Selection markers and expression enhancing elements: novel strategies to create mammalian production cell lines
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MODIFIED DHFR GENES CAN SERVE AS ‘MAINTENANCE’ MARKER TO CONVEY LONG-TERM STABILITY OF PROTEIN EXPRESSION IN CHO-DG44 CELLS

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ABSTRACT

Maintenance of high levels of protein expression in mammalian cells often requires the continuous presence of toxic selection agents in the culture medium. Here we employ modified Dhfr gene variants to achieve long term stability of protein expression in CHO-DG44 cells, in the absence of selection agents. The modifications consist of altered startcodons that provide attenuated translation efficiencies, such as GTG or TTG instead of ATG. The modified Dhfr genes were coupled to the gene of interest through an IRES sequence. Stability of protein expression was achieved in the absence of primary selection agents such as Zeocin, provided that metabolic products synthesized by the DHFR protein were omitted from the culture medium. The expression levels of both d2EGFP and a monoclonal antibody remained stable over prolonged periods of time. The results show that the Dhfr gene as ‘maintenance’ marker can provide a means to achieve a high level of stability of protein expression in Dhfr minus cells. Whereas protocols that employ DHFR as selection marker commonly use a toxic inhibitor of the DHFR protein, methotrexate, this is not needed in the protocol we develop here.

INTRODUCTION

STAR elements are genomic stretches of DNA that block chromatin-associated repressor activity such as heterochromatin-related proteins. When these STAR elements were applied in protein expression cassettes, protein expression levels were elevated. Constructs with STAR elements induced the formation of many more clones than without STAR elements. To be able to efficiently identify only those colonies with high protein expression levels, we devised a novel, very stringent selection system that creates few, but highly productive mammalian cell lines. In essence, we placed a selection marker with a startcodon that confers attenuated translation initiation frequency, upstream of the gene of interest with a startcodon that confers optimal translation initiation. This modified startcodon could, for instance, be a TTG instead of an ATG, resulting in selection markers such as TTG Zeocin or TTG Neomycin. From the transcribed bicistronic mRNA, the selection marker is translated at a low frequency, and the protein of interest at a high frequency. Incorporation of STAR elements in such constructs resulted in the formation of clones that expressed proteins at high levels. We called this selection system STAR-Select.

Keeping protein expression levels in a TTG Zeocin or TTG Neomycin selected colony at the same high level over a prolonged period of time, however, requires active selection pressure. This can be accomplished by keeping Zeocin or Neomycin in the culture medium, but this is not favored since such agents are both toxic and expensive. Another approach is to use an enzyme that metabolizes one or more essential steps in a metabolic pathway as selection marker. Essential means that they have to be present in the culture medium, since the cell is not able to synthesize specific essential metabolic building blocks itself in order to allow the cell to survive. Examples are the essential amino acids that cannot be synthesized by a mammalian cell and that need to be present in the culture medium to allow the cell to survive. Another example is related to the tetrahydrofolate synthesizing Dhfr gene. The corresponding DHFR protein is an enzyme in the folate pathway. The DHFR protein specifically catalyzes the reduction of dihydrofolate to tetrahydrofolate, a methyl group shuttle required for the de novo synthesis of purines (hypoxanthine), thymidylic acid (thymidine), and the amino acid glycine. To operate, the non-toxic substance folate has to be present in the
culture medium. CHO-DG44 cells lack the DHFR enzyme and the need of these cells for the DHFR protein can be used as selection principle.

Usually, selection of cells through growth in hypoxanthine and thymidine (HT) minus culture medium is combined with killing of non-transfected cells by Neomycin. To achieve that, the neomycin resistance gene is co-transfected to the cells. The origin of this practice lies in the rather slow killing rate of non-transfected wild type cells growing in HT-minus culture medium alone (see Figure 1B). Additional killing of non-transfected cells with the aid of a second selection marker (Neomycin) speeds up the selection of transfected cells. Furthermore, to raise the selection stringency of the Dhfr marker, an inhibitor of DHFR, methotrexate is added during the selection procedure. Upon inhibition of the DHFR enzyme more functional DHFR enzyme is required for the cell to survive. Methotrexate achieves this by inducing massive gene amplification. Because the amplification unit is much larger (over 100 kb) than the size of the Dhfr gene, a specific gene of interest, which is co-linked to the Dhfr gene in the same expression vector, is coamplified. Up to thousands of amplified loci are created and this results in a higher level of transcription of the gene of interest. This, however, does not happen immediately after addition of methotrexate to the culture medium, because gene amplification is a slow process. Methotrexate-treated colonies are first diluted to obtain many, separate colonies that all arise from a single cell. These so-called subclones are then monitored for gene expression. Subsequently, the highest protein-expressing subclones are again subjected to methotrexate-induced gene amplification, followed by another round of subcloning. This time consuming procedure is sometimes repeated up to five times before a suitable subclone is isolated that displays high enough expression levels of the protein of interest.

