Employing epigenetics to augment protein expression in mammalian cells
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Targeting of a histone acetyltransferase domain to a promoter enhances protein expression levels in mammalian cells

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Abstract

Silencing of transfected genes in mammalian cells is a fundamental problem that probably involves the (in)accessibility status of chromatin. A potential solution to this problem is to provide a cell with protein factors that make the chromatin of a promoter more open or accessible for transcription. We tested this by targeting such proteins to different promoters. We found that targeting the p300 histone acetyltransferase (HAT) domain to strong viral or cellular promoters is sufficient to result in higher expression levels of a reporter protein. In contrast, targeting the chromatin-remodeling factor Brahma does not result in stable, higher protein expression levels. The long-term effects of the targeted p300HAT domain on protein expression levels are positively reinforced, when also anti-repressor elements are applied to flank the reporter construct. These elements were previously shown to be potent blockers of chromatin-associated repressors. The simultaneous application of the targeted p300HAT domain and anti-repressor elements conveys long-term stability to protein expression. Whereas no copy number dependency is achieved by targeting of the p300HAT domain alone, copy number dependency is improved when anti-repressor elements are included. We conclude that targeting of protein domains such as HAT domains helps to facilitate expression of transfected genes in mammalian cells. However, the simultaneous application of other genomic elements such as the anti-repressor elements prevents silencing more efficiently.

Keywords: Protein expression; HAT; Anti-repressor elements; Chromatin; Gene silencing
Introduction

Transfection of exogenous genes to mammalian cell lines is a common practice for the large-scale production of therapeutic proteins as well as in research laboratories. It is, however, often difficult to obtain stable mammalian cell lines that express the protein of interest at desirable, high levels. Moreover, expression is often silenced over time. An important cause for this silencing involves chromatin, since the accessibility of chromatin is crucial for a gene to be expressed or not. When integrated in or close to heterochromatin, the transfected gene will be silenced. Silencing can be caused by potent repressors of gene activity such as heterochromatin-associated protein HP1\(^1\) or the chromatin-associated Polycomb group (PcG) proteins\(^2\). Also phenomena such as RNAi can cause silencing of transgenes\(^3\), although it is not clear to which extent RNAi contributes to gene silencing in mammalian cell systems\(^4\). However, the fact that RNAi and HP1-mediated heterochromatinization are mechanistically linked suggests that there is a common underlying mechanism\(^5,6\).

One way to circumvent chromatin-mediated gene silencing is to flank a transgene with genomic elements that improve gene expression. Such elements can be, for instance, MAR elements\(^7\) or the hypersensitive site (HS4) in the locus control region (LCR) of the β-globin gene locus\(^8\). Both elements have successfully been used to improve stable gene expression of an introduced transgene.

Recently, we described a genetic screen to identify genomic elements that efficiently counteract various forms of chromatin-associated repressors\(^9\). Flanking a transgene with these anti-repressor elements, results in higher and more stable protein expression levels.

An alternative way to improve stable gene expression may be targeting factors that facilitate transcription of genes. These could be histone acetyl-transferases (HATs)\(^10,11\) or chromatin-remodelling factors\(^12\). Both classes are thought to make chromatin more accessible for transcription factors, hence facilitating transcription. In fact, for one chromatin-remodelling protein, Brahma, it has been shown that cells transfected with a gene whose expression is driven by a viral long terminal repeat (LTR) show less mosaic expression in the presence of Brahma than in its absence\(^13\). This has been interpreted as such that the presence of Brahma conveys stability to transgene expression.

Here, we tested the effects of HAT proteins and Brahma on the protein expression levels that are driven by the strong viral CMV promoter or the cellular UB6 promoter. We target the factors as LexA-fusion proteins to these promoters and study their effects on protein

Targeting of a histone acetyltransferase domain
expression levels. We find that the HAT domain of the p300 protein\textsuperscript{14} alone is sufficient to exert a positive effect on protein expression levels. The Brahma protein conveys an immediate positive effect, but this is very limited in time. When combined with the previously described anti-repressor elements, the positive effects of the targeted p300HAT domain are enhanced, both in terms of height and stability of expression levels, as well as copy number dependency of protein expression.

