Expression Vectors for Enhanced Transient Gene Expression and Mammalian Cells expressing them

FIELD OF THE INVENTION

This invention relates to new mammalian cells and cell lines, especially CHO and 293 cell lines, which comprise expression vectors encoding truncated EBNA1 genes which enhance transient gene expression. The invention also relates to expression cassettes which include such truncated genes.

BACKGROUND OF THE INVENTION

Mammalian cells are an established expression system in the biotechnology industry for the production of recombinant proteins (r-proteins). In contrast to lower eukaryotes or prokaryotes, mammalian cells provide active r-proteins that possess relevant post-translational modifications. However, in order to obtain sufficient amount of protein for structure/activity analyses or high-throughput screenings, one needs to go through the long and tedious process of stable clone isolation and characterization. Protein production by large-scale transfection is an interesting alternative to the generation of stable clones as it allows the very fast generation of mg to gram quantities of r-protein within few days.

The use of vectors containing the Epstein-Barr virus (EBV) oriP in cell lines stably expressing EBV's EBNA1 protein, such as the HEK293-EBNA1 (293E) cell line (ATCC#CRL-10852) significantly increases protein yield (Durocher et al., 2002). EBNA1 is a multi-functional protein that have been shown to positively regulate many viral promoters present on plasmid DNA when the oriP is present in *cis* (Reisman and Sugden, 1986).

The production of secreted r-protein often needs to be performed in serum-free medium in order to facilitate their purification. Adaptation of the 293E cell line to serum-free medium formulations is not straightforward and is rarely successful. To circumvent this problem, the generation of new 293-EBNA1 cell line from a serum-free medium adapted 293 cell line is preferable (Pham et al., 2003;Pham et al., 2005). However, these new cell lines do not always show optimal growth properties or high transfectabilities in serum-free medium. Also, the isolation of new clones stably expressing full-length EBNA1 is difficult as this protein seems to be cytotoxic to the cells.

Preliminary transient gene expression studies with the commercially available 293F cells adapted to the FreeStyle™ medium showed that this cell line has a good potential for the large-scale r-protein production in serum-free medium. Improvement of this cell line by stably expressing a less cytotoxic but functional EBNA1 protein is needed.

Kennedy, G. and Sugden, B. (2003) *EBNA-1, a Bifunctional Transcriptional Activator* Molecular and Cellular Biology, **23**: 6901-6908 disclose that the ability of EBNA1 to activate transcription from both integrated and transfected templates can be inhibited by a derivative of EBNA1 lacking the amino acids required for activation from integrated templates (aa 65-89). We have found, against previous expectations, that truncations of these amino acids from EBNA1-coding nucleotide sequences can enhance transient gene expression in HEK293 cells to a level similar to EBNA1.

SUMMARY OF THE INVENTION

This invention relates to the unexpected discovery that nucleotide coding sequences coding for a truncated Epstein Barr Nuclear Antigen 1 (e.g. EBNA1t) protein (lacking the Gly-Gly-Ala domain), when in cells of mammalian origin, are associated with increased transient gene expression when compared with control cells. In addition, expression of this truncated EBNA1 gene is more stable and expressed at higher levels than expression of the full-length EBNA1 gene. This results in cell lines with better growth properties and with enhanced transient gene expression. Mammalian cell lines in general are contemplated and human embryonic kidney 293 cells, CHO cells and PER-C6™ cells are of particular interest. This invention also relates to a mammalian cell line such as a 293 cell line stably expressing a processed version of EBNA1t (e.g. 293-6E cells) also showing enhanced transient gene expression compared to EBNA1t, EBNA1 and control cell lines.

Preferably the transfected gene expression is performed in a cell line stably expressing truncated EBNA1. Alternatively, the transfected gene expression is associated with a transiently transfected EBNA1 gene. Also, preferably the EBNA1 nucleotide sequence is truncated to lack most of (i.e. more than 50%, preferably nmore than 75% and, in some embodiments, all) the Gly-Gly-Ala domain. Preferably the nucleotide sequence is less than 70% of a complete EBNA1 coding sequence, especially less than 50% of the complete EBNA1 coding sequence. Alternatively, or as well, one or more of the DNA linking regions LR1 and LR2 can be absent from the truncated sequence. One of the

truncated sequences we have used lacks LR1 and we expect that an equivalent sequence lacking LR2 (with or without LR1 present) to serve a similar purpose. The nucleotide sequence can be included in an expression vector, such as a pTT vector or any other vectors containing a complete or partial Epstein Barr Virus (EBV) oriP sequence, allowing expression of the gene.