Here, we tested the usefulness of the DHFR enzyme as selection/maintenance marker in the context of the STAR-Select system, in which methotrexate-induced gene amplification is avoided.

**RESULTS**

**Experimental set-up**

As a first step to assess the possibilities to use the DHFR enzyme as selection/maintenance marker we tested the effects of HT minus medium on the viability of wild type CHO-DG44 cells. Tryphan blue staining was used to monitor the viability of cells. Figure 1B shows that after one week approximately 50% of the CHO-DG44 cells had died and after two weeks approximately 70% cells had died in HT minus medium. In contrast, addition of the standard concentration for selection, 400 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-DG44 than removal of the essential metabolic components hypoxanthine and thymidine.

We wanted to avoid the use of the toxic methotrexate as well as the time consuming procedure of multiple rounds of methotrexate-induced gene amplification and subcloning. We therefore modified the commonly used strategy in two important ways: 1) we first established high protein-producing, stable colonies through selection by the Zeocin selection agent (TTG Zeo as marker) and 2) only then we switched to HT-minus culture medium to analyze long-term protein expression levels. With this novel procedure we aimed to circumvent both the use of the toxic methotrexate and the need for multiple rounds of gene amplification and subcloning.

We created a plasmid harboring the mouse Dhfr gene. The modified TTG Zeocin (TTG Zeo) selection marker preceded the d2EGFP reporter gene (Figure 2). We placed the Dhfr
Figure 1. The metabolic pathway in which DHFR is involved and the effects of culturing in the absence of HT on CHO-DG44 wild type cells.
A. DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate. This in turn is a metabolic building block for the synthesis of hypoxanthine, thymidine and glycine. B. Killing curve of wild type CHO-DG44 cells by Zeocin and in culture medium devoid of hypoxanthine and thymidine (-HT). Tryphan blue staining monitored cell viability for 14 days. The percentage of viable cells is indicated.

The gene behind the d2EGFP reporter gene, separated by an IRES sequence. We used the so-called ‘attenuated’ IRES sequence. A wild type IRES sequence contains 12 ATGs and translation of a downstream-coupled gene of interest always starts at the last two, most 3' located ATGs of the IRES sequence. In contrast, these 3' located two ATGs are removed in an ‘attenuated’ IRES. This results in a less preferred site for internal ribosomal entry and thus less efficient translation initiation of the gene that is located downstream of the IRES sequence. Another difference with the non-attenuated, wild type IRES is that translation with the attenuated IRES sequence starts at the startcodon of the coupled gene of interest. Therefore, an attenuated IRES allows testing of different startcodon modifications of the downstream-coupled Dhfr gene.

Different modified translation initiation codons were introduced in the Dhfr gene. We created an ATG, GTG and TTG Dhfr gene (Figure 2). The GTG translation initiation codon is less efficient than the ATG, and the TTG translation initiation codon is again less efficient than the GTG codon. In principle, the GTG Dhfr derived mRNA would thus result in less translation of the DHFR enzyme than the ATG Dhfr derived mRNA. Likewise, the TTG Dhfr derived mRNA would result in less translation of the DHFR enzyme than the GTG Dhfr derived mRNA. In both cases higher bicistronic mRNA levels would be required than with an ATG Dhfr gene to obtain sufficient levels of the marker protein in order for the cell to survive.
The expression cassette was flanked by a STAR 7/67/7 element combination, thus creating the following plasmids: STAR7/67-CMV promoter-TTG Zeo-d2EGFP-IRES-XGT Dhfr - STAR7 (Figure 2).