**Experimental protocol**

*Transient transfection of constructs*
Constructs expressing either the LexA-binding protein only or LexA fused to a DNA-associated protein (P/CAF, Brm and p300HAT: aa934-1652) were co-transfected into Chinese Hamster Ovary cell line CHO-K1 (ATCC CCL-61) with a plasmid containing LexA-binding sites upstream of the CMV promoter and the DsRED gene using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen). One day after the transfection, the DsRED signal was measured on an Epics XL flowcytometer (Beckman Coulter). In another experiment, stable colonies were established after transfection with the plasmid containing LexA-binding sites upstream of the CMV promoter and the DsRED gene. Four stable colonies were chosen with different DsRED expression levels. These four colonies were transiently transfected with the plasmids expressing the LexA-fusion proteins. After one day, the DsRED signal was measured.

*Stable d2EGFP expression in mammalian cell lines*
The Chinese hamster ovary cell line CHO-K1 (ATCC CCL-61) is cultured in HAM-F12 medium + 10% fetal calf serum containing 2mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C/5% CO\textsubscript{2}. Cells are transfected with the plasmids using Lipofectamine\textsuperscript{TM} 2000 (Invitrogen) as described by the manufacturer. Briefly, cells are seeded to culture vessels and grown overnight to 70–90% confluence. Lipofectamine\textsuperscript{TM} 2000 is combined with plasmid DNA at 2.5μl/μg DNA and added to the cells. After 6 h, the transfection mixture is replaced by fresh medium, and the transfected cells are incubated further. The following day, cells are seeded in serial dilutions into medium containing zeocin (100 μg/ml). Approximately 12 days after transfection individual colonies become visible, and colonies are isolated and transferred to 24-well plates in medium containing
zeocin. When grown to ~70% confluence, cells were transferred to six-well plates. Stable clones were expanded for one to two weeks in six-well plates before the d2EGFP signal was determined on an Epics XL Beckman Coulter flowcytometer. The mean of the d2EGFP signal was taken as measure for the level of d2EGFP expression. d2EGFP signals in the clones were measured after period of two weeks.

**Determination of copy numbers**

Indicated stable individual CHO clones transfected with d2EGFP containing plasmids were isolated and assayed for d2EGFP expression. Genomic DNA was purified and the copy number of both the d2EGFP gene and the β-actin gene were determined by a competitive PCR protocol9. The resulting autoradiogram was analyzed by densitometry to determine the relative strength of the d2EGFP DNA bands. The blot was re-hybridized with a probe for actin, and the ratio between the d2EGFP and actin signal was taken as the relative copy number.

**Results**

**Chromatin-associated factors do not transiently influence CMV-driven gene expression levels**

Several chromatin-associated proteins, alone or as part of large protein complexes, have been implicated in positively regulating gene expression. Two classes are histone acetyltransferase (HATs) and chromatin-remodelling factors such as Brahma. A convenient way to assess the influence of these factors on gene activity would be to test their effects in a transient gene expression assay. To do this, we tested a reporter gene construct containing LexA-binding sites upstream of the CMV promoter that drives the DsRED reporter gene. This construct was co-transfected with different constructs encoding a fusion protein between LexA and proteins of interest. We tested the Brahma protein15, the entire HAT protein P/CAF16 and the domain of the p300 HAT protein that confers HAT activity17. As shown in Fig. 1A, we did not see any effect of these LexA-fusion proteins on transient, CMV-driven gene expression levels. We verified whether the LexA-fusion proteins were expressed and found that in all cases antibodies against LexA showed intense nuclear staining, indicating that the proteins were expressed in the proper place (results not shown).
An alternative approach is to first stably transfect the cell line with the DsRED reporter construct, select stable colonies and use these colonies for transient transfection with the plasmids expressing the LexA-fusion proteins. We selected four different stably transfected DsRED colonies, with different DsRED expression levels. In Fig. 1B and C, we show the results of two such colonies with relatively low (clone #6) and high (clone #9) DsRED expression levels, respectively. In neither case did transient expression of the LexA-fusion proteins result in significant effects on DsRED expression levels. Also in the other two selected colonies, no effects were observed (results not shown).
Taken together, these results exclude the possibility to efficiently access potential effects of chromatin-associated factors on gene expression levels, when these factors are transiently expressed.