Stable cell lines including such expression vectors with truncated EBNA1 nucleotide coding sequences comprise an aspect of the invention.

According to one aspect of the invention, we provide new stable serum-free 293F-EBNA1 cell lines, including full-length of truncated versions of EBNA1.

The use of EBNA1t reduces the difficulty of obtaining stable clones (apparent deleterious effects of over-expressing the full-length EBNA1 protein). To our knowledge, no reports describing stable 293-EBNA1t cell lines exist. Also, by isolating and characterizing a stable 293F-EBNA1t cell line (clones 6E), we observed another new further truncated and functional form of EBNA1, of even shorter amino acid sequence length than EBNA1t (location of truncation not yet identified).

According to another aspect of the invention we provide a series of new truncated EBNA1t expressed proteins (including EBNA1c).

The following aspects of the invention are described in detail below.

1. The new 293FEt cell line, where Et is a truncated version of the EBNA1 protein e.g. EBNA1t described below and in the figures.
2. The new 293-6E cell line expressing a processed form of EBNA1t protein
3. The new truncated EBNA1 protein, EBNA1c consisting of LR2+NLS+DBD domains
4. Using transient EBNA1 (full-length or truncated) expression in *trans* to increase protein production in EBNA1 (full-length or truncated) and non-EBNA1 cell lines
5. The use of an EBNA1t or EBNA1c expression cassette in the pTT vector or other oriP-containing vectors (expression in *cis*) to increase protein production in EBNA1 and non-EBNA1 cells.
6. New truncated EBNA1 protein consisting of LR1+NLS+DBD domains.

The invention further relates to a process for *in vitro* production of a protein which process comprises:

(a) transfecting a mammalian cell with an expression vector coding for said protein, said mammalian cell having been transfected with a truncated EBNA1 expression vector of the invention;

(b) culturing a transfected cell resulting from (a) to yield said protein.

BRIEF DESCRIPTION OF THE DRAWINGS

In drawings which illustrate the invention, Figure 1 shows transient SEAP expression in 293F cells following co-transfection of various amounts of pTT/EBNA1t vector. Figure 2 shows stable or transient EBNA1 constructs expression in 293 cells. Figure 3 shows transient GFP expression in various 293F-EBNA1 clones or pools. Figure 4 shows a Western Blot analysis of EBNA1 expression in various 293F clones. Figure 5 shows transient human placental secreted alkaline phosphatase (SEAP) expression in various EBNA1 clones. Figure 6 shows the growth of various 293F-EBNA1 clones following transfection (hpt = hours post-transfection). Figure 7 shows the growth curve of 293-6E cells compared to 293F cell in 125 ml shaker flasks. Figure 8 shows the amino acid sequence of EBNA1 (SEQ ID NO: 1) with various parts of the sequence identified in the Figure. Figure 9 shows the amino acid sequence of full-length EBNA1 protein (SEQ ID NO: 1) and EBNA1t (underline) (SEQ ID NO: 2) and EBNA1c (bold) (SEQ ID NO: 3) truncated versions. The first amino acid of the new EBNA1c protein is a methionine (as indicated above the glycine residue). Figure 10 shows the schematic structure of various EBNA1 constructs. Figure 11 (A-C) shows DNA sequence of full-length EBNA1(SEQ ID NO: 4), and truncated EBNA1 (EBNA1t (SEQ ID NO: 5) and EBNA1c (SEQ ID NO: 6)). Figure 12 shows transient EBNA1t and EBNA1c expression in 293F cells compared to 293F or 293-6E cells. Figure 13 shows the effect of co-expressing EBNA1t or EBNA1c on transient SEAP expression in 293F cells. Figure 14 shows examples of proteins transiently expressed in 293-6E cells.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to nucleotide coding sequences coding for a truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein which, when in cells of a mammalian cell line, are associated with increased transfected gene expression when compared with cells of a control cell line comprising a complete EBNA1 coding sequence. By "truncated" we mean a sequence which is less than the full EBNA1 nucleotide sequence. As shown in Figures 8 and 10 there are identified components of the full EBNA1 sequence. These

