Selection markers and expression enhancing elements: novel strategies to create mammalian production cell lines
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ANALYSIS OF SEVERAL HUMAN CELLULAR PROMOTERS REVEALS FAVORABLE CHARACTERISTICS OF THE HUMAN $\beta$-ACTIN PROMOTER

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**ABSTRACT**

For the production of recombinant proteins in mammalian cells, often the viral CMV promoter is employed. In order to find other strong promoters, we isolated and analyzed eleven human cellular promoters and compared them with the CMV promoter. These human cellular promoter regions have some structural aspects in common, such as an untranslated exon and intron. We focused on the ability of the promoters to induce stably transfected clones in three different mammalian cell lines and analyzed the expression levels of several reporter genes in such clones. The human cellular promoters differed vastly in their ability to induce stably transfected clones and high protein expression levels. We found that in particular the human β-actin and ubiquitin C promoters were able to induce substantially higher protein expression levels in CHO derivatives than the CMV promoter. The cellular promoters also reacted very differently to sub-optimal growth conditions, such as serum reduction and confluent growth. Overall, the human β-actin promoter appeared to be the most robust promoter, in the three cell lines we tested. We conclude that the β-actin promoter seems to be at least as good a choice as the CMV promoter for the production of proteins in mammalian cells.

**INTRODUCTION**

Viral promoters are often considered as promoter of choice for the production of recombinant proteins in mammalian cells. The promoter regions of the Simian Virus 40 (SV40), Rous Sarcoma Virus (RSV) and human Cytomegalovirus (CMV) immediate early gene are the most commonly used viral promoters. These promoters have been used for decades, both in basic research and in industrial settings. In particular the CMV promoter is popular since it is considered a reliable promoter that confers high protein expression levels\(^1\). Over the years, however, several human cellular promoters have been found to also convey high expression levels of recombinant proteins in mammalian cells. Since the production of therapeutic proteins in large-scale production processes is a profitable and important aspect of the biotechnology industry, it seems worthwhile to compare characteristics, promoter strength and behavior of viral and human cellular promoters. However, such a comprehensive comparison in mammalian cell lines that are relevant for the industrial production of therapeutic proteins, has not been published. In this study we isolated and characterized several human cellular promoters and compared them with the CMV promoter. We focused in particular on the question whether these human cellular promoters are able to confer high expression levels of recombinant genes in stably transfected mammalian cell cultures.

We compared the CMV promoter with the promoters of the following human genes: β-actin and γ-actin\(^2,3\), ubiquitin B and C (UBB and UBC)\(^4,6\), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)\(^7\), histone H3 (hisH3)\(^8\), and the ribosomal proteins (RPS21, RPL41, RPL32, RPL8 and RPLP1)\(^9\). These genes encode proteins that are respectively involved in cytoskeleton formation, protein degradation, carbohydrate metabolism, chromatin formation, and the formation of ribosomal subunits. As such they encode so-called housekeeping proteins, proteins that are required for the maintenance of basal cellular functions\(^10,11\). Furthermore, housekeeping genes are supposed to be constitutively expressed in a species- and tissue-independent way. Most of these genes are thought to be cell cycle-independently expressed, with the possible exception of the ribosomal proteins\(^12,13\). These therefore provide interesting properties to a promoter to drive the expression of recombinant genes in different culture cells.
The promoters that we selected belong to genes that convey high messenger RNA (mRNA) expression levels of their respective genes, as determined by Su et al.\textsuperscript{14}. As a further criterion, we selected promoters that belong to single-copy genes. We preferred to study these promoters, since high mRNA expression levels can be expected to be due to high promoter activity, although also differences in mRNA stability can play a role. We hypothesized that, as a consequence, such promoters would also be able to confer high mRNA expression of recombinant genes.

The human cellular promoter regions that we selected for this study have also structural aspects in common. They possess introns in their 5’ untranslated regions, with the exception of the RPLP1 promoter. In Figure 1B the size and location of untranslated exons, introns and 5’ flanking sequences of the studied human cellular promoters are shown. The presence of an intron has been envisioned to enable high promoter activity; for instance, enhancer activity has been described to be located in the intron of the promoter region of the human β-actin gene\textsuperscript{15}. Furthermore, efficient cytoplasmic transport and translation of the mRNA in eukaryotic cells depends on splicing\textsuperscript{16}. For this reason, most expression vectors now include at least one intron sequence, which is usually located between the promoter and the cDNA coding sequence. Because of the presence of introns in our selected human cellular promoters, this is also accomplished in our expression vector. Finally, the studied human cellular promoter regions possess regions of high evolutionary conservation. These homologous regions could suggest that these particular DNA regions are important for the proper functioning of the promoter\textsuperscript{17}. Although most of these human cellular promoters have been described previously, they were never the subjects of a systematic comparison in mammalian cell lines relevant for industrial therapeutic protein production, as we present here.