**Chromatin-associated factors positively influence CMV-driven gene expression levels when tested in stably transfected clones**

We next tested whether targeted Brahma, P/CAF and the p300HAT domain (aa 934–1652) influence CMV-driven gene expression levels in stably transfected cell lines. We constructed plasmids that encompass on one plasmid, both the d2EGFP reporter gene and the gene encoding the LexA-fusion protein. These constructs were designed in such a manner that both expression units are placed in a divergent orientation. In this way, LexA-binding sites are present between the two promoters that drive the respective expression units (Fig. 2). Placing LexA-binding sites for both the CMV-d2EGFP unit and the SV40-LexA-Fusion unit may have the advantage that positive effects of the LexA-fusion proteins can create a loop that keeps both expression units open for transcription. For efficient selection, we coupled the Zeocin resistance gene to the d2EGFP expression unit by means of an internal ribosome entry site (IRES). The d2EGFP-IRES-Zeo configuration warrants that during selection of colonies, only cells can survive if both the Zeocin resistance gene and the d2EGFP gene are expressed.

We found that targeting of the p300HAT domain, the Brahma protein and the P/CAF protein all result in higher d2EGFP expression levels in stably transfected colonies than when the LexA-binding domain alone was targeted (Fig. 2A). As above, we verified whether the LexA-fusion proteins were expressed and found that in all cases antibodies against LexA showed intense nuclear staining, indicating that the proteins were expressed in the proper place (results not shown). These first measurements were performed three weeks after transfection. To study whether these protein expression levels remained stable over a longer time period, we measured the d2EGFP expression levels again two weeks later. The average expression of clones transfected with LexA-p300HAT had remained stable (p300HAT day 21, X(11) = 131 versus day 35, X(11) = 121) (Fig. 2B). In comparison, the expression levels in Brahma-transfected clones were much less stable (Brahma day 21, X(10) = 89 versus day 35, X(10) = 27) (Fig. 2C). Also the d2EGFP expression levels in P/CAF-transfected clones became significantly lower (P/CAF day 21, X(10) = 40, versus day 35, X(10) = 15) (Fig. 2D). Finally, the already low expression levels in LexA-transfected clones became slightly lower (LexA day 21, X(10)=25, versus day
Of Brahma, it has been shown that the lack of detectable endogenous Brahma is of importance for gene silencing\textsuperscript{13}. We therefore compared the endogenous Brahma protein levels in CHO with the Brahma protein levels in the C33A and SW620 cell lines, which express Brahma at an undetectable and high level\textsuperscript{13}. As shown in Fig. 2C in CHO-K1, Brahma was expressed at an intermediate level lower than in SW620, but higher than in C33A, in which no Brahma protein could be detected. We, therefore, do not believe that lack of Brahma protein is a reason for the rapid decline in the d2EGFP expression levels in the colonies that have been transfected with LexA-Brahma.

We conclude that the initial positive effects of LexA-Brahma and LexA-P/CAF on protein expression levels are limited to a very small period of time. In contrast, expression levels in clones transfected with LexA-p300HAT remained stable between three and five weeks after transfection. Furthermore, in comparison with LexA, LexA-Brahma and LexA-P/CAF transfected clones, the expression levels in LexA-p300HAT transfected clones were also highest (Fig. 2B). These results prompted us to further focus on the effects of LexA-p300HAT.

35,X(10)=19 (Fig. 2A). Hence, five weeks after transfection, the expression levels in clones transfected with LexA-Brahma or LexAP/CAF were as low as in the control clones, transfected with LexA alone.

