Effects of pCIneo and pCEP4 expression vectors on transient and stable protein production in human and simian cell lines

Janet H. Parham*, Tom Kost & Jeff T. Hutchins

GlaxoSmithKline Discovery Research, 5 Moore Drive, Research Triangle Park, NC 27709 USA

(* E-mail: jhs23232@gsk.com)

Received 10 January 2001; accepted 21 May 2001

Key words: episomal vectors, Epstein Barr nuclear antigen, human hepatocyte growth factor, secreted placental alkaline phosphatase, transfection

Abstract

To support and meet the demand for recombinant proteins early in the drug discovery process, much work has been directed toward improving the methods used for transient gene transfection and expression. A factor which could potentially affect the outcome of experiments is the choice of the expression vector. Conventional vectors such as pCIneo and pcDNA3 have been used frequently. Each of these places the gene of interest under the control of the CMV promoter. An interesting alternative is provided by episomal vectors. For example, the pCEP4 vector contains the gene coding for the Epstein Barr nuclear antigen as well as the EBNA ori P sequence. This combination allows for the episomal replication of the plasmid. In preliminary experiments, we compared transient secreted placental alkaline phosphatase production in 8 cell lines from 3 different species using the pCIneo vs. pCEP4 vectors and found the utility of the pCEP4 vector to be limited to the human 293 EBNA cell line. In this paper, we have compared the two vectors in six cell lines of simian and human origin, measuring the transient production of secreted placental alkaline phosphatase and human hepatocyte growth factor. In general, the pCEP4 vector produced higher amounts of both proteins in transient transfections. Results were particularly pronounced in the HEK 293 and 293 EBNA cell lines. Stable pools of cells (uncloned) expressing human hepatocyte growth factor were isolated using pCIneo and pCEP4 and protein production levels were compared to those seen in transient transfections. Stable expression with pCEP4 was found to produce the highest levels of human hepatocyte growth factor in 3 of 4 cell lines. Finally, electroporation and FuGENETM6 (Roche, Indianapolis IN) as transfection methods were compared measuring transient production of secreted placental alkaline phosphatase, human hepatocyte growth factor, and green fluorescent protein. FuGENE produced higher protein concentrations in less time than electroporation for all 3 proteins.

Abbreviations: EBNA, Epstein Barr Nuclear Antigen; GFP, green fluorescent protein; HHGF, human hepatocyte growth factor; SPAP, secreted placental alkaline phosphatase.

Introduction

In 1985 Yates, Warren and Sugden described the creation of a plasmid which contained a 2.9 kb fragment encoding the EBNA-1 gene, a hygromycin resistance cassette, and the cis-acting EBNA ori P. This episomal expression plasmid was used to measure transfection frequencies and plasmid copy numbers/cell in several different cell types. They found that cell lines from three species (human, monkey, and dog) were able

to autonomously replicate the plasmid as an episome, without chromosomal integration. Cell lines of rodent origin were also able to produce drug resistant colonies; however, in these cell lines the vector sequences were integrated into chromosomal DNA. If the EBNA gene was not present in a cell line, then both the ori P and the EBNA-1 gene were required in the expression plasmid in order for the cells to maintain the plasmid as an episome.

Vidal (1990) found the effectiveness of the episomal vectors to be very cell line dependent, even among human lines. He showed that HeLa cells maintained an average copy number of 30 copies/cell compared to the A431 cell line which had an average copy number of 10 copies/cell, even though both cell lines were EBV negative. In HeLa the plasmids were maintained as episomes; whereas in A431, the plasmids were integrated and underwent some rearrangements. Overexpression of the EBNA-1 protein was shown to be detrimental to the health and growth of both cell types.

Horlick et al. (1997) demonstrated the utility of episomal plasmids in 293 EBNA cells, expressing human corticotropin-releasing hormone receptor. They found that 1–10% of the cells survived the selection process. These surviving cells were allowed to expand and populations were pooled for receptor production. These pooled populations were found to have about 7 copies/cell of the episomal plasmid compared to 6–13 copies when the cells were clonally isolated. Clonally isolated CHO lines expressing the same plasmid were shown to contain 3 integrated copies of the plasmid/cell and produced 2–3 fold less receptor than the pooled 293 EBNA populations.