**RESULTS**

**Cloning of the human cellular promoters in the STAR-Select vector**

The human cellular promoters that we studied all contain DNA stretches with a high degree of evolutionary conservation. Figure 1A shows an example of BLAT results of the human β-actin promoter that was used in this study. All isolated human cellular promoter regions possessed a relatively long 5’ non-coding region, with an untranslated exon and an intron (with the exception of RPLP1) as common structural features (Figure 1B). The indicated human cellular promoters (Figure 1B) were isolated by polymerase chain reaction (PCR) on human genomic DNA and they were compared to the CMV immediate-early promoter/enhancer. The human cellular promoter regions contain introns and for a proper comparison with the CMV promoter, an intron known to enhance the stability of mRNA was placed between the CMV promoter and the reporter gene\textsuperscript{18}.

The promoters were cloned in the STAR-Select vector that conveys a high stringency of selection pressure\textsuperscript{19} (Figure 1C). In a STAR-Select vector, the Zeocin selection marker is modified to use a startcodon that confers attenuated translation initiation frequency. This modified selection marker is placed upstream of the d2EGFP reporter gene, which, in contrast, has a startcodon that confers optimal translation initiation (Figure 1C). Due to the difference in translation initiation frequency of the startcodons, translation from the bicistronic mRNA will be initiated most frequently at the d2EGFP reporter gene, whereas the selection marker will be translated at a much lower frequency. This warrants a relatively low amount of selection marker protein in comparison with the amount of reporter protein. For cell survival, however, a threshold amount of selection marker protein is required. To achieve that, high amounts of bicistronic mRNA have to be produced, which in turn leads to a high expression level of the reporter protein. This system thus couples high selection stringency to high expression levels
Figure 1. Structural aspects of the selected human cellular promoters.

A. The 3.5 kb β-actin promoter was submitted to BLAT. Shown is a typical result of such a search. Arrows indicate the regions of high evolutionary conservation between the indicated species. At the bottom, the size and structural features of the β-actin promoter are shown. The numbers indicate the nucleotide positions relative to the ATG start codon.

B. The structural features of the eleven human cellular promoters we isolated are shown. Indicated are the noncoding 5' flanking sequence (stippled), an untranslated 5' exon (solid black) and intron (grey). The lengths of the bars are proportional, the β-actin promoter being 3.5 kb long.

C. The indicated human cellular promoters were cloned in the STAR-Select vector that employs a high stringency of selection pressure. This construct consists of a specific promoter, a modified Zeocin resistance gene (TTG Zeo) and the d2EGFP reporter gene. Indicated STAR elements (7 and 67) are used to flank the entire construct.
of a protein of interest\textsuperscript{19}. In the STAR-Select vector, STAR elements\textsuperscript{20} are applied to augment gene expression and thus mRNA expression levels, to enable colony formation under very stringent selection conditions. In fact, when STAR elements are not included in the construct, expression levels are often too low to produce enough selection marker protein, resulting in no colony formation\textsuperscript{19}.

As reporter gene we used d2EGFP, an unstable variant of EGFP (enhanced green fluorescent protein) that is characterized by a much shorter half-life compared to EGFP\textsuperscript{21}. d2EGFP is taken as reporter gene because this warrants a rapid turnover rate of the protein. As a consequence, the measured expression levels of d2EGFP protein probably better reflect promoter activity than when the more stable, and thus accumulating EGFP protein is used as reporter gene.

**Different human cellular promoters induce a wide range of colony numbers and d2EGFP expression levels in CHO-K1 cells**

We tested the STAR-Select expression vectors with the various cellular promoters by stably transfecting CHO-K1 (Chinese Hamster Ovary) cells. For the stable transfections, equal amounts of DNA (3 μg) were used in case of each construct. For the analysis of promoter activity, two experimental read-outs were chosen. First, we examined the number of colonies that formed under identical selection conditions with each promoter. Secondly, the d2EGFP expression levels in these stable clones were measured. Figure 2 shows that the CMV, β-actin and RPS21 promoters induced most colonies in CHO-K1 cells, ranging between 110 and 120 colonies. The RPL41, RPL8, RPL32 and UBC promoters induced fewer colonies, ranging from 35 to 90. Finally, the γ-actin, UBB, GAPDH, hisH3 and RPLP1 promoters induced less than 25 colonies. Since equal micrograms of DNA were used for these transfections and since the promoters differ in size, different molar amounts were transfected. However, the promoters were cloned in vectors with a backbone of 10.7 kilobase (kb), and therefore the size differences between the vectors due to the different lengths of the promoters were limited. In fact, the resulting vectors ranged in size from 11.3 to 16.3 kb, corresponding to 0.28 and 0.4 picomole of transfected DNA, respectively. This can not explain the up to fourfold differences in colony numbers. Moreover, major differences in colony number were observed with similar-sized constructs (between 12.1 and 12.5 kb), containing the γ-actin, UBB, UBC, RPS21 or RPLP8 promoters (Figure 2). Finally, inclusion of the β-actin promoter created one of the largest vectors (14.2 kb), and this vector induced the highest number of colonies, in the upper range of 110 to 120. Differences in vector size and thus different molar amounts of transfected DNA are therefore unlikely explanations for the rather high heterogeneity in colony formation. Instead, we presume that the observed differences in colony formation reflect differences in promoter activity.

