Employing epigenetics to augment protein expression in mammalian cells
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Various expression augmenting DNA elements benefit from STAR-Select, a novel high stringency selection system for protein expression

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Abstract

The creation of highly productive mammalian cell lines often requires the screening of large numbers of clones and even then, expression levels are often low. Previously, we identified DNA elements, anti-repressor or STAR elements that increase protein expression levels. These positive effects of STAR elements are most apparent when stable clones are established under high selection stringency. We therefore developed a very high selection system, STAR-Select, that allows the formation of few, but highly productive clones. Here we compare the influence of STAR and other expression augmenting DNA elements on protein expression levels in CHO-K1 cells. The comparison is done in the context of the often-used co-transfection selection procedure, and in the context of the STAR-Select system. We show that STAR elements, as well as MAR elements induce the highest protein expression levels with both selection systems. Furthermore, in trans co-transfection of multiple copies of STAR and MAR elements also results in higher protein expression levels. However, highest expression levels are achieved with the STAR-Select selection system, when STAR elements or MARs are incorporated in a single construct. Our results also show that the novel STAR-Select selection system, which was developed in the context of STAR elements, is also very beneficial for the use of MAR elements.
Introduction

The creation of mammalian cell lines that express a therapeutic protein at high levels is often a painstaking process. Usually, large numbers of clones are generated of which the vast majority express very low protein levels. This has led to the introduction of robotics to screen and analyze large numbers of clones (e.g. Biospectra Clone screener). Another approach is to incorporate cis-acting DNA elements in the protein expression cassettes to augment promoter activity. These DNA elements have in common that they have no effect on transient gene-expression levels, this in contrast to enhancers, indicating a mechanism involving chromatin structure.

One of the first identified and best-studied elements is the chicken cHS4 insulator, a 1.2 kb long DNaseI Hypersensitive site (cHS4) of the chicken β-globin LCR. Incorporation of the cHS4 insulator into a construct conveys stability of protein expression over time, but induces only modest increases of protein expression in CHO-K1 cells.

Ubiquitous Chromatin Opening Elements (UCOEs) forms another class of DNA elements that employ promoter regions of ubiquitously expressed housekeeping genes. The idea behind this is that housekeeping genes are always active at a high level, and that regulatory elements in the vicinity of these promoters could be used to induce higher promoter activity. For instance, the dual, divergently expressed promoters of the HNRPA2B1 and CBX3 genes have been shown to convey higher protein expression levels in a number of mammalian cell lines.

Matrix Attachment Regions (MARs) are DNA elements that bind to the nuclear matrix and they are thought to create chromatin loops in which genes are co-ordinately regulated. Both positive and negative influences of MAR elements on protein expression levels have been reported.

Finally, anti-repressor elements, which were dubbed STAR elements, have been identified that block chromatin-associated repressors, such as heterochromatin-related proteins. When these STAR elements were applied in protein expression cassettes, increased numbers of expressing colonies were established and protein expression levels were enhanced.

No analysis has been reported that directly compares the effects of these DNA elements on protein expression levels in a similar experimental set-up. Here we report such analysis and we tested three parameters. In the first place we tested whether DNA elements, other than STAR elements, also act as anti-repressor elements. To test this we employed a screen that...
uses a targeted chromatin-associated repressor to a promoter\textsuperscript{19,21}. In the second place we cloned all DNA elements upstream and downstream of a protein expression cassette that encompasses the CMV promoter and the d2EGFP reporter gene. These constructs were transfected to CHO-K1 cells and selection was executed by means of a co-transfected selection marker, a commonly used procedure. Finally, we cloned all DNA elements upstream and downstream of a protein expression cassette that encompasses the CMV promoter, a modified Zeocin selection gene and the d2EGFP reporter gene. This last set-up constitutes a novel, very stringent selection system that allows the creation of few, but highly productive clones\textsuperscript{22}.