include DNA Linking Regions 1 and 2, a Transcription activation domain, a Nuclear Localization Signal and a DNA Binding and Dimerization region. Truncated sequences of the invention preferably contain the DNA Binding and Dimerization region along with the Nuclear Localization Signal and one or more DNA Linking Regions. Figure 1 shows that transient SEAP expression can be increased significantly by co-expression of EBNA1t protein. Similar increase can be observed using full length EBNA1 protein (not shown). This Figure also shows that transient SEAP expression does increase by augmenting EBNA1t expression. However, it seems that over expressing full-length EBNA1 is difficult to achieve in mammalian cells. This is illustrated in Figure 2 where stable expression of full-length EBNA1 in the commercially available cell line HEK293-EBNA1 (formerly available at Invitrogen or available at ATCC #CRL-10852) or in our best SFE clone (SFE41;(Pham et al., 2003)) is significantly lower than in 293FEt bulks (lanes 6 and 7) or 293-6E cells (lanes 8 and 9). In addition, expression of full-length EBNA1 in 293F cells (bulk) in also very low (lanes 4 and 5). Note that while truncated forms of EBNA1 increases with time in these bulks (lanes 6 vs 7 and lanes 8 vs 9), expression of full length EBNA1 drops with time (lanes 4 vs 5), indicating that overexpression of full-length EBNA1 may have negative effects on cell physiology. Unexpectedly, a major and smaller form of EBNA1t was observed in clone 6E (lanes 8 and 9).

EBNA1t was amplified by PCR using forward (ACGGAATTCGCCGCCACCATGTCTGAC GAGGGGCCA) (SEQ ID NO:7) and reverse (GAGGAAGGGCAGGA GTGAGAATTCCCT) (SEQ ID NO:8) primers and cloned at the EcoRI site of pIRES-Neo vector (Clontech). We made the 293FEt cell line (including the 293-6E clone) following transfection of 293F cells with the plRES-EBNA1t-Neo vector and selection with 25 ug/ml geneticin. Stable clones were isolated by limiting dilution and clones selected based on EBNA1 expression using the rat monoclonal antibody 1H4 (Grasser et al., 1994)

Figure 1 shows that expressing EBNA1t increases transient SEAP expression in a dose-dependent manner. 293F cells were transfected with 100% pTT-SEAP vector (CTRL) or with mixtures of 99 to 60% pTT-SEAP and 1 to 40% of pTT-EBNA1t respectively. With 60% pTT-SEAP and 40% pTT-EBNA1t, the expression level of SEAP was increased by 3-fold over control.

Figure 2 shows EBNA1 expression levels in various stable HEK293 cell lines or following transient transfection. Stable expression of full-length EBNA1 in HEK293-EBNA1 cell line (lane 1) and 293-SFE cell line (lane 2). Lane 3: 293F cells (no EBNA1 expression). Expression of full-length EBNA1 in 293F cells following transfection and G418 selection for 2 months (lane 4) and 4 months (lane 5). Note that expression of full-length EBNA1 decreases with time in this non-clonal cell population. Expression of truncated EBNA1 (EBNA1t) in 293F cells following transfection and G418 selection for 2 months (lane 6) and 4 months (lane 7). Note that expression of EBNA1t increases with time in this non-clonal cell population. Expression of the new form of EBNA1t in clone 6E derived from 293F-EBNA1t after 4 months in culture in the presence of G418 and 1% serum (lane 8) or G418 in serum-free medium (lane 9). Transient expression of full-length EBNA1 (lane 10) or EBNA1t (lane 11) in 293F cells.

The precise nature of the new EBNA1t protein remains to be solved. Detection of EBNA1 was performed using a rat monoclonal antibody (clone 1H4). The two bands seen at Mr 200 and above are not-specific.

Figure 3 shows transient GFP expression in various EBNA1 cell lines. Cells (cultured for 3 months under G418 selection following transfection) were transfected with pTT-GFP and GFP expression was measured 3 days later by flow cytometry. The 293F-EBNA1t clone 6E shows the highest GFP expression. Transfection efficiency was between 40% and 65% for all clones.

Figure 4 shows EBNA1 expression levels in various 293 clones. All clones were cultured in the presence of 25 μg/ml geneticin. Expression of EBNA1 in clone 6A can be detected with longer exposure time.

Figure 5 shows that when various 293F-EBNA1 stable clones were transfected with pTT/SEAP, the clones expressing truncated EBNA1 coding sequences showed enhanced SEAP expression when measured 5 days later (clones 6E, 11 and 13) when compared to clones expressing the full length (clones 1A and 2B) or another uncharacterized truncated form of EBNA1 (clone 6A). In the context of this invention, SEAP is an example of a recombinant protein. Genetic material coding for a protein or polypeptide of choice can be used in place of SEAP coding sequences and, indeed, this is an aim of this invention (see Figure 14 for additional examples).

Figure 6 shows that cell growth and viability does not appear to be affected when truncated EBNA1 nucleotide sequences are stably overexpressed. Cells were fed with 0.5% TN1 24 hpt (Pham et al., 2005) and counted 6 days after transfection.