As second read-out we analyzed the d2EGFP expression levels in the established, stably transfected colonies. Up to 24 stably transfected colonies (or less if not sufficient colonies had formed) were isolated, propagated and the d2EGFP expression levels were determined by flow cytometry (FACS) (Figure 2). The average levels of d2EGFP in all colonies, as well as expression levels in the individual clones are shown. The d2EGFP expression levels varied greatly among the different promoters and between colonies. The γ-actin promoter induced the highest average d2EGFP expression in all colonies (864), but with this promoter a total of only 23 colonies were formed. Furthermore, the average d2EGFP expression levels induced by the β-actin (503), UBC (554) and ribosomal promoters (ranging between 444 and 570) were relatively high, as compared to the average d2EGFP expression levels induced by the CMV promoter (333). In contrast, the two colonies that survived the transfection with the hisH3 promoter showed lower d2EGFP expression (average 98). Finally, the γ-actin, UBC and RPLP8 promoters induced the highest-d2EGFP expressing individual clones (Figure 2).
Figure 2. Different human cellular promoters induce a wide range of colony numbers and d2EGFP expression levels in CHO-K1 cells. The upper diagram shows the mean GFP fluorescence values in individual, stably transfected CHO-K1 clones, which are indicated by dots and are indicative of d2EGFP expression levels. The horizontal bars indicate the average d2EGFP expression levels of all clones with the same promoter. For example, the γ-actin promoter induced the highest average d2EGFP expression as well as the highest d2EGFP expression level in an individual clone. The bars in the lower diagram indicate the total number of stably transfected CHO-K1 colonies, established after each transfection with the indicated promoter constructs. Equal amounts of DNA were transfected and the same selection pressure was applied. The β-actin, RPS21, and CMV promoter induced most colonies, whereas the γ-actin, UBB, GAPDH, hisH3, and RPLP1 promoters induced fewest colonies.

In summary, the various human cellular promoters induced considerably different numbers of stable colonies as well as different d2EGFP expression levels in these colonies. Based on these results we omitted the UBB, GAPDH, hisH3 and RPLP1 promoters from the analysis for further study. These promoters induced only few colonies and in case of the hisH3 promoter, d2EGFP expression levels in these colonies were very low as well.
Human cellular promoters react differently to reduced serum levels and complete cell culture confluence in CHO-K1 cells

So far, promoter activities were tested in CHO-K1 cells that were exponentially growing in rich-serum medium. We next tested the behavior of the promoters under sub-optimal growth conditions, that is, under reduced serum levels and complete confluence conditions; conditions that can be important in industrial processes. Cells were initially seeded and grown under normal serum conditions (9,1%), but 24 hours prior to FACS analysis, this medium was replaced by medium containing 0,5% serum. The average d2EGFP expression level of all clones growing under reduced serum conditions was compared to the average d2EGFP expression level of the same clones, growing under normal conditions (Figure 3A). In case of the γ-actin and ribosomal protein promoters, the average d2EGFP expression levels dropped considerably in reaction to reduced serum conditions, varying from 29% to 43% reduction. The CMV and β-actin promoters were less affected by reduced serum conditions, a respective reduction of 12% and 13% in average d2EGFP expression levels, and the UBC promoter was not affected at all.

Next, cells were grown to complete confluence and the cells were maintained in this confluent status for another 24 hours before the FACS analysis was performed. The average d2EGFP expression level of all confluent clones was compared to the average d2EGFP expression level of the same, but still exponentially growing clones (Figure 3B). With all promoters a great reduction in the average d2EGFP expression levels was observed when cells were grown to complete confluence, but the CMV promoter showed the smallest reduction (38%) (Figure 3B). A reduction in average d2EGFP expression levels ranging between 54% and 65% was observed with the β-actin, γ-actin and UBC promoters. Finally, the greatest reduction (from 71% to 83%) was observed with the four ribosomal protein promoters.

In summary, the CMV, β-actin and UBC promoters were least affected by sub-optimal growth conditions, as defined by reduced serum and complete confluence. The ribosomal protein promoters were most affected by these growth conditions. We therefore decided to omit the ribosomal protein promoters from further analysis.

Varying colony numbers and d2EGFP expression levels induced by a selection of the human cellular promoters in CHO-DG44 and PER.C6 cells

So far, all experiments were performed in CHO-K1 cells. In order to examine how the selected promoters behave in other cell lines, constructs containing the CMV, β-actin, γ-actin and UBC promoters were stably transfected to CHO-DG44 cells, a cell line that the industry often uses for the production of therapeutic proteins. The CMV, β-actin and UBC promoters were also transfected to PER.C6 cells, a human cell line used for recombinant protein production. As with CHO-K1, same amounts of DNA were used for these stable transfections.

