, but there are at least two major drawbacks. Obviously one would like the cells to express the secreted therapeutic proteins at a high level, since this implies effectively more end product in a single production run that usually lasts several weeks. A major problem in industrial production of therapeutic proteins is that the expression levels per cell (specific productivity) do not remain stable during such a production run. To keep the expression levels stable over time requires a constant selection pressure on the cells. This can and is commonly achieved by keeping the antibiotic selection agent in the culture medium. However, selecting stably transfected colonies in a laboratory and propagation of cells under constant selection pressure in a petri dish is one thing. It is another matter to grow these cells in up to 20,000 liter, large-scale reactors that are the standard for protein production at an industrial scale. Antibiotics such as Zeocin are very expensive, even for use in a laboratory setting. Keeping sufficient levels of antibiotics in a large-scale reactor is vastly expensive and results in an increase of the so-called costs-of-good. Costs-of-good signify the entire costs to produce the end product: the therapeutic protein as medicine in a bottle. It is therefore common practice not to add antibiotics during large-scale production runs and to accept the lower total amount of end product as negative consequence.

Another drawback of using antibiotics in large-scale industrial production of therapeutic proteins is their toxicity. Keeping antibiotics up to the end in a production run implies very costly additional purification steps. The Federal Food and Drug Administration (FDA) in the USA has set very strict guidelines for removing antibiotics. In fact, the use of penicillin and other β-lactam antibiotics is strictly forbidden for production of therapeutics and the use of antibiotics in general is discouraged. The Federal Food and Drug Administration (FDA) in the USA has set very strict guidelines for removing antibiotics. In fact, the use of penicillin and other β-lactam antibiotics is strictly forbidden for production of therapeutics and the use of antibiotics in general is discouraged. It is therefore considered to be very important to find means to circumvent the use of antibiotics, particularly during the large-scale production runs. One means to overcome the need for traditional antibiotics is the use of metabolic markers. As explained above, these involve steps in metabolic pathways that are essential for certain cells. For instance, certain amino acids cannot be synthesized by a cell and are thus called essential. The cell cannot
synthesize the amino acid because it lacks a particular enzyme in the metabolic pathway to synthesise the amino acid. These enzymes are used as selection marker. Two metabolic markers have been commonly used for this purpose: the tetrahydrofolate synthesizing \textit{Dhfr} gene\textsuperscript{11,12} and glutamine synthetase (\textit{GS})\textsuperscript{13,14}.

The DHFR protein is an enzyme in the folate pathway that converts dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the de novo synthesis of purines (hypoxanthine), thymidylic acid (thymidine), and the amino acid glycine. Certain CHO cells lack this enzyme. Adding the \textit{Dhfr} gene to a protein expression plasmid can result in supplementing the needed DHFR enzyme and thus the \textit{Dhfr} gene can serve as selection marker. To achieve that, the precursor folate needs to be present in the culture medium that is devoid of the end products hypoxanthine, thymidine and glycine. Similarly the \textit{GS} gene is involved in the synthesis of the essential amino acid glutamine. With glutamate and ammonia in the culture medium that is devoid of glutamine, the \textit{GS} gene can serve as selection marker.

There are, however, several restrictions to the use of both \textit{Dhfr} and \textit{GS} genes. In the first place, only a limited number of mammalian cell lines lack either the \textit{Dhfr} or \textit{GS} genes. In the case of the \textit{Dhfr} gene these are \textit{Dhfr} minus CHO cells\textsuperscript{15}. For instance CHO-DG44 is such a cell line and it is not surprising that this is the cell line of choice in the biotechnology industry for the production of therapeutic proteins. The reason for that is not, however, that CHO-DG44 is a cell line with many exceptional desirable characteristics, but simply the fact that it is \textit{Dhfr} minus. With the \textit{GS} gene this is even more problematic. Only the mouse hybridoma cell line NS0 is \textit{GS} minus and can be properly used with the \textit{GS} gene as selection marker. However, NS0 is known to be a very poor growing cell line and that has a capacity for only very low specific productivity of therapeutic proteins. These problems are in part resolved by the addition of toxic inhibitors of either the DHFR or GS enzymes: respectively methotrexate and methionine sulfoxime\textsuperscript{13,16,17}. Addition of these inhibitors to cells that are \textit{Dhfr}+ or \textit{GS}+ results in the inhibition of the endogenous DHFR and GS proteins. This inhibition subsequently requires the addition of exogenously DHFR/GS enzymes, which are provided by the transfected protein expression plasmids. Clearly we are back where we started: the addition of and constantly required presence of toxic substances to the culture medium that we wanted to avoid in the first place.