Figure 7 shows the growth characteristic of the 293FEt-clone 6E (lower panel) compared to the parental 293F cell line (upper panel). Maximum viable cell density is about $3.5x10^6$ cells/ml for the clone 6E compared to $4.2x10^6$ cells/ml for the 293F cell line.

Figure 8, Figure 9, Figure 10 and Figure 11 are best reviewed together. They show the amino acid sequence and schematic structure of EBNA1 constructs and the relationship to the EBNA1 DNA sequences (Figures 11 A-C).

Figure 8 shows the EBNA1 full length protein (641 aa, 56.4 kDa) (Accession number: NC_001345) with its main features. Figure 9 highlights the differences between EBNA1, EBNA1t and EBNA1c and the amino acid level. EBNA1t truncated protein (underline: 417 aa, 42.5 kDa) and EBNA1c further truncated protein (bold: 306 aa, 32.5 kDa). The first amino acid of the new EBNA1c protein is a Methionine (as indicated above the Glycine residue).

Figure 12 contrasts transient expression of two truncated EBNA1 constructs with 293F and 293-6E cells. Cells were transfected with pTT/EBNA1t or pTT/EBNA1c vectors and EBNA1 expression was detected 3 days later by Western blot. Non-transfected 293F cells and 293-6E cells are also shown as controls.

Figure 13 shows 293F cells co-transfected with pTT/SEAP and pTT/EBNA1 constructs. 293F cells were co-transfected with a mixture of 50% pTT-SEAP vector with pTT/EBNA1t, 50% pTT/EBNA1c, or 50% salmon sperm DNA (stuffer DNA). SEAP expression was measured 5 days later.

Figure 14 shows examples of proteins transiently expressed in 293-6E cells. 293-6E cells were transfected with pTT vectors encoding various secreted proteins and culture medium (20 microliters) was harvested 5 days after transfection and analyzed by SDS-PAGE and Coomassie staining.

Reference List

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Reisman, D. and Sugden, B., 1986. trans activation of an Epstein-Barr viral transcriptional enhancer by the Epstein-Barr viral nuclear antigen 1. Mol. Cell Biol. 6, 3838-3846.

The above-described embodiments of the present invention are intended to be examples only. Alterations, modifications and variations may be effected to the particular embodiments by those of skill in the art without departing from the scope of the invention, which is defined solely by the claims appended hereto.

CLAIMS:

1. A nucleotide coding sequence coding for a truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein, said coding sequence, when stably expressed in a mammalian cell line, being associated with increased transfected gene expression when compared with a control cell line not expressing EBNA1.

2. The nucleotide sequence of claim 1 wherein said transfected gene expression is associated with a transiently transfected gene.

3. The nucleotide sequence of claim 1 wherein said nucleotide sequence is truncated to lack most of the the Gly-Gly-Ala domain.

4. The nucleotide sequence of claim 1 which is less than 70% of a complete EBNA1 coding sequence.

5. The nucleotide sequence of claim 1 which is less than 50% of a complete EBNA1 coding sequence.

6. The nucleotide sequence of claim 1 which codes for SEQ ID NO: 2.

7. The nucleotide sequence of claim 1 which codes for SEQ ID NO: 3.

8. A stable mammalian cell line comprising the coding sequence according to claim 1.

9. The stable mammalian cell line of claim 8 wherein said cell line is a CHO, PER-C6 or 293 cell line.

10. The stable mammalian cell line of claim 9 stably expressing a truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein which is less than 70% of a complete EBNA1 protein.

11. The stable mammalian cell line of claim 9 stably expressing a truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein which is less than 50% of a complete EBNA1 protein.

12. A truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein which is less than 70% of a complete EBNA1 protein.

13. The truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein of claim 12 consisting essentially of SEQ ID NO: 2.

14. A truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein which is less than 50% of a complete EBNA1 protein.

15. The truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein of claim 14 consisting essentially of SEQ ID NO: 3.

16. An expression vector comprising the coding sequence of claim 1.

17. A pTT vector comprising the coding sequence of claim 1.

18. A stable mammalian cell comprising the nucleotide sequence of claim 1.

19. A stable 293 cell line comprising the expression vector of claim 16.

20. A stable 293 cell line comprising the expression vector of claim 17.

21. A stable 293 cell line expressing a truncated Epstein Barr Nuclear Antigen 1 (EBNA1) protein lacking its Gly-Gly-Ala domain.

22. A process for *in vitro* production of a protein which process comprises:

(a) transfecting a mammalian cell with an expression vector coding for said protein, said mammalian cell having been transfected with the expression vector of claim 16;

(b) culturing a transfected cell resulting from (a) to yield said protein.