**Results**

*Testing which DNA fragments operate as anti-repressor or STAR element.*

Previously, we reported a screening method for the isolation of genetic elements that counteract gene repression by chromatin-associated repressor proteins. A targeted fusion protein between the LexA protein and the heterochromatin protein HP1\textsuperscript{23} represses the SV40 promoter driven Zeocin resistance gene. This selection vector was called pSelect. When random DNA is inserted between the LexA binding sites and the SV40 promoter, the Zeocin resistance gene is repressed and cells die when subjected to Zeocin (Fig. 1A). By creating and testing a large library of human genomic DNA fragments, we identified and isolated \textasciitilde 70 DNA fragments from surviving clones. These DNA fragment apparently were able to block HP1-mediated repression, resulting in expression of Zeocin resistance gene and subsequent survival when grown in the presence of Zeocin (Fig. 1A). We called these DNA elements anti-repressor or STAR elements\textsuperscript{19}. In a similar manner we now tested whether other known DNA elements that are claimed to enhance protein expression levels, also act as anti-repressor element in this system. Previously, we found that only DNA elements smaller than 3.5 kb can be tested in this system. The targeted LexA-HP1 fusion protein can not adequately cover distances longer than 3.5 kb and therefore does no longer sufficiently repress the downstream located SV40 promoter\textsuperscript{24}. As a result, some cells can survive the selection, leading to false positive clones\textsuperscript{24}. We, therefore, restricted our analysis to DNA elements smaller than 3.5 kb. As reported previously, all isolated STAR elements were substantially smaller than 3 kb\textsuperscript{19}. 
The 1.2 kb cHS4 insulator encompasses a so-called core element that is ~250 bp long and that conveys most of the insulating activity of the cHS4 element. Two different configurations of the cHS4 elements have been reported as being optimal for improving gene expression, hence we tested both configurations\textsuperscript{25-28}. These are either a tandem of six times the core element (6 x cHS4 core, Fig. 1B) or a tandem of two full-length cHS4 elements (2 x cHS4, Fig. 1B). The cHS4 element was cloned in both configurations between the LexA binding sites and SV40 promoter of the Zeocin resistance gene.

In a similar manner we cloned the 2.96 kb long chicken lysozyme MAR element\textsuperscript{29} in the pSelect vector. The lysozyme MAR element has most frequently been employed to augment gene expression. Recently, however, another MAR element, located on the human X-chromosome, has been described to be more potent than the lysozyme MAR in terms of protein expression enhancing properties (patent application no. WO 2005/040377). Hence we also included the X-chromosome MAR in our analysis. We cloned the 3.35 kb X-chromosome MAR element by PCR on human genomic DNA and verified the identity of the product by sequencing.

Two UCOEs have been reported to positively influence gene expression. The functional domain of the so-called TBP UCOE has been mapped to a 25 kb long DNA fragment\textsuperscript{12}. Further fine-mapping indicates that for full functionality still a 5.5 kb fragment is required (US patent 6,881,556 B2). In either case, these fragments are too long to be tested in the anti-repression assay. Furthermore, unlike as with the X-chromosome MAR elements, no claims have been made of whether the TBP UCOE displays more potent expression augmenting properties than the HNRPA2B1 UCOE. Therefore, we only isolated and analysed the HNRPA2B1 and CBX3 UCOE, which has been characterized in more detail\textsuperscript{12}. Functional activity of the HNRPA2B1 and CBX3 UCOE has been shown to be confined to a 8.3 kb long DNA fragment. Within this larger fragment, a 2.0 kb DNA fragment still encompasses more than 90% of the activity of the longer fragment. Hence we isolated a 2.65 kb long HNRPA2B1 and CBX3 UCOE by excising the corresponding DNA fragment from a human BAC clone (RP11-9305). This 2.65 kb DNA fragment fully contains the reported 2.0 kb DNA fragment\textsuperscript{12}. The size of this fragment allows testing in the anti-repressor assay.

Finally, we compared two STAR elements throughout the analysis. In the first place we used the previously reported STAR element 7\textsuperscript{19}. Extensive analysis of multiple STAR elements in the novel selection system described below, showed that not all STAR elements are equally potent in augmenting gene expression (unpublished observations), as noted
before in selection systems employing co-transfection of the selection marker\textsuperscript{19}. For instance, the previously described STAR element 40\textsuperscript{19}, is much less potent than for instance STAR element 7 (unpublished observation). Of the previously\textsuperscript{19} described STAR elements we here compare only STAR elements 7 and 44. In addition, we compared STAR element 67, because in recent studies we found that combining STAR element 67 with other STAR

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**Figure 1. Which expression augmenting DNA fragments can be classified as anti-repressor or STAR element?**

A) Experimental strategy to identify anti-repressor or STAR elements. The heterochromatin protein 1 (HP1) protein was targeted as LexA fusion protein to repress the Zeocin selection gene. DNA fragments are cloned between the LexA binding sites and the Zeocin selection gene. Addition of Zeocin to the culture medium results in cell death when this DNA fragment is a random DNA fragment (upper panel). Cells survive when an anti-repressor or STAR element is present (lower panel).