Given the problems with metabolic markers as discussed above, we sought to identify other metabolic markers that do not have the described drawbacks. One such marker can be potentially found in the metabolic pathway of L-threonine synthesis. All mammalian cells lack the \textit{thrB}/\textit{thrC} genes and L-threonine is therefore a more ‘universal’ essential amino acid for a wide variety of mammalian cell lines. As we have shown above, the combination of \textit{thrB}/\textit{thrC} can indeed be used as selection marker in for instance CHO cell lines. There are, however, also restrictions to the use of the \textit{thrB}/\textit{thrC} genes as selection marker. In the first place, direct selection of stable cell lines is not possible with this marker combination. Cells apparently can survive quite a long time in culture medium without L-threonine. The reason for that is not known, but it could be very trivial that a cell does not need much L-threonine in the first place. But this property implies that non-tranfected cells survive too long to allow efficient identification and isolation of transfected colonies. Non-transfected cells simply overgrow the cell population. This means that transfected cells have to be initially selected as stable colonies by another selection agent, in our case Zeocin (and Neomycin). Only after this initial selection is finished, cells can be switched to culture medium devoid of L-threonine and this results in the desired stability of protein expression. This therefore leaves a role for the \textit{thrB}/\textit{thrC} genes as ‘maintenance’ marker, but not as marker for direct selection.

How bad is this for the potential use of the \textit{thrB}/\textit{thrC} genes as marker genes? The depicted situation is not uncommon. Also the \textit{Dhfr} gene is usually not used as single selection marker. Cells do not very rapidly die in culture medium devoid of hypoxanthine and thymidine (HT)
(see Chapter 6), which also hampers the use of the $Dhfr$ gene for direct selection. It is therefore common to concomitantly select with Neomycin, in combination with HT minus medium. After the initial selection phase, the Neomycin is omitted. The use of this antibiotic is thereby at least avoided in the industrial production phase. Similarly, we envision that Zeocin or Neomycin is only used in the initial, laboratory phases, and that the maintenance of high protein expression levels conveyed by the $thrB/thrC$ genes is used during the production phases.

The $thrB/thrC$ marker genes do not suffer from the limited use of the $Dhfr$ and $GS$ markers in few mammalian cell lines and therefore do not require the addition of toxic inhibitors. This advantage goes even further. As outlined above, there are two ways to achieve ‘maintenance’ of protein expression with the $thrB/thrC$ genes. This can either be the change to L-threonine minus medium and the addition of a low (1 mM) and non-toxic concentration of the precursor L-homoserine. The second option is to leave the L-threonine in the culture medium and add a higher (5 mM) concentration of L-homoserine. Both ways result in stable protein expression levels over prolonged periods of time. Obviously the second protocol is favourable since it avoids the need to switch to expensive ‘designer’ medium and only requires the addition of (inexpensive) L-homoserine. We did not note negative side effects of 5 mM L-homoserine to stably transfected cells, although at this concentration growth of non-transfected wild type cells is hampered.

We hypothesized that the intermediate product, O-phospo-L-homoserine is toxic. Strictly spoken we do not know this. It is, however, tempting to consider this to be the right assumption. Cells that are in L-threonine-containing medium transfected with the enzyme thrB (that converts L-homoserine to O-phospo-L-homoserine) very rapidly die after the addition of 1 mM L-homoserine. Furthermore, simultaneous expression of the thrC enzyme (that converts O-phospo-L-homoserine to L-threonine) results in cell survival and we observed no toxicity of L-homoserine at all. Finally, raising the concentration of L-homoserine to 5 mM (with L-threonine present in the culture medium) probably shifts the balance in the enzymatic reactions, requiring higher tricistronic mRNA levels and thus maintenance of the high expression levels of the protein of interest.