**Modified Dhfr genes convey highly stable protein expression**

The above-described plasmids were transfected to (Dhfr minus) CHO-DG44 cells and we selected stably transfected colonies with Zeocin (400 μg/ml Zeocin). We isolated approximately twelve colonies. Two weeks after isolation and propagation of the clones we quantified d2EGFP expression by flowcytometry (FACS). As shown in Figure 2A, the average d2EGFP value in the colonies was between 350 and 500 (day 1) for the three plasmid encompassing ATG, GTG or TTG Dhfr, when measured in the presence of 400 μg/ml Zeocin.

**Figure 2.** Modified STAR-Select Dhfr markers convey a high degree of stability of protein expression. A. Three constructs were made, all containing an expression cassette driven by the CMV promoter and surrounded by STARs 7 and 67 upstream and STAR 7 downstream. The expression cassettes consist of the TTG Zeo selection marker, the d2EGFP gene and a modified Dhfr gene. Results with the different Dhfr modifications are labeled as ATG Dhfr, GTG Dhfr and TTG Dhfr. Stably transfected CHO-DG44 clones were isolated and propagated before d2EGFP values were measured. In all three experimental set-ups, measurements were performed under maximum Zeocin selection pressure (day 1), and in the presence of HT products in the culture medium. After this first measurement cells were split and cultured for 65 days under different selection conditions, as indicated with plusses and minuses. After 65 days d2EGFP values were determined. The d2EGFP values per colony are shown. The horizontal bars signify the average d2EGFP values of the colonies. B. The average doubling time (in hours) of the clones as described in A are shown.
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 culturing conditions:
- Zeocin selection (400 μg/ml Zeocin only);
- No selection (no Zeocin);
- HT selection (no Zeocin and without HT products, but with 0.01 mM of the precursor folate present in the medium).

After 65 days we again measured the d2EGFP values. As shown in Figure 2A, continuous selection on 400 μg/ml Zeocin resulted in rather stable high d2EGFP expression levels, in all three TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr derived colonies. In contrast, without Zeocin selection, the average d2EGFP value in all three TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr derived colonies dropped to very low levels. A distinct picture for colonies established with the three different plasmids emerged only after the switch to HT-minus selection. d2EGFP values did not remain stable in case of the ATG Dhfr, but remained high in the case of GGT/TTG Dhfr genes and even increased to a respective average of 700 and 1057 (Figure 2A).

We interpret these result as that the ATG Dhfr gene produces too much DHFR enzyme in the bicistronic mRNA to create a stringent selection system. In contrast, the GTG and TTG modified Dhfr genes produce just enough DHFR enzyme to allow cell survival, but to achieve that, more mRNA has to be produced to warrant such a level of DHFR protein. Since the Dhfr and d2EGFP transcripts are coupled, this automatically implies higher d2EGFP protein expression levels.

We noted that the GTG/TTG Dhfr induced clones grew slower after switching to HT minus medium than in either 400 μg/ml Zeocin medium or with no selection. We therefore measured all growth rates of the above-described colonies. In Figure 2B we show that no differences were found in the ATG Dhfr induced colonies, under any selection condition. In contrast, in either GTG or TTG Dhfr induced colonies in HT minus medium, the doubling times of the cells increased to an average of approximately 32 +/- 6 and 39 +/- 3 hours respectively.

The data show that coupling an appropriate variant of the Dhfr gene through an attenuated IRES sequence to the d2EGFP gene allows a high degree of stability of d2EGFP expression in CHO-DG44 cells. This occurs in culture medium without the toxic Zeocin and from which only essential metabolic end products are removed. Initial selection on Zeocin through the modified TTG Zeo selection marker allows the efficient establishment of colonies with high d2EGFP expression levels. Subsequently, a change of culture medium (removing Zeocin and HT) is required to maintain high d2EGFP expression levels. However, in the cases that the d2EGFP expression increased after the switch to HT minus medium (GTG/TTG Dhfr) we also noted a rather severe drop in the growth rates of the cells.