We conclude that the initial positive effects of LexA-Brahma and LexA-P/CAF on protein expression levels are limited to a very small period of time. In contrast, expression levels in clones transfected with LexA-p300HAT remained stable between three and five weeks after transfection. Furthermore, in comparison with LexA, LexA-Brahma and LexA-P/CAF transfected clones, the expression levels in LexA-p300HAT transfected clones were also highest (Fig. 2B). These results prompted us to further focus on the effects of LexA-p300HAT.
p300HAT-induced enhancement of protein expression levels is only stably over prolonged time periods in the context of flanking anti-repressor elements

We repeated the experiment in which LexAp300HAT is targeted to the CMV promoter, and compared the clones that were stably transfected with a plasmid that only contains the expression unit d2EGFP-IRES-Zeo (Fig. 3). We found an increase in the average d2EGFP expression levels, when measured ~six weeks after transfection (Control day 40, X(11) = 55 versus p300HAT day 40, X(12) = 122) (Fig. 3). These first measurements were performed while the clones were still under Zeocin-selection pressure. Next we tested the stability of expression after the selection pressure was removed. After removal of Zeocin...

Figure 3. Increases in CMV-driven protein expression levels, induced by p300HAT, are not stable over prolonged time periods.

Indicated constructs encoding either the CMV-driven d2EGFP reporter protein alone (A) or together with the LexA-p300HAT fusion protein (B) were transfected to CHO-K1 cells. As in Fig. 2, stably transfected clones were isolated, d2EGFP protein expression levels were measured (the d2EGFP-fluorescence signal in the flowcytometer is taken as measure for d2EGFP protein expression), selection was removed and clones were grown further. The expression levels in individual clones, of which the number is given in the X-axis are indicated. Measurements were 40 days after transfection, and colonies are sorted according to increasing expression levels (black bars). Individual colonies are followed 120 days after transfection (white bars). At the bottom of the charts, the mean of the d2EGFP expression levels of all colonies is stated. For example, the mean of the d2EGFP expression levels in the 12 analyzed stable colonies transfected with LexA-p300HAT (B) after 40 days is 122, hence stated as 40 days; X(12) = 122.
from the culture medium, we measured d2EGFP expression levels every two weeks, up to four months after transfection. After these four months, the d2EGFP expression levels in virtually all control clones were dropped to very low levels. In contrast, after four months, some of the LexA-p300HAT transfected clones maintained high d2EGFP expression levels (Control day 120, X(11) = 28 versus p300HAT day 120, X(12) = 79) (Fig. 3). However, after four months, in most of the LexA-p300HAT transfected clones there was a substantial decline in d2EGFP expression levels. In Fig. 2, colonies were measured under continuous selection pressure and only up to two months. From Fig. 3, it becomes clear that when selection pressure is removed and expression levels are followed over a period longer than two months, targeting of LexA-p300HAT does not result in stable expression in all clones.

The detailed time course of measurements indeed showed that expression levels in LexA-p300HAT transfected clones slowly became lower in time (data not shown). This result prompted us to examine whether we could improve the stability of expression by flanking the constructs with anti-repressor elements that we previously identified to stabilize protein expression levels. These anti-repressor elements were identified in a genetic screen that was designed to isolate DNA fragments, which are able to counteract chromatin-associated repressors such as Polycomb group (PcG) proteins and the HP1 protein. Previously, we showed that inclusion of these elements renders protein expression more stable over a long time period. We therefore flanked the constructs with either anti-repressor element 4 or 7 (Fig. 4). We found that both elements 4 and 7 positively influenced d2EGFP expression levels in terms of height and stability. Measurements one month after transfection showed that application of elements 4 and 7 results in higher expression levels (p300HAT day 35, X(10) = 120; p300HAT 7/7 day 35, X(10) = 168 and p300HAT4/4 day 35, X(10) = 215) (Fig. 4). We attribute this increase in d2EGFP expression levels to the action of the respective anti-repressor elements, as shown previously. These differences became more apparent, when the d2EGFP expression levels were compared approximately four months after transfection (p300HAT day 110, X(10) = 62; p300HAT 7/7 day 110, X(10) = 164 and p300HAT 4/4 day 110, X(10) = 231). The average d2EGFP expression levels in LexA-p300HAT transfected clones dropped a factor two, as in the experiment in Fig. 3. In contrast, no significant decreases in d2EGFP expression levels were observed in the clones that contain constructs with anti-repressor elements 4 and 7, along with the LexA-p300HAT protein. Therefore, after approximately four months in culture, the average expression levels in LexA-p300HAT 4/4 was a factor 4 higher in comparison with expression levels of colonies with LexA-p300HAT alone. On an average, the p300HAT 7/7
plasmid produces eight-fold more GFP-protein than the control plasmid, whereas the p300HAT plasmid produces only two-fold more d2EGFP protein.