B) Several, indicated DNA fragments were cloned between the LexA binding sites and the Zeocin selection gene. Only when STAR elements 7, 44 or 67 were present, cells survived after addition of Zeocin, as indicated by plusses. When the other indicated DNA fragments were present, cells died after addition of Zeocin, as indicated by minuses.
Expression augmenting DNA elements in STAR-select

elements has an additive, positive effect on gene expression levels\textsuperscript{22} (Patent application no. EP1600510).

All pSelect constructs were transfected to human U-2 OS cells that contain the LexA-HP1 expressing plasmid, and stable pools of cells were established. Subsequently, expression of LexA-HP1 fusion protein was induced and Zeocin was added to the culture medium. This resulted in the death of cells transfected with the 2 x cHS4, 6 x cHS4 core, the UCOE, the lysozyme MAR and the X-chromosome MAR. In contrast, cells containing the pSelect vector with STAR elements 7, 44 or 67, all survived when grown in the presence of Zeocin (Fig. 1B). STAR elements 7 and 44 containing cells were faster growing and formed bigger colonies in comparison with STAR element 67 containing cells (Fig. 1B). This may indicate a higher strength of STAR elements 7 and 44 to block chromatin-associated repression. Such differences have been noted before when the original STAR screen was performed\textsuperscript{19}. This result shows that the cHS4 insulator, the UCOE and the MAR elements cannot be classified as anti-repressor or STAR element and probably operate by different mechanisms than the anti-repressor STAR elements.

\textit{STAR elements and MAR elements induce high protein expression levels in the context of co-transfection selection.}

We cloned the 2 x cHS4 insulator, the lysozyme MAR and X-chromosome MAR up- and downstream of a expression cassette encompassing the CMV promoter and the d2EGFP reporter gene. The 2 x cHS4 configuration is most often used to test effects on reporter gene expression\textsuperscript{9,30}. The UCOE was cloned only upstream of the CMV promoter, following the described configuration\textsuperscript{13}. We also created two constructs with STAR elements. We used a combination of the STAR elements 7 and element 67\textsuperscript{22}, placed upstream of the expression cassette and STAR element 7 downstream of the cassette. The configuration of two STAR elements upstream of the expression cassette and one STAR element downstream of the cassette was found previously to be optimal\textsuperscript{22}. In the second construct we placed the combination of STAR elements 44 and element 67 upstream of the expression cassette and element 44 downstream of the cassette. The constructs were co-transfected with a plasmid containing the neomycin resistance gene to Chinese Hamster Ovary (CHO) K1 cells. After ~ two weeks up to 19 colonies were isolated and after another ~ three weeks d2EGFP expression levels in the individual colonies were analyzed.

We did not find that incorporation of neither the 2 x cHS4 insulators nor the UCOE resulted in the establishment of colonies with significantly higher d2EGFP expression levels in
comparison to the control. The mean d2EGFP fluorescence signal of the control colonies was 31, of the 2 x cHS4 colonies 55 and of the UCOE colonies 27 (Fig. 2A). In contrast, incorporation of both the lysozyme and the X chromosome MAR resulted in a marked increase in d2EGFP expression levels, from the mean signal of 31 in the control colonies to 182 in the lysozyme MAR colonies and 162 in the X-chromosome MAR colonies. However, greatest increases were observed with constructs containing the STAR elements. Incorporation of 7/67 STAR elements resulted in a mean d2EGFP signal of 363 and incorporation of 44/67 resulted in a mean d2EGFP signal of 387.