A modified Dhfr gene confers stable monoclonal antibody expression

Above, we used the d2EGFP gene as reporter gene. Next we applied the above-explained Dhfr gene configurations to test whether the observed improvement of stability can also be accomplished with a monoclonal antibody as reporter gene. This experimental set-up constitutes a more complex level of gene regulation, since a monoclonal antibody consists of two polypeptide chains that are thus encoded by two genes. In the configuration we use, these two genes are present on one plasmid, both genes being under the control of a separate promoter. Here we coupled the Dhfr gene to only one expression cassette and tested whether this suffices to improve the stability of expression.

We coupled the GTG/TTG Dhfr variants to the expression cassette encoding the light chain (LC) of the monoclonal antibody directed against the epithelial cell adhesion molecule.
Figure 3. Incorporation of one modified Dhfr gene is sufficient to induce a high degree of stability of monoclonal antibody expression levels.
A. Stably transfected CHO-DG44 clones with the indicated GTG Dhfr and TTG Dhfr constructs were isolated in the presence of Zeocin and Neomycin and cells were propagated before anti-EpCAM expression levels were measured on day 1. After that, cells were split and cultured under different selection conditions, as indicated with plusses and minuses. Shown are the anti-EpCAM expression levels per colony. The horizontal bars signify the average anti-EpCAM values of the colonies. B. The average doubling time (in hours) of the clones as described in A are shown.

EpCAM (Figure 3). The TTG Zeo selection marker was placed upstream of the light chain-encoding gene and the GTG/T TG Dhfr marker was placed downstream of the light chain, coupled through the weakened IRES sequence. The second cassette, containing the heavy chain-encoding gene was placed downstream of the cassette containing the light chain-encoding gene. Upstream of the heavy chain-encoding gene we placed the TTG Neomycin selection marker. Multiple STAR elements were applied in this configuration. Since we observed that the ATG Dhfr variant did not result in stabilizing d2EGFP expression after the switch to HT selection (Figure 2), we only focused on the GTG/T TG Dhfr variant. Thus the following two constructs were made (Figure 3): STAR7/67-CMV-TTG Zeo-LC EpCAM-IRES-GTG/T TG Dhfr-Star67-CMV-TTG Neo-HC EpCAM-STAR7. These two constructs were transfected to CHO-DG44 cells and stably transfected clones were isolated (in the presence of 400 µg/ml Zeocin and 500 µg/ml Neomycin) and cells were further cultured for two weeks before culture medium was sampled and anti-EpCAM values were determined, by using an ELISA.
The average anti-EpCAM values in 20 colonies with TTG Zeo-LC EpCAM-IRES-GTG Dhfr-TTG Neo HC EpCAM were 9.1 pg/cell/day (day 1, Figure 3), and in 20 colonies with the TTG Dhfr variant 8.2 pg/cell/day (day 1, Figure 3). In both cases cells grew in the presence of 400 µg/ml Zeocin, 500 µg/ml Neomycin and HT products. As with the d2EGFP as reporter gene (Figure 2), after these measurements the cells were split and further cultured under three culturing conditions:

- Zeocin/ Neomycin selection (400 µg/ml Zeocin and 500 µg/ml Neomycin);
- No selection (no Zeocin or Neomycin);
- HT selection (no Zeocin, Neomycin and without HT products, but with 0.01 mM of the precursor folate present in the medium).

After 65 days we again measured the anti-EpCAM values. The average anti-EpCAM value in the TTG Zeo-LC EpCAM-IRES-GTG/TTG Dhfr-TTG Neo-HC EpCAM colonies under Zeocin and Neomycin selection were now 6.1 and 7.3 pg/cell/day respectively (Figure 3). Without Zeocin and Neomycin selection, but in the presence of HT products, these values dropped to 1.7 and 1.1 pg/cell/day respectively (Figure 3). Finally, the average anti-EpCAM values in the TTG Zeo LC EpCAM IRES GTG/TTG Dhfr-TTG Neo HC EpCAM colonies under HT selection conditions (without Zeocin and Neomycin selection and in the absence of HT products) increased to 12.1 and 16.0 pg/cell/day respectively (Figure 3).

As with d2EGFP as reporter gene, we noted that the GTG/TTG Dhfr induced EpCAM clones grew slower after switching to HT minus medium than before the switch. As shown in Figure 3B in both GTG or TTG Dhfr induced anti-EpCAM producing colonies the doubling times of the cells increased dramatically, to approximately 36 +/- 9 and 48 +/- 7 hours respectively.