The stable episomal system was recently included in a review article by Geisse and Kocher (1999). As defined by Geisse and Kocher, this is a borderline stable system, 'being transient with respect to integration events and stable in terms of production rates over long periods of time'. In this system antibiotic resistant populations are generated under selection. The episomes are thus maintained stably for several months, allowing for the rapid generation of populations of 293 EBNA cells expressing the protein of interest. They compared protein yields from several different expression systems, with the stable episomal system comparing quite favorably to other systems in terms of yield, versatility, and time required to reach the production phase.

The studies cited above did not evaluate the efficacy of using episomal vectors for transient expression. In an effort to achieve maximal protein yield in expression experiments, we have compared the effects of the pCIneo vector to the episomal pCEP4 vector on the transient production of SPAP and HHGF in several cell lines of human and simian origin. HHGF transient expression was then compared to stable (nonclonal) expression with pCIneo and pCEP4. The episomal vector in HEK 293 and 293 EBNA cells proved to be quite versatile, in that it is very efficient in producing

high levels of protein in transient expression experiments as well as in stable expression experiments.

Materials and methods

DNA constructs

pCEP4 is an expression vector commercially available from Invitrogen (Carlsbad CA). pCEP4 contains the EBNA-1 gene, the EBNA ori P, the CMV promoter, and a hygromycin resistance cassette. It can be used for stable or transient transfections. pCIneo, an expression vector available from Promega (Madison, WI), can also be used for stable or transient transfections. It carries an SV40 minimum origin of replication, a neo resistance cassette under the control of the SV 40 enhancer and early promoter, as well as the CMV promoter. Stable cell lines made using pCIneo as the expression vector would generally have the DNA construct integrated into the host cell genome, except possibly in Cos-7 where it could be maintained episomally. In human or simian cell lines, the pCEP4 vector allows for the episomal replication of the plasmid. The 1.6 kb SPAP fragment and the 2.2 kb HHGF fragment were inserted into the multi cloning site of each vector. The 730 bp Green Lantern fragment (Life Technologies, Gaithersburg MD) was cloned into the multi cloning site of pCEP4. Plasmid DNA was purified using Qiagen (Valencia CA) giga prep kits.

Cell lines

The cell lines used were as follows:

- Cos-7 (African green monkey kidney, stably expressing SV 40 large T antigen; ATCC)
- HCT 116 (human colon adenocarcinoma, ATCC)
- RKO (human colon adenocarcinoma; Boyd et al., 1988)
- HEK 293 (human embryonic kidney, ATCC)
- 293 EBNA (HEK 293 stably expressing Epstein Barr nuclear antigen, Invitrogen)
- Vero (African green monkey kidney, ATCC)

Transfections

Cells were plated on the day prior to transfection at 1×10^6 cells onto 100 mm culture dishes (6 ml of media) for transient transfections and at 0.4×10^6 cells into 6-well dishes for stable transfections (2 ml

Figure 1. SPAP production was measured in six cell lines over a 10 day time course. The values shown in this figure represent the maximal value observed for each cell line and each vector. The day with the highest measured SPAP activity was not the same for all cell lines.

of media). FuGENETM6 (Roche, Indianapolis IN) was used for transfections. For 100 mm dishes 18 μ l of FuGENE and 6 μ g of SPAP or HHGF construct DNA were added to 0.3 ml of serum free medium; for 6-well dishes, each well received 0.1 ml serum free medium with 6 μ l of FuGENE and 2 μ g DNA. Medium was not changed following transfections. For transient transfections, media samples were taken on days 1, 3, 7 and 10 following transfection. For stable transfections, the cells were passed into 100 mm dishes with either G418 (250 μ g ml⁻¹ for HEK 293 and 1000 μ g ml⁻¹ for HCT 116 and Vero) or hygromycin selection (150 μ g ml⁻¹ for HEK 293, HCT 116, Vero and 300 μ g ml⁻¹ for 293 EBNA) 2 days following transfection. Cells were generally kept on selection for 11 days and subcultured as needed. However, the HCT 116 and HEK 293 were on hygromycin selection for 18 days. After the selection period, cells were plated at 1×10^6 cells onto 100 mm dishes. Media samples were taken on days 1, 3, 7 and 10 following plating.