As shown in Figure 4A, the β-actin promoter induced most colonies (54) in CHO-DG44 cells. Fewer colonies were formed in case of the CMV (34) and UBC (28) promoters. As in CHO-K1, only few colonies (2) formed in case of the γ-actin promoter and we therefore decided to omit the γ-actin promoter for further analysis. Up to 24 colonies of the stably transfected CHO-DG44 cells were isolated and propagated in order to determine d2EGFP expression levels. Striking differences in d2EGFP expression levels, induced by the different promoters were observed (Figure 4A). Up to four-fold higher average d2EGFP expression levels were observed with the β-actin (942) and UBC (1012) promoters as compared to the CMV (266) promoter. Furthermore, both the β-actin and the UBC promoter induced many high-expressing individual clones, this in contrast to the CMV promoter (Figure 4A). In fact, the average d2EGFP expression levels induced by the β-actin and UBC promoters coincided with the d2EGFP value in the highest-expressing colony induced by the CMV promoter.
Figure 3. The selected promoters react differently to reduced serum levels and complete cell culture confluence in CHO-K1 cells.

A. The bars represent average d2EGFP expression levels of all stably transfected CHO-K1 clones (up to 24), induced by the indicated promoter. The same clones were compared for growth under normal (9.1% serum) (solid black bars) and reduced (0.5% serum for 24 hours) serum conditions (striped bars). The relative reduction of d2EGFP expression levels is shown. Only the activity of the UBC promoter was not affected by growth on reduced serum, whereas the activities of the γ-actin and the ribosomal protein promoters were most severely affected by growth on reduced serum. B. CHO-K1 cells were grown either to sub-confluence (~70-80%) (solid black bars) or to complete confluence after which the cells were maintained for another 24 hours before FACS analysis was performed (striped bars). The average d2EGFP expression levels of all clones with the same promoter are represented by the bars. The activities of all promoters were affected negatively. The CMV promoter was least affected, while the greatest reduction was observed with the ribosomal protein promoters.

Striking differences in colony formation were also observed in PER.C6 cells. The β-actin (26) promoter induced 5-fold more colonies than the CMV (5) promoter, whereas the UBC promoter induced no colonies at all (Figure 4B). All colonies induced by either the CMV or the β-actin promoter, were isolated and propagated. The average d2EGFP expression levels were higher with the β-actin (337) promoter than with the CMV (219) promoter. Furthermore, more high-expressing colonies were formed in case of the β-actin promoter, when considering d2EGFP expression levels in the individual clones.

In conclusion, the selected human cellular promoters showed similarities in behavior in CHO-DG44 and PER.C6 cells as compared to the CHO-K1 cells. In general, the β-actin promoter induced more colonies with higher d2EGFP expression levels than the CMV promoter. However, the differences in d2EGFP expression levels between the various promoters were more extreme in the CHO-DG44 cells than in the CHO-K1 cells. A striking difference between the CHO-K1 and PER.C6 cells is the inability of the UBC promoter to induce colony formation in PER.C6 cells.

Effects of complete cell culture confluence and reduced serum levels on the different promoters in CHO-DG44 cells

Above, we examined the behavior of the selected promoters under reduced serum and complete confluence conditions in CHO-K1 cells. Next, we compared the effects of these conditions on the CMV, β-actin and UBC promoters in CHO-DG44 cells. As above, cells were grown to complete confluence and FACS analysis was performed after another 24 hours.

The average d2EGFP expression level of all confluent CHO-DG44 clones was compared to the average d2EGFP expression level of the same, but still exponentially growing clones (Figure 5A). With all three promoters a reduction in the average d2EGFP expression levels was observed when cells were grown to complete confluence, but the reduction was least
Figure 4. Comparison of promoter activities in CHO-DG44 and PER.C6 cells. The bars in the lower diagrams indicate the total number of stably transfected CHO-DG44 (A) or PER.C6 (B) colonies, established after each transfection with the indicated promoter constructs. Equal amounts of DNA were transfected and the same selection pressure was applied. In either cell line the β-actin promoter induced most colonies. In PER.C6 cells, the UBC promoter induced no colonies. The mean GFP fluorescence values in the individual, stably transfected CHO-DG44 (A) and PER.C6 (B) clones are shown as dots in the upper diagrams. The horizontal bars indicate the average d2EGFP expression levels of all clones with the same promoter. In CHO-DG44 the β-actin and UBC promoters induced the highest average d2EGFP expression and the β-actin promoter induced the highest d2EGFP expression level in an individual clone. In PER.C6 cells, the β-actin promoter induced the highest average d2EGFP expression, as well as the highest d2EGFP expression level in an individual clone.

Dramatic in case of the β-actin promoter (31%) (Figure 5A). A reduction of 43% was observed for both the CMV and the UBC promoter.