In conclusion, as with d2EGFP we thus observed a severe drop in anti-EpCAM values in the absence of Zeocin and Neomycin, but in the presence of HT products. In the absence of Zeocin, Neomycin and HT products, the anti-EpCAM values remained stable or increased. This indicates that the expression levels of the DHFR protein, due to the impaired translation efficiency of the GTG/TTG Dhfr mRNA is low enough to provide high selection stringency. This selection pressure, in the absence of any toxic agents, is high enough to maintain high protein expression levels over time. The data also show that coupling one Dhfr variant to only one chain of the EpCAM monoclonal antibody gene is sufficient for maintenance of protein expression over a prolonged period of time. Unfortunately, as with d2EGFP as reporter gene, we noted that the increased anti-EpCAM expression per cell (specific activity) on HT minus medium coincided with reduced cell growth.

Stability of protein expression by the modified Dhfr genes is not the result of gene amplification

It is a well-known fact that using the DHFR enzyme as metabolic selection marker results in gene amplification. Many thousands of copies of the gene of interest can be integrated in the genome of the CHO cells. This phenomenon is highly boosted by the use of the toxic DHFR inhibitor methotrexate. Only in the presence of increasingly higher concentrations of methotrexate the activity of the DHFR enzyme becomes so low that the cells need to amplify the entire locus, including the Dhfr gene, for survival. In the current experiments we did, however, not use methotrexate to inhibit the DHFR enzyme activity. Only the hypoxanthine and thymidine products were removed from the culture medium, and this was sufficient to achieve stability of protein expression. We therefore wondered whether the employment of the DHFR selection marker with a startcodon that confers attenuated initiation as used in our experimental set-up also resulted in gene amplification.

We isolated DNA from the colonies that were described above in Figure 2, on the same day (65) that the d2EGFP values were measured. With this DNA we determined the copy
numbers of the d2EGFP reporter gene. The average d2EGFP copy numbers in the TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr colonies under Zeocin selection were 42, 23 and 32 respectively (Figure 4). In the same clones, but without Zeocin selection, the respective d2EGFP copy numbers were 37, 24 and 27 respectively (Figure 4). As is to be expected, the copy number did not significantly change by simply removing the selection pressure on cells.

Finally, the average d2EGFP copy number in the TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr colonies under HT selection conditions (without Zeocin and Neomycin selection and in the absence of HT products) was 40, 27 and 32 respectively (Figure 4). Therefore, although the d2EGFP expression levels were increased with the GTG/TTG Dhfr variants (Figure 2), no significant increase in copy numbers in these clones was observed. We conclude from these data that gene amplification, which always results from the inhibiting action of methotrexate on the DHFR protein, is not responsible for keeping the d2EGFP expression levels stable over time.

**Direct selection by using modified Dhfr genes is not possible**

The above-described experiments all involve initial selection by agents such as Zeocin and Neomycin and subsequent switching to HT selection. Although many protocols follow an analogous route by simultaneous selection by Neomycin and inhibition of the DHFR enzyme by methotrexate, we tested whether we could use the modified Dhfr genes for direct selection of transfected CHO-DG44 cells. We therefore transfected the TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr plasmids to CHO-DG44 cells and cultured them from the beginning in HT-minus medium. No Zeocin was added to this culture medium. We found that the ATG Dhfr variant resulted in a monolayer of cells, which were not green when monitored under a fluorescence microscope. The GTG/TTG Dhfr variants induced only very small colonies and these were not green either (data not shown). We therefore conclude that direct selection on HT minus medium solely in combination with modified Dhfr genes is not possible.