Above experiments were all performed in CHO-K1 cells. We repeated the experiments in the human PER·C6 cell line\textsuperscript{18}. We found that transfection with the construct that encode the LexA-p300HAT and the d2EGFP reporter gene results in the establishment of colonies with elevated d2EGFP expression levels, particularly when also anti-repressor elements are incorporated (data not shown). Therefore, the beneficial effects of the p300-HAT on reporter gene expression are not limited to one specific cell line.

**Figure 4. Anti-repressor elements stabilize CMV-driven protein expression levels over prolonged time periods.**

Indicated constructs encoding the CMV-driven d2EGFP reporter protein and the LexA-p300HAT fusion protein (A), in the absence or presence of anti-repressor elements 7 (B) or 4 (C), were transfected to CHO-K1 cells. As in Fig. 2, stably transfected clones were isolated, d2EGFP protein expression levels measured (the d2EGFP-fluorescence signal in the flowcytometer is taken as measure for d2EGFP protein expression), selection was removed and clones were grown further. Indicated are the expression levels in individual clones, of which the number is given in the X-axis. Measurements were 35 days after transfection and colonies are sorted according to increasing expression levels (black bars). Individual colonies are followed 110 days after transfection (white bars). At the bottom of the charts, the mean of the d2EGFP expression levels of all colonies is stated. For example, the mean of the d2EGFP expression levels in the 10 analyzed stable colonies transfected with LexA-p300HAT (A) after 35 days is 120, hence stated as 35 days; X(10) = 120.
**Targeted p300HAT protein has also positive effects on the cellular UB6 promoter**

We also tested whether LexA-p300HAT, alone or in combination with an anti-repressor element, has an effect on a strong cellular promoter. For this, we used the UB6 promoter. Cells were transfected with a plasmid that only contains the expression unit d2EGFP-IRES-Zeo (UB6 Control) (Fig. 5A). Stable clones were compared with clones in which LexA-p300HAT is targeted to the UB6 promoter (UB6-p300HAT) (Fig. 5B) or have in addition anti-repressor element 7 included to flank the entire construct (UB6-p300HAT-7/7) (Fig. 5C). One month after transfection, we found no increase in the average d2EGFP expression levels when LexA-p300HAT is included, and we found a two-fold increase when the anti-

![Diagram showing constructs and expression levels](image)

**Figure 5. p300HAT, in combination with an anti-repressor element, enhances UB6 driven protein expression levels over prolonged time periods.**

Indicated constructs encoding the UB6-driven d2EGFP reporter protein alone (A), or together with the LexA-p300HAT fusion protein (B) and/or anti-repressor element 7 (C), were transfected to CHO-K1 cells. As in Fig. 2, stably transfected clones were isolated, d2EGFP protein expression levels measured (the d2EGFP-fluorescence signal in the flowcytometer is taken as measure for d2EGFP protein expression), selection was removed and clones were grown further. Indicated are the expression levels in individual clones, of which the number is given in the X-axis. Measurements were 35 days after transfection, and colonies are sorted according to increasing expression levels (black bars). Individual colonies are followed 110 days after transfection (white bars). At the bottom of the charts, the mean of the d2EGFP expression levels of all colonies is stated. For example, the mean of the d2EGFP expression levels in the 12 analyzed stable colonies transfected with LexA-p300HAT (B) after 35 days is 72, hence stated as 35 days; \( X(12) = 72 \).
repressor element 7/7 is included as well (UB6 Control day 35, X(12) = 70; UB6-p300HAT day 35, X(12) = 72 and UB6-p300HAT 7/7 day 35, X(12) = 157) (Fig. 5). After approximately four months in culture, without selection pressure, the d2EGFP expression levels in UB6-p300HAT-transfected colonies and UB6-p300HAT-7/7-transfected clones remained stable, whereas the expression levels in the control clones dropped significantly (UB6 Control day 110, X(12) = 31, UB6-p300HAT day 110, X(12) = 63 and UB6-p300HAT-7/7 day 110, X(12) = 207). Therefore, after approximately four months in culture, the difference in average expression levels in LexA-p300HAT became a factor 2 higher in comparison with expression levels of control clones. Inclusion of anti-repressor element 7, in addition to the p300HAT domain, results in average expression levels that are a factor 6 to 7 higher than in control clones.