For comparison of electroporation vs. FuGENE, cells to be transfected with FuGENE were plated one day prior to transfection at 3×10^6 cells per 100

mm dish. Transfections of HHGF, SPAP, and GFP (all in the pCEP4 expression vector) were performed as described above. Cells to be electroporated (3×10^6) were trypsinized, electroporated and plated immediately onto 100 mm dishes. Conditions for the electroporation of 293 EBNA cells have been previously described (Parham et al., 1998). Cells were harvested for GFP analysis on day 2 following transfection or electroporation. Media samples for HHGF and SPAP analysis were taken on days 1, 3, 6 and 9.

SPAP and HHGF assays and GFP analysis

SPAP was assayed using a modified version of the assay described by Berger et al. (1998) which measures the hydrolysis of p-nitro-phenyl phosphate substrate. Unknown samples were read against a standard curve containing known amounts of calf intestinal alkaline phosphatase (Life Technologies, Gaithersburg MD) in a SpectraMax 250 spectrophotometer; the data was analyzed using SpectraMax Pro software.

HHGF was analyzed using a human HGF assay kit purchased from R and D Systems (Minneapolis MN). Unknown samples were measured against a standard

Figure 2. HHGF production was measured in six cell lines over a 10 day time course. The values shown in this figure represent the maximal value observed for each cell line and each vector. The day with the highest measured HHGF concentration was not the same for all cell lines.

curve containing known amounts of HHGF in a SpectraMax 250 spectrophotometer and data was analyzed using Spectra Max Pro software.

GFP expression was measured in a FACStar Plus_{tm} (Becton Dickinson, San Jose CA). Data was expressed as the percent of cells expressing GFP and fluorescence intensity as measured by mean equivalents of soluble fluorochrome (MESF). Methods for the quantitation of MESF have been previously described (Parham et al., 1998).

Results and discussion

Comparison of transient SPAP and HHGF production using pCIneo and pCEP4 in cell lines of human and simian origin

Based on the findings from earlier studies which demonstrated that the utility of the pCEP4 vector was limited to 293 EBNA (Parham and Hutchins, 1999), we extended the comparison of the pCIneo and pCEP4 vectors to include additional cell lines of

human and simian origin as well as the expression of proteins other than SPAP. For these experiments we looked at Cos-7, Vero, HCT 116, RKO, HEK 293, and 293 EBNA. Production of SPAP and HHGF were measured over a 10 day time course. The method of transfection used was FuGENE, a cationic lipid compound.

As shown in Figure 1, optimal SPAP activity levels were higher with pCEP4 than pCIneo in 5 of the 6 cell lines, HCT 116 being the only exception. The most impressive increases were seen in HEK 293 (7.8-fold increase) and 293 EBNA and RKO, both with 2.6-fold increases. SPAP activity levels generally were still increasing on day 10. When HHGF was expressed, the pCEP4 produced higher levels only in HEK 293 (2.5-fold) and 293 EBNA (1.7-fold) (Figure 2). In HCT 116 and RKO the protein levels were almost undetectable with both vectors.

The two proteins expressed are quite different in their properties and there does appear to be some effect of the protein when looking at the benefit derived from using the pCEP4 vector. However, in general it

Figure 3. HHGF production was measured in four cell lines over a 10 day time course. The values shown in this figure represent the maximal value observed for each cell line under each of the four different expression regimens. Note that the results with the stable transfections represent pooled populations rather than clonal populations for both the pCIneo as well as the pCEP4.

appears that use of the pCEP4 vector can be beneficial when used in human or simian lines. The most pronounced enhancement is still seen with the HEK293 and 293 EBNA cell lines which have been the cell lines customarily used with these vectors. HCT 116 and RKO expressed poorly with both proteins when compared to the other four cell lines. Cos-7 cells express SV40 TAG. However, transfection of these cells with pCIneo, which contains the SV40 minimum origin of replication, did not lead to the production of higher protein levels than those seen with pCEP4.

Comparison of transient expression to stable expression of HHGF using pCIneo and pCEP4 in four cell lines

Transient expression of HHGF using pCIneo and pCEP4 was compared to stable expression in the HCT 116, HEK 293, 293 EBNA, and Vero cell lines. A stable transfection of 293 EBNA using G418 selection was not performed since the cells already possess a G418 resistance cassette. After selection for a pool of G418 (pCIneo) or hygromycin (pCEP4) resistant cells

(11 days for 293 EBNA and Vero, 18 days for HCT 116 and HEK 293) the cells were plated at the same density and were sampled at the same time points as transiently transfected cells. As shown in Figure 3, stable transfection of the cells with pCIneo produced low levels of HHGF. In contrast stable transfection with pCEP4, produced the highest HHGF levels of any of the 4 transfection regimens in 3 of the 4 cell lines. The effect again was particularly pronounced in HEK 293 and 293 EBNA. The stable episomal method produced about 3-fold and 4-fold more HHGF respectively in these two cell lines.