DISCUSSION

Stability of protein expression is an important biotechnological issue. When a therapeutic protein is produced at an industrial scale, this involves growth to large numbers of cells in large volumes (up to 10-20,000 liters). When due to instability of protein expression the production levels decline during the last growth phases in such bioreactors, this is very costly and inefficient. One way to achieve stability of protein expression over prolonged periods of time is to keep the cells under continuous selection pressure. However, keeping cells in large volumes under continuous selection pressure also requires large quantities of expensive selection agent. Another issue is that the selection agents need to be removed from the final product, which is an important regulatory and cost issue. It is therefore preferable to grow cells under non-selective conditions, even if expression levels eventually become lower in course of time. Ideally, it would be preferable if protein expression remains stable in the absence of any selection pressure. Previously, we reported that incorporation of STAR elements in a gene construct conveys prolonged stability of gene expression\(^1\). In the mean time we developed a very stringent selection system, STAR-Select, which allows isolation of only a few clones that on average display very high protein expression levels\(^5\). One drawback of this system, in comparison with the previously reported data is, however, that the long-term stability of protein expression is significantly lower when the selection pressure is removed completely. Applying minimal, but constant selection pressure is needed to achieve stable protein expression levels. We therefore searched for means to improve the expression levels obtained with STAR-Select, while avoiding the presence of selection agents in the culture medium. Here we show that the use of a modified metabolic marker could be a straightforward way to achieve long-term stability of protein expression. In fact only a simple shift in the culture medium is needed, which normally also must take place to remove the selection agents. To our knowledge, such ‘passive’ use of a metabolic marker to force the cell to maintain long-term transgene expression has not been reported before.

The usual protocol in which the \(Dhfr\) marker is used as selection marker involves a dual selection procedure. Non-transfected cells are killed by the concomitant use of the Neomycin resistance gene. Furthermore, the toxic methotrexate agent inhibits the activity of the DHFR enzyme, resulting in gene amplification. Up to thousands of copies of the \(Dhfr\) encompassing locus can be amplified, to such an extent that the amplified locus is visible by light microscopy. This results in inherently unstable cells, both genetically and metabolically\(^16\). As explained above, the procedure also requires time-consuming and costly rounds of gene amplification, subcloning and screening of many clones to isolate a clone that displays the desired high protein-expressing quality\(^17\). The continuous presence of toxic methotrexate is required in order to achieve stability of protein expression over prolonged periods of time. When methotrexate is removed from the cell culture, the cell tends to return to a previous state, with a gradual loss of the gene copy number. Obviously, this is accompanied by lower protein expression levels, not a desired outcome\(^18,19\). These effects are considered as major drawbacks of \(Dhfr\) as selection marker.

With our approach we achieved high-producing colonies after a single round of high stringency selection, induced by the modified, TTG Zeo selection marker. This already avoids the time consuming rounds of gene amplification and subcloning. Furthermore, our experiments show that gene amplification is not the reason that we achieve stable protein expression levels. This circumvents the unwanted side effects of genetic instability of cells that are due to the highly amplified gene loci as well as the need for methotrexate. Methotrexate is needed neither to achieve high protein expression, nor to maintain these high levels. In the procedure we developed here, the only way to achieve stable protein expression levels was to first select stable colonies by means of another selection agent, being Zeocin or Neomycin.
Although this two-step process is not unlike to the commonly used methotrexate-induced gene amplification procedure, in our case we can omit both Zeocin and Neomycin after obtaining high-protein expressing colonies, and with only one subsequent switch to HT minus medium (without methotrexate). No costly and time-consuming subcloning is needed.

Our approach is, unfortunately, not without drawbacks either. Direct selection on HT minus medium, only by employing a modified \(Dhfr\) gene still remains complicated. No viable, protein-expressing colonies emerged either with the GTG or TTG \(Dhfr\) variant, indicating that the GTG/TTG translation initiation codons create selection markers that are too stringent for cells to survive at all. Cells survived with the GTG/TTG \(Dhfr\) variants only after initial Zeocin selection, and protein expression levels increased after the switch to HT minus medium. This probably reflects higher mRNA levels that are needed to produce enough DHFR enzyme for the cell to survive. However, these cells showed retarded cell growth, which is definitely an unwanted side effect and we therefore consider both the GTG/TTG \(Dhfr\) variants as too stringent for practical use. One option would be to find and employ translation initiation codons that are less stringent than the GTG codon, to further decrease the selection stringency. Such codons should, however, still provide a higher translation initiation stringency than the ‘wild type’ ATG codon, which provides a stringency which is certainly too low for creating a usable maintenance marker. Apparently, finding the precise selection stringency is of utmost importance for creating an efficient selection system. When the selection stringency is too low, protein expression levels do not remain stable over time; when the selection stringency is too high, protein expression levels remain stable, but the growth of cells is retarded.