![Diagram](image)

**Figure 6. The anti-repressor element improves the copy number dependency of protein expression levels.**

Indicated constructs encoding the UB6- driven d2EGFP reporter protein alone (A), together with the LexA-p300HAT fusion protein (B) and/or anti-repressor element 7 (C), were transfected to CHO-K1 cells. After approximately three months in culture (without Zeocin selection), genomic DNA was isolated from indicated stable clones. The copy number of the d2EGFP construct was determined. The relative copy numbers were plotted against the d2EGFP signals and the regression line and the correlation coefficients were added.
Copy number dependency and the incorporation of p300HAT and anti-repressor elements into the constructs

An important question is whether the transfected reporter genes are expressed in a copy number dependent fashion\(^{19}\). We, therefore, determined the copy number of the integrated UB6-control, UB6-p300HAT and UB6-p300HAT-7/7 vectors. In each case, 12 to 14 colonies were analysed. As shown in Fig. 6, no correlation (correlation coefficient R\(^2\) = 0.137) between the copy number of integrated plasmids and the expression level of d2EGFP was observed in control clones (UB6 Control) (Fig. 6A). An increase in the correlation coefficient was found in clones containing plasmid with LexA-p300HAT alone (R\(^2\) =0.333) (Fig. 6B). The best correlation coefficient was found, however, when both LexA-p300HAT and anti-repressor element 7 were included in the constructs (R\(^2\) = 0.833) (Fig. 6C). It is however, important to note that doubling of the number of copies does not signify doubling in protein expression levels. Therefore, although there is a better correlation coefficient in clones with LexA-p300HAT and anti-repressor element 7, it cannot be concluded that complete copy number dependency is achieved. The results do indicate though that in p300HAT-7/7 colonies, more d2EGFP protein is expressed per copy of p300HAT 7/7 plasmid, as is shown by the steeper slope of the curve.

Discussion

We report that targeting of the HAT domain of the p300 protein to promoters positively influences stable gene expression in transgenic cell lines. In contrast, targeting of another histone acetyltransferase, P/CAF or the chromatin-remodelling factor, Brahma to these promoters does not result in long-term enhancement of the expression level of the reporter protein. A number of observations can be discussed. None of the tested LexA-fusion proteins worked in a transient fashion. For the p300HAT-domain, this has been reported previously\(^{20}\). Only when targeted to a minimal promoter, but not a strong viral promoter, the LexA-p300HAT fusion protein significantly enhanced expression levels. We found the same to be true in our experimental set-up (data not shown). Our results showing only a positive effect on protein expression levels in stably transfected clones indicate that these proteins exert their action through chromatin structure, as expected. Most of the effects on expression levels become apparent when clones are cultured for prolonged time, particularly in the absence of selection pressure.
In the negative control clones, expression levels drop to significantly lower levels than in LexA-p300HAT transfected clones. This can, however, not conceal the fact that the d2EGFP expression levels decrease in some LexA-p300HAT clones also. This is particularly true for CMV promoter-driven expression. An explanation may be that the effects (histone acetylation) of p300HAT alone are simply insufficient to keep the promoter open for transcription, and that silencing of the promoter occurs nonetheless. This last explanation may also be the reason for the very limited effects of targeted LexA-Brahma and LexA-P/CAF. When measured two weeks after transfection, there is a significant positive effect of these targeted-fusion proteins on d2EGFP expression levels. However, only weeks later these positive effects are completely obliterated. Brahma has previously been reported to have a positive effect on mosaic expression pattern in clones transfected with LTR-driven reporter constructs. Overexpression of Brahma in these clones results in a significantly less mosaic expression pattern, which is interpreted as an indication for more stable gene expression in such a colony. It is not clear, however, how the expression patterns in these clones behave after prolonged time periods. Also, differences may exist amongst promoters. In the cited study, it was found that the used MuLV-LTR probably contains an endogenous binding site for Brahma-containing protein complexes. This is entirely unknown for the CMV promoter, which we tested in the current study. In line with this notion, we found that targeting of LexA-p300HAT to the UB6 promoter results in very stable UB6-driven expression levels, even after four months in culture. This may indicate that p300HAT acts more efficiently on the UB6 promoter than on the CMV promoter.