Figure 2. Effects of DNA elements on the expression levels of a d2EGFP reporter gene.
A) Effects on gene expression levels in the context of a co-transfection selection procedure. Several DNA elements were cloned to flank an expression cassette consisting of a CMV promoter and the d2EGFP reporter gene. The different DNA elements are described below the graph and indicated as triangle. The control construct contained no flanking DNA elements. These constructs were transfected to CHO-K1 cells and selection took place with Neomycin. The Neomycin selection marker, which was under the control of the human β-actin (HBAP) promoter, was located on a different plasmid that was co-transfected with the d2EGFP reporter gene constructs. The d2EGFP value in the individual clones and the mean d2EGFP fluorescence values in these clones are indicated.

B) Effects on gene expression levels in the context of a novel selection system. The same DNA elements as in A were cloned to flank an expression cassette consisting of a CMV promoter, a modified Zeocin resistance gene (TTG Zeo) and the d2EGFP reporter gene. These constructs were transfected to CHO-K1 cells and selection took place with Zeocin. The d2EGFP value in the individual clones and the mean d2EGFP fluorescence values in these clones are indicated.
The results show that in the context of co-transfection of a selection marker, STAR elements have the largest positive impact on d2EGFP expression levels and that both tested MAR elements also induce a marked increase of d2EGFP expression levels. In this experimental set-up the cHS4 insulator and UCOE have no significant influence on d2EGFP expression levels (p=0.3265 respectively p=0.6725).

**STAR elements and MAR elements induce high protein expression levels in the context of a novel, stringent selection system.**

We recently developed a novel, very stringent selection system to rapidly create few, but highly productive mammalian cell lines. In short, we placed a selection marker with a start codon that confers attenuated translation initiation frequency upstream of the gene of interest with a start codon that confers optimal translation initiation. 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 a construct induced the formation of clones that expressed proteins at very high levels.

We cloned the 2 x cHS4 insulator, the lysozyme MAR and the X-chromosome MAR up- and downstream of the CMV promoter--TTG Zeo d2EGFP cassette. The UCOE was cloned only upstream of the CMV promoter. Similarly, we cloned the combination of STAR elements 7 and element 67 upstream of the CMV promoter--TTG Zeo d2EGFP cassette and STAR element 7 downstream of the cassette. In the second construct we placed the combination of STAR elements 44 and element 67 upstream of the CMV promoter--TTG Zeo d2EGFP cassette and STAR element 44 downstream of the cassette. The constructs were transfected to CHO-K1 cells. After ~ two weeks up to 24 colonies were isolated and after another ~ three weeks d2EGFP expression levels in the individual colonies were analyzed.

As in the co-transfection selection procedure, we did not find that incorporation of neither the 2 x cHS4 insulator nor the UCOE resulted in the establishment of colonies that had significantly higher d2EGFP expression levels. The mean d2EGFP fluorescence signal of the control colonies was 81, the 2 x cHS4 colonies 108 and the UCOE colonies 86 (Fig. 2B). Again both the lysozyme and X-chromosome MAR induced marked increases in d2EGFP expression levels (Fig. 2B), from the mean signal of 81 in the control colonies to 804 in the lysozyme MAR colonies and 903 in the X-chromosome MAR colonies. Also with the novel selection system greatest increases were observed with constructs containing the STAR elements. Incorporation of 7/67 STAR elements resulted in a mean d2EGFP...
signal of 1195 and incorporation of 44/67 STAR elements resulted in a mean d2EGFP signal of 1323. The peak fluorescence signal with the lysozyme and X-chromosome MAR was 1356 and 1508 respectively, whereas with the 7/67 and 44/67 STAR elements the peak fluorescence signal was 2195 and 2483 respectively.

The results show that in the context of the novel selection system, STAR elements have the largest positive impact on d2EGFP expression levels, but also that both tested MAR elements induce a marked increase of d2EGFP expression levels. Furthermore, no significant differences between the two different MARs were observed in terms of impact on d2EGFP expression levels (p=0.3510). The same is true for the 7/67 or 44/67 STAR element combinations (p=0.5098). Finally, also in this experimental setup the cHS4 insulator and the UCOE had no significant effect on d2EGFP expression levels (p=0.7175 respectively p=0.9339).