To test the behavior of the three promoters under reduced serum conditions, the CHO-K1 cells were grown on 0,5% serum for 24 hours (see above), but in CHO-DG44 we used more extreme conditions. The cells were seeded and 72 hours prior to FACS analysis, normal medium (containing 4,6% serum) was replaced by serum-free medium. The average d2EGFP expression level of all clones growing under serum-free conditions was compared to the average d2EGFP expression level of the same clones, growing under normal conditions (Figure 5B). A surprising, but slight increased average d2EGFP expression (6%) was observed in case of the β-actin promoter. In contrast, a decrease of 25% and 42% respectively in average d2EGFP expression was observed in case of the UBC and CMV promoters (Figure 5B). Still, even under these reduced serum conditions, the β-actin and UBC promoters induced much higher d2EGFP expression levels than the CMV promoter. Finally, also under less extreme conditions (on 0,5% serum for 48 hours), average d2EGFP expression levels increased 19% in case of the β-actin promoter and decreased with 39% in case of the CMV promoter, while the expression remained unaltered in case of the UBC promoter (data not shown).

Next we selected six of the above-described clones that were induced by the three different promoters. The clones were weaned to adapt to serum-free medium and suspension growth. With all three promoters, similar d2EGFP expression levels were maintained in the suspension growing cells (Figure 5C). Moreover, in suspension, the β-actin and UBC promoters still induced higher d2EGFP expression levels as compared to the CMV promoter.
The behavior of the promoters under sub-optimal growth conditions was rather similar in CHO-K1 and CHO-DG44 cells. However, in case of the β-actin and UBC promoters, a greater decrease of d2EGFP expression levels was observed in CHO-K1 than in CHO-DG44 cells when grown to complete confluence. Furthermore, the β-actin promoter reacted differently to reduced serum levels, showing a decrease in d2EGFP expression levels in CHO-K1 and an increase in CHO-DG44.

The CMV, β-actin and UBC promoters induce different hEPO expression levels in CHO-K1 and CHO-DG44 cells

So far, we used d2EGFP as reporter gene, which is not secreted. However, therapeutic proteins need to be secreted by the cell. We therefore examined human erythropoietin (hEPO) as reporter protein. hEPO is an important therapeutic protein that is being produced in cultured cells on an industrial scale. We cloned the hEPO gene into the STAR-Select vector with the CMV, β-actin and UBC promoters and compared hEPO expression levels in stably transfected CHO-K1 and CHO-DG44 clones. In CHO-K1 cells, 58 and 64 colonies formed
respectively with the CMV and β-actin promoters and 24 colonies formed with the UBC promoter. We isolated and propagated up to 24 colonies for each promoter to determine hEPO expression levels. Average hEPO expression levels were higher in case of the β-actin (16.0 pg/cell/day) and UBC (14.4 pg/cell/day) promoters, as compared to the CMV (12.3 pg/cell/day) promoter (Figure 6A). Moreover, the highest-expressing clones of the cells transfected with the β-actin (28.4 pg/cell/day) and UBC (27.1 pg/cell/day) promoters secreted more hEPO than those transfected with the CMV (18.7 pg/cell/day) promoter (Figure 6A).

In CHO-DG44 cells, both the CMV and the β-actin promoter induced 38 colonies and the UBC promoter induced 22 colonies. In these clones, a ~3-fold higher average levels of hEPO secretion was induced by the β-actin (5.6 pg/cell/day) or UBC (6.7 pg/cell/day) promoters as compared to the CMV (2.1 pg/cell/day) promoter (Figure 6B). The β-actin and UBC promoters induced the highest hEPO values (9.2 and 12.4 pg/cell/day respectively), as compared to the CMV (6.9 pg/cell/day) promoter (Figure 6B).

In summary, both in CHO-K1 and in CHO-DG44 cells the β-actin and UBC promoters performed better than the CMV promoter, as monitored by hEPO expression levels. The differences in induced hEPO expression levels by the different promoters were more outspoken in the CHO-DG44 cells than in the CHO-K1 cells.

The CMV, β-actin and UBC promoters induce different anti-EpCAM antibody expression levels in CHO-DG44 cells

We finally examined the effects of the different promoters on the expression levels of a monoclonal antibody. We cloned the DNA sequences encoding the light and heavy chain of the monoclonal antibody directed against the epithelial cell adhesion molecule (EpCAM) in our STAR-Select vector with the CMV, β-actin and UBC promoters. The vectors contained the two expression cassettes on one plasmid (Figure 7A). In each construct, the same promoter was used for both light and heavy chain expression cassettes. In this way we were able to compare anti-EpCAM antibody expression by the CMV, β-actin and UBC promoters in CHO-DG44 cells.