Another question is why does a related histone acetyltransferase such as P/CAF not operate well, whereas the p300HAT domain alone is sufficient for mediating positive effect on protein expression? This is puzzling, since the HAT domains of p300 and P/CAF are closely related. The answer may be that the entire P/CAF protein encompasses other protein domains that interfere negatively with the transcription machinery on a long-term basis. This appears to be a feasible explanation, since we also tested the entire p300 protein as LexA-fusion protein and found this full-length protein had limited or no effects on expression levels (unpublished observation). This suggests that only protein domains that exert a certain function, such as histone acetylation, are useful and sufficient for achieving stable, long-term effects on gene expression. We found that not many chromatin-associated factors are able to influence protein expression levels in stably transfected cells. Along with the HATs and Brahma, we tested the human homolog of the Ash1 protein, a known trithorax group protein, implicated in epigenetic activation of gene activity. Ash1 has been
shown to be a histone methyltransferase, and targeting Ash1 to a promoter results in activation of gene activity in Drosophila\textsuperscript{21}. We, however, did not find any effect of targeting the human Ash1 homolog to the CMV promoter\textsuperscript{22}. We tested different portions of the human Ash1 protein in view of the result that of the p300 HAT protein, only the HAT domain exerted a positive influence. However, portions of the human Ash1 protein encompassing the predicted histone methyltransferase activity domain did not exert a positive effect on protein expression levels (data not shown). Therefore, the above-discussed conclusion that only a domain with HAT or HMTase activity should be able to positively influence protein expression levels does not hold in this case. We further tested whether targeting of a human G/T mismatch DNA glycosylase (MCDG) had positive effects on CMV-driven protein expression levels. This protein has a capacity to demethylate stably integrated promoters and has been reported to de-methylate and strongly enhance a hormone-regulated promoter\textsuperscript{23}. However, no effects were observed of targeted LexA-MCDG on the CMV-driven d2EGFP expression levels in stably transfected colonies. We conclude that only a domain with known histone acetyltransferase activity does significantly influence protein expression levels driven by either the strong viral CMV promoter or the strong cellular UB6 promoter. How does this work? The rational for this study to target a HAT domain to a promoter was the assumption that this domain will acetylate N-terminal histone tails on or in the vicinity of the promoter region. Acetylation of histones is generally accepted to have a positive influence on transcription. However, our results indicate that the positive effects of targeting the p300HAT domain are limited in terms of long-term stability, particularly in the context of the CMV promoter. It is therefore interesting that inclusion of anti-repressor elements to the constructs conveys more stability to the protein expression levels. The anti-repressor elements have a beneficial effect on both stability and height of expression levels, this above the reported effect of the p300HAT domain alone. Placing both the LexA-p300HAT gene and anti-repressor elements on one construct results in higher expression levels. This raises the question whether these elements work through a mechanism involving attraction of histone acetyltransferases. The modest increases of a factor two to three may, however, indicate that we deal with distinct molecular mechanisms. If anti-repressor elements operate through targeting of endogenous HATs, one might expect larger, synergistic effects when both the anti-repressor elements and the targeted HAT domain are present. Such a working mechanism has been suggested for the HS4 insulator.
The endogenous HS4 insulator is characterized by distinct patterns of histone acetylation, up- and downstream of the insulator\textsuperscript{24}. This suggests that the HS4 insulator is a target site for endogenous HAT activity. In agreement with this, inclusion of the HS4 insulator in a construct induces high histone acetylation levels in the promoter region\textsuperscript{24}. We will address the question of histone modifications in further studies to see whether the application of anti-repressor elements results in the induction of differential histone modification patterns.

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**References**