**Effects of in trans co-transfection of MAR and STAR elements.**

Recently it was reported that co-transfection of MAR elements that were located on a separate plasmid has a large beneficial effect on expression levels of a reporter gene. We performed similar, in trans experiments with both the lysozyme MAR element and STAR 7 element. These elements were cloned into the pBluescript plasmid, creating pBS-MAR and pBS-STAR 7 respectively. Either plasmid was co-transfected with the control TTG Zeo d2EGFP plasmid, in a molar ratio of 1:2 (Fig. 3A). This ratio was taken from the quoted study. As a control we co-transfected the TTG Zeo d2EGFP control plasmid with the empty pBS plasmid, also in a molar ratio of 1:2. After ~ two weeks up to 24 colonies were isolated and after another ~ three weeks d2EGFP expression levels in the individual colonies were analyzed.

With the control TTG Zeo d2EGFP plasmid we found only four surviving colonies that expressed d2EGFP. The mean d2EGFP fluorescence signal of the control colonies was 61 (Fig. 3A). Co-transfection of the control plasmid with the empty pBS plasmid resulted in no colonies at all. However, co-transfection of the control plasmid with pBS-MAR resulted in d2EGFP expressing colonies. Five colonies emerged with an average d2EGFP signal of 677. Co-transfection of the control plasmid with pBS-STAR 7 resulted in more d2EGFP expressing colonies. Ten colonies emerged with an average d2EGFP signal of 1033. These results confirm that in trans co-transfections of either MAR or STAR elements have a beneficial effect on expression levels of a reporter gene.
We also co-transfected pBS-MAR or pBS-STAR 7 with TTG Zeo d2EGFP constructs that were flanked with respectively MAR or STAR elements, as described in the previous section. These constructs were the MAR - TTG Zeo d2EGFP - MAR (cis MAR-MAR) and the STAR 7/67 - TTG Zeo d2EGFP – STAR 7 (cis STAR-STAR) plasmids (Fig. 3B). These plasmids were transfected alone, or co-transfected with the empty pBS, pBS-MAR or with pBS-STAR 7 (Fig. 3B).

Figure 3. Effects of in trans co-transfection of MAR and STAR elements on the expression levels of a d2EGFP reporter gene.
A) The X-chromosome MAR and STAR 7 were cloned in pBluescript, to create pBS-MAR or pBS-STAR 7 (as described below the graph and indicated as triangle). These constructs were in trans co-transfected with a reporter gene construct consisting of the CMV promoter, the TTG Zeo resistance gene and the d2EGFP reporter gene. The constructs were co-transfected to CHO-K1 cells as indicated and selection took place with Zeocin. The d2EGFP value in the individual clones and the mean d2EGFP fluorescence values in these clones are indicated.
B) As in A, but now the reporter gene construct consisted of either a MAR or STAR 7/67 flanked CMV promoter, TTG Zeo- d2EGFP gene expression cassette. In case of the MAR flanked construct, pBS-MAR was in trans co-transfected, in case of the STAR 7/67 flanked construct, pBS-STAR 7 was in trans co-transfected.
Transfection with the MAR - TTG Zeo d2EGFP – MAR plasmid alone resulted in (Fig. 3B) 19 d2EGFP expressing colonies, with an average d2EGFP signal of 898. Co-transfection with the empty pBS resulted in a decrease in the number of d2EGFP expressing colonies (five) and a decrease in the average d2EGFP signal (490) (Fig. 3B). Co-transfection with the pBS-MAR resulted in seven d2EGFP expressing colonies, with an average d2EGFP signal of 582 (Fig. 3B).

A similar picture was found with co-transfection of STAR 7/67 - TTG Zeo d2EGFP – STAR 7 and either the empty pBS or pBS-STAR 7 plasmid. Transfection with the STAR7/67 - TTG Zeo d2EGFP – STAR 7 plasmid alone resulted in 24 d2EGFP expressing colonies, with an average d2EGFP signal of 1243 (Fig. 3B). These were the highest d2EGFP values achieved in this set of experiments, and with the largest number of colonies, thereby replicating the results presented in Fig. 2. Co-transfection with empty pBS plasmid resulted in a decrease in the number of d2EGFP expressing colonies (eight) and a decrease in the average d2EGFP signal (674) (Fig. 3B). Co-transfection with pBS-STAR 7 resulted in ten d2EGFP expressing colonies, with an average d2EGFP signal of 827 (Fig. 3B).