![Figure 6](image)

**Figure 6.** Comparison of promoters in CHO-K1 and CHO-DG44 cells, as monitored with hEPO as reporter gene. CHO-K1 (A) and CHO-DG44 (B) cells were transfected with constructs driven by the indicated promoters and with hEPO as reporter gene. Dots indicate the hEPO expression levels in the individual CHO-K1 (A) or CHO-DG44 (B) clones, stated as specific activities (pg secreted hEPO/cell/day). The horizontal bars indicate the average hEPO expression levels of all clones with the same promoter. In both cell lines the β-actin and UBC promoters induced the highest average hEPO expression levels, as well as the highest individual hEPO-expressing clones, as compared to the CMV promoter.
**Figure 7.** The β-actin and UBC promoters induce higher anti-EpCAM antibody expression levels than the CMV promoter in CHO-DG44 cells.

A. Structure of the anti-EpCAM antibody expressing construct. The DNA sequences encoding the light and heavy chain of the monoclonal antibody against EpCAM were cloned in our STAR-Select vector. The vector contains two expression cassettes and the same promoter is used for both light and heavy chain expression cassettes. Indicated STAR elements (7 and 67) are used to flank the entire construct. B. CHO-DG44 cells were transfected with constructs driven by the indicated promoters and with anti-EpCAM antibody as reporter gene. Dots indicate the anti-EpCAM expression levels in the individual CHO-DG44 clones, stated as specific activities (pg secreted anti-EpCAM/cell/day). The horizontal bars indicate the average anti-EpCAM expression levels of all clones with the same promoter. The β-actin and UBC promoters induced the highest average anti-EpCAM expression levels, as well as the highest anti-EpCAM expressing clones, as compared to the CMV promoter.

The CMV and β-actin promoters induced most colonies (22 and 24 respectively), as compared to the UBC promoter (12). All clones were isolated and propagated and anti-EpCAM antibody secretion was analyzed. As shown in Figure 7B, a ~2-fold higher average antibody specific productivity was observed with the β-actin (17.1 pg/cell/day) and UBC (21.4 pg/cell/day) promoters as compared to the CMV (9.0 pg/cell/day) promoter. The β-actin and the UBC promoters induced the highest anti-EpCAM expressing clones (24.3 and 30.5 pg/cell/day, respectively), as compared to the CMV (16.5 pg/cell/day) promoter (Figure 7B).

In summary, the β-actin and UBC promoters were able to confer higher expression levels of the anti-EpCAM antibody than the CMV promoter. As described above, this was also the case for d2EGFP and hEPO expression levels.

**DISCUSSION**

The industrial production of complex therapeutic proteins in mammalian cells can be very difficult and even relatively small increases in expression levels are often viewed as valuable. One obvious means to positively influence protein expression levels is selecting the strongest possible promoter in a particular cell line. Most often, viral promoters such as the SV40 and CMV promoters are used. One important reason is that these promoters behave consistently in a range of different cell lines. The CMV promoter is viewed as one of the strongest promoters...
available, whereas the SV40 promoter is relatively weak. We found in fact that the SV40 acted as minimal promoter in the PER.C6 cell line (data not shown). This was one of the reasons to not incorporate the SV40 promoter in this analysis, but to focus on the CMV promoter instead. The CMV promoter is considered a strong promoter in a wide range of cell types. However, large variations in CMV promoter activity have been reported\textsuperscript{24-28}. Various cellular promoters have been described to drive strong gene expression in mammalian cells. In particular cellular promoters that belong to the broad class of housekeeping genes are of interest, because housekeeping genes are supposed to be constantly expressed at a high level, in a range of different cell types. The promoters of the $\beta$-actin and ubiquitin C genes have been described as strong cellular promoters\textsuperscript{5,29}. Cellular promoters provide not often employed alternatives for the viral promoters. In this study we compared several human cellular promoters with the CMV promoter in mammalian cells that are relevant for industrial therapeutic protein production.

The promoters that we studied belong to housekeeping genes that are constitutively expressed at a high level\textsuperscript{14}. However, high expression is not always the result of high promoter activity. For instance, the majority of 5S rRNA genes are organized in clusters of tandem repeated units and have copy numbers from 160 to 20,000 per haploid genome\textsuperscript{30-33}. As a consequence, the high 5S mRNA levels are not due to the strength of an individual 5S promoter. Therefore we selected promoters that belong to single-copy genes. Thus, the high mRNA expression levels of those genes are likely to be due to strong promoter activity. For those reasons, we expected these human cellular promoters to also be able to induce high expression of recombinant genes. The human cellular promoters we selected all contain sites of high evolutionary homology. The lengths of the isolated 5’ flanking sequences were determined on the basis of the regions of conservation. Almost all selected promoters contain an intron and an untranslated exon region. Of either characteristic we speculated that they might be important for the proper functioning of the promoter\textsuperscript{15-17}. These characteristics gave focus to our selection of human cellular promoters. However, our results show that for instance the hisH3 and GAPDH promoters performed very poorly, both in terms of induced colonies and protein expression levels. All promoters were chosen on the basis of the same criteria (Figure 1B), but this clearly did not provide good predictive criteria for promoter strength, since large variations in performance were observed.