The stable method of transfection using pCEP4 could offer certain advantages over transient transfections with pCEP4: 1) higher levels of protein can be produced using this method; 2) this method doesn't require the use of large amounts of expensive transfection reagents. A disadvantage of the stable episomal system would be that it does take about two weeks longer to reach the production stage than does the transient method. The advantage of this method over stable transfection with non-episomal vectors is that the necessary and time-consuming step of clon-

Figure 4. The effects of electroporation and FuGene as transfection methods were evaluated in 9 day time courses which measured both SPAP and HHGF production. Both genes were cloned into the pCEP4 expression vector.

ing for high expressers is eliminated. It is unknown whether one could obtain even higher levels of protein production by cloning these stably transfected cells. However, if this was done, it would negate one of the advantages of this system, which is the generation of protein-producing cells in two weeks as compared to the 4–6 weeks ordinarily needed to generate clonal populations. One caveat of this system in general would be that its utility appears to be limited to two cell lines – HEK 293 and 293 EBNA.

Comparison of electroporation and FuGene as transfection methods

SPAP and HHGF production and GFP transfection efficiencies and intensities using pCEP4 were compared in 293 EBNA cells with electroporation and FuGENE as the transfection methods. Previously in our laboratory electroporation was the predominantly used method (Parham et al., 1998). As shown in Figure 4, FuGENE produced higher levels of both SPAP and HHGF much more quickly than did electroporation. With both methods, 85% of the cells expressed

GFP. However, the fluorescence intensity was 2.3-fold higher with FuGENE (data not shown). From the GFP data, it can be seen that electroporation can be quite efficient at getting the plasmid into the cells. The main difference between the two methods is that with electroporation significant numbers of cells are lost and the remaining cells experience a lag phase while the cells recover from the process. With FuGENE, there is no significant cytotoxicity and the cells can proceed directly into protein production with no lag phase.

Conclusions

From these studies, we have concluded that transient transfection with pCEP4 can be a very efficient method for protein production, especially when used in HEK 293 or 293 EBNA cells. This vector also offers the flexibility for use in performing stable transfections which can produce even higher levels of protein than those seen with transient transfections. Based on results presented in this paper, FuGENE is now used

rather than electroporation as the method of choice for transfections in our laboratory.

Acknowledgements

We would like to acknowledge Tom Littleton and Sam Witherspoon for performing the FACS analysis of GFP.

References

- Berger J, Hauber J, Hauber R, Geiger R & Cullen, BR (1988) Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* 66: 1–10.
- Boyd D, Florent G, Kim P & Brattain M (1988) Determination of the levels of urokinase and its receptor in human colon carcinoma lines. *Canc Res* 48: 3112–3116.
- Geisse S & Kocher HP (1999) Protein expression in mammalian and insect cell systems. *Methods in Enzymology* 306: 19–41.
- Horlick RA, Sperle K, Breth LA, Reid CC, Shen ES, Robbins AK, Cooke GM & Largent BL (1997) Rapid generation of stable cell lines expressing corticotropin-releasing hormone receptor for drug discovery. *Protein Expr And Pur* 9: 301–308.
- Parham JH, Iannone MA, Overton LK & Hutchins JT (1998) Optimization of transient gene expression in mammalian cells and potential for scale-up using flow electroporation. *Cytotechnology* 28: 147–155.
- Parham JH & Hutchins JT (1999) Comparison of SPAP production following transient transfection with SPAP constructs containing pCIneo vs. pCEP4 vectors. 4th Gene Delivery and Cellular Protein Expression Conference Abstract.
- Yates JL, Warren N & Sugden B (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature* 313: 812–815.
- Vidal M, Wrighton C, Eccles S, Burke J & Grosveld F (1990) Differences in human cell lines to support stable replication of Epstein-Barr virus-based vectors. *Biochim Biophys Acta* 1048: 171–177.