In conclusion, we find that in trans co-transfection with either MAR or STAR element together with a control TTG Zeo construct has a beneficial effect on the expression level of a reporter gene. In contrast, in trans co-transfection of a MAR or STAR element with respectively an in cis MAR-MAR or STAR-STAR construct does not result in an increased number of colonies nor in increased d2EGFP expression levels. Finally, the in cis STAR7/67 - TTG Zeo d2EGFP – STAR 7 plasmid induces the highest colony numbers as well as the highest d2EGFP expression levels.

**Discussion**

In recent years several DNA elements have been claimed to be beneficial for gene expression. A problem with these reports is, however, that the employed expression systems are very diverse (reviewed in 4). This excludes a fair comparison between these diverse classes of DNA elements. In this study we compare several elements and do this in the context of two different selection systems. Furthermore, we test whether the reported DNA elements have anti-repressor properties. From this last analysis it appears that DNA elements such as the cHS4 insulator, UCOE and MARs can not be classified as anti-
repressor or STAR elements, as defined by the anti-repressor assay we developed\textsuperscript{19,21}. This indicates that these elements operate in a different manner. In some cases this is not difficult to envision. By definition, Matrix Attachment Regions (MARs) are DNA elements that bind to the nuclear matrix, a proteinous structure in the cell nucleus. As such they are thought to be involved in creating higher order structuring of chromatin in topologically independent loops\textsuperscript{15,16}. One hypothesis is that the expression of multiple genes that reside in such loops are co-ordinately regulated. Hence MARs may have a predominant structural function in gene organization. In contrast, Ubiquitous Chromatin Opening Elements (UCOEs) consist of divergent promoters of ubiquitously expressed housekeeping genes. It can therefore be envisioned that potentially direct effects of UCOEs on promoters differ mechanistically from more indirect effects of MARs on gene regulation through a structural, gene-organizing manner. The same reasoning can be followed for anti-repressor elements that were identified and isolated by their virtue to survive in a selection system that uses chromatin-associated repression. The diverse identification backgrounds of the distinct DNA fragments already suggest different modes of operation.

How do claims hold up that these DNA elements elevate gene expression? Neither the cHS4 insulator in different configurations, nor the UCOE have a beneficial effect on gene expression in our experimental set-up. The cHS4 insulator was previously shown to induce only modest increases of transgene expression in CHO-K1 cells\textsuperscript{10}. An explanation could be that the cHS4 insulator is derived from the β-globin LCR, whose action is erythropoietic cell line specific. Several reports indeed show beneficial effects of the cHS4 insulator in erythropoietic related cell lines\textsuperscript{9,25,26,33}. We do, however, not have such a potential explanation for the lack of effects of the UCOE in our experimental set-up, particularly because the used, small DNA fragment was shown to retain full functionality\textsuperscript{12}.

In two different selection systems we find that only STAR elements and MARs indeed have a profound, positive effect on gene expression levels. Overall the STAR elements have the most potent, beneficial effects on gene expression, in both tested selection systems. We have compared two different MARs because several reports claim positive and negative effects of MARs, which can largely be attributed to the use of different MARs in different cell lines. Hence we compared two MARs to see how consistent results are amongst this class of elements, and in our hands the two different MARs behave similarly. Secondly, the X-chromosome MAR has been reported to be superior to the lysozyme MAR (patent application no. WO 2005/040377). We do, however, not observe significant differences in
the effects of the two MARs on reporter gene expression levels. The same is actually true for the two different 7/67 and 44/67 STAR elements combinations we tested.

To really assess the large increase in reporter gene expression, induced by STAR elements and MARs, one should compare the control expression levels with the often-used co-transfection selection system and the recently reported, novel selection system which was developed in the context of STAR elements. In this system, a selection marker with a start codon that confers attenuated translation initiation is placed upstream of the gene of interest with a start codon that confers optimal translation initiation. Due to this configuration, the system is forced to express a bicistronic mRNA at very high levels to produce enough resistance marker protein for cell survival. Due to the optimal translation initiation codon, the presence of high levels of the bicistronic mRNA simultaneously results in a high level of the downstream translated protein of interest. We developed this system in the context of anti-repressor or STAR elements that were previously shown to enhance expression levels of proteins in mammalian cells. For instance, incorporation of 44/67 STAR elements in the novel selection system resulted in a mean d2EGFP signal of 1323, versus a mean d2EGFP signal of 31 in the control colonies derived from co-transfection selection. Hence the novel selection system combined with STAR elements results in large increases of d2EGFP expression levels. In this context it should be emphasized that MAR elements are commonly used only in the co-transfection selection system. One of our main conclusions is, therefore, that also MAR elements can greatly benefit from the novel high stringency selection system.