Our first analysis of colony formation showed that most cellular promoters induced equal or fewer colonies than the CMV promoter. For instance the $\gamma$-actin promoter did induce only few colonies. However, many of these colonies showed high protein expression levels. The fact that promoters such as the $\gamma$-actin promoter can induce very high protein expression levels seems at odds with the observation that they induce so few colonies. This may be related to the observation we made that the highest transient d2EGFP expression levels were induced by the CMV promoter, as compared to any human cellular promoter we tested (data not shown). The transient d2EGFP expression levels were routinely measured one day after transfection, in all cell lines that we tested. In the initial phases of our analysis these observations appeared to point to a superiority of the CMV promoter over the tested human cellular promoters. However, we consistently observed that after several days, d2EGFP expression levels induced by for instance the $\gamma$-actin promoter started to increase. As shown throughout this study, in stably transfected clones, protein expression levels induced by the $\gamma$-actin promoter were superior to the levels induced by the CMV promoter. In itself this is a warning against restricting oneself to transient expression studies while analyzing promoter characteristics. It may also explain the low colony numbers induced by the $\gamma$-actin promoter. During the very first days enough Zeocin selection marker protein must be made for the cell to survive. If the initial promoter activity is low, this might lead to massive cell death and the establishment of a low colony number as result.
Whatever the underlying reason for this phenomenon, it may be very attractive to work with promoters that only induce few colonies of which most show high expression. Only few colonies need to be analyzed to find one that shows high protein expression levels. Of course this characteristic has to be balanced by obtaining enough colonies for analysis in the first place. To take the example of the γ-actin promoter once more; in two independent experiments in CHO-DG44 cells, the γ-actin promoter induced only two colonies. Clearly this number does not provide a solid basis for an elaborate analysis. Also the UBC promoter induced high protein expression levels in the CHO-K1 and CHO-DG44 cell lines, but showed reduced colony formation. Moreover, the UBC promoter was unable to induce any colony formation in PER.C6 cells at all. In particular this last observation is puzzling, because one might expect a housekeeping gene promoter to be able to induce expression in a human cell line as well. However, the UBC promoter did not give transient d2EGFP expression with the tested TTG Zeo STAR-Select construct, nor with other, less stringent selection systems (data not shown). This makes the UBC promoter less attractive as a general promoter of choice for any cell type. In fact, in three different cell lines, of all studied human cellular promoters, only the β-actin promoter performed very well. The higher expression levels induced by the β-actin promoter, as compared to the CMV promoter, were most striking in CHO-DG44 cells, this in the context of multiple reporter genes.

We also analyzed how the different promoters performed under less favorable culture conditions that can be important in industrial processes. Promoter activities were examined under reduced serum conditions and in a situation where growth was impaired. There are indications that the ribosomal proteins are expressed in a cell cycle-dependent fashion\textsuperscript{12,13}. This may relate to our observation that in particular the ribosomal protein promoters did not perform well in CHO-K1 cells that were grown to full confluence. In contrast, ubiquitin is induced when cells are exposed to stressful conditions, such as heat shock\textsuperscript{34}, starvation\textsuperscript{35}, UV-irradiation and TPA-treatment\textsuperscript{36} and other stress-inducing agents\textsuperscript{37}. This could explain our observations that the UBC promoter performed very well under reduced serum conditions. However, the problem of reduced expression induced by the CMV promoter in less favorable culture conditions can clearly not be fully overcome by the human cellular promoters that we studied. On the other hand, the fact that the β-actin and UBC promoters induced higher expression than the CMV promoter in serum-free, in suspension growing CHO-DG44 cells, is still a promising characteristic for the usefulness of these cellular promoters in an industrial setting.

The β-actin promoter consistently performed well in varying conditions and therefore seems to be the best overall candidate cellular promoter, with the UBC promoter as second best. Both promoters are being offered in commercially available plasmids: the β-actin promoter by Invivogen and the UBC promoter by Invitrogen. In either case the commercially available cellular promoter regions are substantially smaller in the 5’ flanking sequence than the promoter regions we used. Initial comparisons indicate not many differences between the commercially available β-actin and UBC promoters and the longer versions we isolated (data not shown). We isolated these longer versions to include the evolutionary conserved homologous regions of the promoters, as indicated in Figure 1B. However, as pointed out above for the hisH3 and GAPDH promoters, the presence of, for instance, the 5’ flanking sequences appear not to provide good predictive criteria for promoter strength.