Still, we conclude that the most promising application of the \(Dhfr\) gene as selection marker without the use of toxic methotrexate, gene amplification and the obligatory subcloning is as ‘maintenance’ marker. Hence, a first phase in which colonies are established through selection with Zeocin (or in combination with Neomycin) remains essential. Only after enough cells are propagating in an established clone, the cells appear to be able to handle the very stringent selection pressure conveyed by the GTG/TTG \(Dhfr\) genes. A further search for means to decrease the selection stringency provided by the GTG/TTG \(Dhfr\) genes may result in a better balance between keeping protein expression levels stable, without the unwanted side effects of hampered cell growth.

**MATERIALS AND METHODS**

**Vector constructions**

We cloned the modified \(Zeocin\) resistance gene TTG Zeo upstream of the d2EGFP reporter gene and different \(Dhfr\) gene modifications downstream of the d2EGFP gene, coupled through a weakened IRES sequence. Including the following primers made the ATG, GTG TTG \(Dhfr\) gene modifications:

- GATTAGGATCCACCATTGACCCATTGAACCGTACTCATC, \(\text{DHFR-BamH1-ATG-Forward}\)
- GATTAGGATCCACCATTGACCCATTGAACCGTACTCATC, \(\text{DHFR-BamH1-GTG-Forward}\)
- GATTAGGATCCACCATTGACCCATTGAACCGTACTCATC, \(\text{DHFR-BamH1-TTG-Forward}\)

The reverse primer for all three modifications was:

- AAGCTTGAATTCTTAGTCTTCTTCTCTCTGTAGACTTC, \(\text{DHFR-EcoRI-Reverse}\)

Finally, we cloned the combination of STAR elements 7/67 upstream of the expression cassette and STAR element 7 respectively downstream of the cassette.
Transfection and analysis of colonies

CHO-DG44 cells were grown in IMDM medium (Invitrogen), supplemented with 4.6% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 100 μM sodium hypoxanthine (Invitrogen), 16 μM thymidine (Invitrogen), and 10 mM MgCl2 at 37°C / 5% CO2. Hypoxanthine and thymidine (HT) were not added to the medium when culturing in the absence of those substances was required. 0.4·10⁶ CHO-DG44 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 Zeocin (Invitrogen) at a concentration of 400 μg/ml alone or in combination with 500 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 or anti-EpCAM expression levels, 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 HT) with Zeocin (and Neomycin); medium lacking HT (which contains 0.01 mM folate). After 65 days d2EGFP expression levels were determined again. Values were visualized using Graphpad Prism 5 for Windows.

For determining survival rates of CHO-DG44 wild-type cells, 0.2·10⁶ cells were seeded in 6-well plates in medium containing 400 μg/ml Zeocin or in medium lacking HT. Daily, viability of cells was monitored by trypan blue staining for a period of two weeks.

For determining growth rates of CHO-DG44 subclones, 5·10⁴ cells were seeded in duplo in 6-well plates. After 24 hours, cells were counted. After another 48 hours, cells were counted again. With these numbers, doubling times were calculated.

ELISA

For anti-EpCAM measurements, equal numbers of CHO-DG44 cells (0.1·10⁶) were seeded in six-well plates three days prior to cell counting and collection of the medium. To perform an ELISA, 96 well-plates were coated with mouse anti-human Ig κ antibody (BD Pharmingen™). Samples were added to wells at varying dilutions and an anti-human-IgG HRP conjugate (BD Pharmingen™) was used for detection. Finally, an OPD (o-Phenylenediamine dihydrochloride; Sigma) substrate solution was used, after which the antibody concentration was determined by comparing optical density at 492 nm with that of the known antibody standard (human IgM; Accurate Chemical).

Determination of copy numbers

Stable CHO-DG44 clones transfected with the TTG Zeo d2EGFP IRES ATG/GTG/TTG Dhfr plasmids were isolated and assayed for d2EGFP fluorescence levels. Cells were split and further cultured under three culturing conditions. After 65 days, d2EGFP fluorescence levels were determined again. Genomic DNA was purified and the copy number of the d2EGFP gene was determined using real-time PCR. The ratio between the d2EGFP and actin signal was taken as the relative copy number.
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