One final drawback of the $thrB/thrC$ marker combination is that it is a combination. Two genes are needed and this puts a restriction on its practical use. In the experimental set-up that we employed this is not much of a problem since we placed the genes in a tricistronic configuration. However, when a monoclonal antibody is produced this is less easy. Placing the genes encoding the light and heavy chains in one expression unit can be achieved by using an IRES sequence. Adding the $thrB/thrC$ genes to such a construct would require four open reading frames in one tetracistronic mRNA. We do not know whether this is practical feasible at all. Another means might be to use different expression units for both genes encoding the light and heavy chains, placed on one plasmid. In that scenario the $thrB$ gene could be coupled to the light chain gene through an IRES sequence and the $thrC$ gene to the heavy chain gene through an IRES sequence. Alternatively, the light/heavy chain and $thrB/thrC$ gene combinations could be placed on different plasmids and co-transfected. This is a feasible scenario, as we showed in Figure 2. It remains to be seen how these scenarios work for the production of monoclonal antibodies. Answers to such questions will be important though to assess the possibility whether the $thrB/thrC$ marker combination will become a valuable biotechnological tool.
MATERIALS AND METHODS

Vector constructions

The *E.coli* thrB and thrC coding regions were synthesized by GENEART. Together with the encephalomyocarditis virus (EMCV) IRES (Clontech)9, these genes were cloned into our STAR-Select vector².

Cell culture, transfections and analysis of clones

CHO-K1 cells (CCL-61; American Type Culture Collection (ATCC)) were grown in HamF12 medium (Invitrogen), supplemented with 9.1% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen) at 37°C / 5% CO₂. For culturing in the absence of L-threonine, special HamF12 medium lacking L-threonine (designed and produced by Invitrogen) and dialyzed FBS (Invitrogen) was used. L-homoserine (Sigma) was diluted in water and used at a final concentration in the culture media of 1 or 5 mM. For transfections, 0.3·10⁶ CHO-K1 cells were seeded in 6-well culture plates 24 hours prior to transfection. Cells were transfected with 3 μg of plasmid DNA using LipofectamineTM 2000 (Invitrogen) as described by the manufacturer. Selection involved 100-150 μg/ml Zeocin (Invitrogen) and 250 μg/ml Neomycin (Invitrogen). Approximately 12 days after transfection, individual colonies became visible and these were isolated and propagated in 24-well plates in medium containing Zeocin (and Neomycin). When grown to ~70% confluence, cells were transferred to 6-well plates. Cells were continued to grow in 6-well plates for another one to two weeks before FACS analysis or ELISA was performed. The d2EGFP expression levels were determined on an Epics XL Beckman Coulter flowcytometer. At this point of time, after determining d2EGFP expression levels or performing an ELISA, cells were split to the various media. Cells were continued to grow for 65 days in the various HamF12 media: medium without antibiotics; medium (containing L-threonine) with Zeocin (and Neomycin); medium (containing L-threonine) with 1 or 5 mM L-homoserine; medium lacking L-threonine and containing 1 mM L-homoserine. After 65 days d2EGFP or hEPO expression levels were determined again. Values were visualized using Graphpad Prism 5 for Windows.

For determining survival rates of CHO-K1 wild-type cells, 0.2·10⁶ cells were seeded in 6-well plates in medium containing 150 μg/ml Zeocin, in medium lacking L-threonine, or in medium containing 1 or 5 mM L-homoserine. Daily, viability of cells was monitored by tryphan blue staining for a period of two weeks. For determining survival rates of stable clones containing either the thrB or the thrC gene, 0.2·10⁶ cells were seeded in 6-well plates in medium containing L-threonine and 1 mM L-homoserine. Approximately every 4-8 hours, viability of cells was monitored by tryphan blue staining for a period of two days.

ELISA

For hEPO measurements, equal numbers of cells (0.1·10⁶) were seeded in six-well plates three days prior to cell counting and collection of the medium. The amount of secreted hEPO was determined using an ELISA-kit (Quantikine IVD kit; R&D systems; Ref DEP00). The antibody concentration was determined by comparing optical density at 415 nm with that of the known antibody standard, as supplied in the ELISA-kit.
REFERENCES


10. Center for Biologics Evaluation & Research (CBER). Points to consider in the characterisation of cell lines used to produce biologicals (1993).