In conclusion, our analysis shows that human cellular promoters such as the β-actin and to a lesser extend also the UBC promoter are as good as or even better suited for protein production in different mammalian cell lines than the most commonly used CMV promoter.
MATERIALS AND METHODS

Vector constructions

The human CMV immediate-early promoter/enhancer from pcDNA3 (Invitrogen) was used and an intron was placed between the CMV promoter and the reporter gene\(^{18}\). The human cellular promoters were isolated by PCR on human genomic DNA. At the 5’ end of each primer, we positioned a DNA sequence that could be cut by appropriate restriction enzymes for efficient cloning into our STAR-Select vector\(^{19}\). We used the following primers for the respective promoters (F: forward; R: reverse):

\(\beta\)-actin promoter:
F: 5’ GATCACGGCGTGCCCCAGTGGCACGCTCCGAAAGCCTCCCTACAGGGCAAAG 3’
R: 5’ GATCACCAGTGGTGAGCTCGCAGAATAGCGGAGCCTGGCT 3’

\(\gamma\)-actin promoter:
F: 5’ GATCACGGCGTAATCCAGCAGCGCAGCAAAGGAAGATTGCAGTGC 3’
R: 5’ GATCACGGCGTGCTCCGAACTGCCCAGGGAAAGATGAGCTTGAGGAC 3’

UBC promoter:
F: 5’ GATCACCCGCTCATGCCCTCGCTGAGGAGTTGAGGACC 3’
R: 5’ GATCAGCGCTCTGTCAAAAAACGAAAAAGCCAAAAACGCGGCAATTTAGCGGAC 3’

UBB promoter:
F: 5’ GATCACGGCGTAATCCAGCAGCGCAGCAAAGGAAGATTGCAGTGC 3’
R: 5’ GATCAGCGCTCTGTCAAAAAACGAAAAAGCCAAAAACGCGGCAATTTAGCGGAC 3’

GAPDH promoter:
F: 5’ GATCACGGCGTCACAATGTCAATAGCGTCACTAGTTGGAGAAACCTGC 3’
R: 5’ GATCACCAGGATGCTGCTGAGGATGCTGAGG 3’

hisH3 promoter:
F: 5’ GATCACGGCGTGAGGCTGGAGAATCGCTTGTATTCAGGAGG 3’
R: 5’ GATCACCAGGATGCTGCTGAGGATGCTGAGG 3’

Cell culture, transfection, and analysis of clones

CHO-K1 cells (CCL-61; American Type Culture Collection (ATCC)) were grown in HAM F12 medium (Invitrogen), supplemented with 9.1% fetal bovine serum (FBS) (Invitrogen), 2 mM glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin.
(Invitrogen) at 37°C / 5% CO₂. CHO-DG44 cells were grown in HAM F12/DMEM (1:1) medium (Invitrogen), supplemented with 4.6% 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. PER.C6 cells were grown in DMEM medium (Invitrogen) supplemented with 9.1% FBS (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), and 10 mM MgCl₂ at 37°C / 10% CO₂. For transfections, 0.3·10⁶ CHO-K1 cells, 0.4·10⁶ CHO-DG44 cells, or 1.0·10⁶ PER.C6 cells were seeded in culture vessels ~24 hours prior to transfection. Cells were transfected with 3 μg of plasmid DNA using Lipofectamine™ 2000 (Invitrogen) as described by the manufacturer. In brief, Lipofectamine™ 2000 was combined with plasmid DNA at 3 μl/μg DNA in case of CHO-K1 cells, at 4 μl/μg DNA in case of CHO-DG44 cells, and at 5 μl/μg in case of PER.C6 cells. The mixture was added to the cells, which had grown to 70-90% confluence. After 5 hours, the transfection mixture was replaced by fresh medium. The following day, cells were seeded in serial dilutions into medium containing Zeocin at a concentration of 150 μg/ml Zeocin for CHO-K1 cells, 400 μg/ml for CHO-DG44 cells, and 100 μg/ml for PER.C6 cells. Approximately 12 days after transfection, individual colonies became visible and these were isolated and propagated in 24-well plates in medium containing Zeocin. When grown to ~70% confluence, cells were transferred to six-well plates. Cells were continued to grow in six-well plates for another one to two weeks before FACS analysis was performed. The d2EGFP expression levels were determined with intervals of two weeks on an Epics XL Beckman Coulter flowcytometer. All experiments were repeated at least two times.

For the weaning of CHO-DG44 cells to grow in suspension, the previously described medium for adhesive CHO-DG44 cells was mixed with CD DG44 medium (Invitrogen) supplemented with 8 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM sodium hypoxanthine, 16 μM thymidine, and 44 μM L-tryptophan. The cells were seeded on suspension six-well plates. At first, adhesive medium and suspension medium were mixed at a 9:1 ratio. By increasing the percentage of suspension medium by 10% every three days, the cells adapted to the suspension medium and eventually were able to grow in suspension.

**ELISA**

For hEPO measurements, equal numbers of cells (0.1·10⁶ for CHO-K1; 0.2·10⁶ for CHO-DG44) were seeded in six-well plates three days prior to cell counting and collection of the medium. The amount of secreted hEPO was determined using an ELISA-kit (Quantikine IVD kit; R&D systems; Ref DEP00). The antibody concentration was determined by comparing optical density at 415 nm with that of the known antibody standard, as supplied in the ELISA-kit.

For anti-EpCAM measurements, equal numbers of CHO-DG44 cells (0.2·10⁶) 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 k 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 490 nm with that of the known antibody standard (human IgM; Accurate Chemical).
REFERENCES