We further confirm the reported positive effects of MAR elements when co-transfected in trans, along with a gene of interest. We modified the reported experimental set-up slightly in the sense that we took the TTG Zeo d2EGFP constructs as target reporter construct, and not a set-up in which the selection marker is co-transfected. We did this because we wanted to assess whether our novel selection system could be further boosted by cotransfection in trans. In this set-up we could indeed repeat the in trans effects when the control TTG Zeo d2EGFP construct was used as target construct. Both MAR and STAR elements, located on separate constructs, induced positive in trans effects on both colony numbers and gene expression levels. However, when in cis MAR- or STAR-flanked TTG Zeo d2EGFP constructs were used as target, negative effects were observed, both in terms of colony numbers and in d2EGFP expression levels. This might imply that optimal expression has been reached with the in cis MAR- or STAR-flanked TTG Zeo d2EGFP configuration and that higher d2EGFP expression levels are not possible. If this is true, co-
transfecting of extra pBS-MAR of pBS-STAR plasmids might simply dilute the corresponding in cis MAR- or STAR-flanked TTG Zeo d2EGFP constructs. Since a cell can stably integrate only a limited number of transfected DNA molecules, a lower number of stably incorporated TTG Zeo d2EGFP reporter copies will result in lower d2EGFP expression levels. Whatever the mechanistic explanation, our results indicate that using a single in cis construct that incorporates either STAR or MAR elements in the context of our novel selection system, is the most simple and effective approach to achieve high protein expressing clones.

To summarize, of four types of claimed expression augmenting DNA fragments, both STAR and MAR elements have large effects on reporter gene expression levels. However, both STAR and MAR elements benefit most from our recently developed selection system. Reported in trans co-transfection effects work only in the context of a control construct that provides inferior colony numbers and expression levels. Most efficient is an in cis approach, in which either STAR or MAR elements are combined with our novel selection system. This selection system is so stringent that it eliminates virtually all low-producing clones. When combined with STAR or MAR elements, the selection system holds a promise to become a useful biotechnological tool for the rapid creation of few, but high protein expressing clones.

**Experimental Protocol**

**Vector constructions.**

We cloned a number of potential expression augmenting DNA fragments upstream and downstream of an expression cassette encompassing the hCMV promoter, the modified Zeocin resistance gene TTG Zeo and the d2EGFP reporter gene. These fragments were: a tandem of two 1.3 kbp fragment with the 5’ hypersensitive site of the chicken β-globin locus⁹, the 1.7 kbp long, six times core element of the 5’ HS element³⁴, a 2.96 kbp fragment encompassing the chicken lysozyme MAR element²⁸, a 3.35 kbp fragment encompassing the human MAR element from the X chromosome (patent application no. WO 2005/040377), and the 2.6 kbp long HNRPA2B1 and CBX3 UCOE¹²,¹³ (only upstream). The UCOE was isolated by excising the 2.6 kbp NotI-Kpnl DNA fragment from a human BAC clone (RP11-9305). The human X chromosome MAR was isolated by PCR on human genomic DNA, using the following primers:
We further cloned the combination of anti-repressor elements 7/67 and 44/67 upstream of the expression cassette and anti-repressor element 7 or 44 respectively downstream of the cassette.

Transfection and analysis of clones.

CHO-K1 cells were grown in HAM F12 medium (Invitrogen), supplemented with 9% FBS. 1x10^6 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufactures protocol. One day after the transfection, cells were seeded to petri dishes and selection agent was added to the culture medium (500 μg/ml G418 or 150 μg/ml Zeocin). The cells are cultured for ~2 weeks, until stably transfected cell clones were formed. Clones were picked, transferred to 6 wells plates and expression of d2EGFP fluorescence levels were analyzed using flowcytometry (Beckman Coulter, EpicsXL). All reported d2EGFP expression data were obtained ~5 weeks after transfection.

Statistical analysis.

Statistical analysis was performed using Graphpad Prism 4 for Windows.

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