**Method of expressing recombinant protein in CHO cells**

The present invention relates to a method for expressing a recombinant product gene in a CHO cell line as well as to recombinant CHO host cells and to novel expression vector constructs.

The Chinese Hamster ovary cell (CHO) mammalian expression system is widely used in production of recombinant protein. Apart from lymphoid cell lines such as hybridoma cell lines, it is one of the few cell types allowing for simple and efficient high-density suspension batch culture of animal cell. Furthermore, they allow for very high product yields and are comparatively robust to metabolic stresses whereas lymphoid cells are more difficult to culture at an industrial scale. Given considerable cost of production, it is of utmost importance to maximize the yield of recombinant protein per bioreactor run. Choice of culture medium composition and bioreactor design and operation are parameters that impact yield and may be quite complex to optimize. More predictably, increases in the strength or transcriptional activity of the promoter controlling expression of product protein enhance yield. Incremental increases at the single cell level will translate into considerable improvements of product yield in high-density batch or fed-batch culture showing stationary phase gene expression at cell densities in the range of $10^6$ to $10^7$ cells/ml.

US5,866,359 describes a method of enhancing expression from an already strong hCMV promoter in CHO and NSO cells by co-expressiong adenoviral E1A protein from a weak promoter. E1A is a multifunctional transcription factor which may act on cell cycle regulation and has both independent transcriptional activating and repressing functional domains. The finetuning of E1A expression to appropriate low level expression is crucial for success of the co-expression approach in order to achieve the ideal balance in between gene transactivation whilst avoiding any negative impact on cell cycle progression. As a disadvantage, apart from careful choice of the promoter driving E1A expression, this system blocks part of the protein synthesis capacity of the cell with E1A expression rather than expressing the recombinant protein of interest.

WO 95/17516 describes use of the murine immunoglobulin gamma 2A locus for targetting

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an expression vector construct to a highly active gene locus in lymphoid cells of the B-cell lineage, e.g. widely used NS0 myeloma cells. NSO cells essentially are a tumor cell line of murine plasma or B-cells. Only in B-cells, the chromatin harboring the immunoglobulin loci is in its fully active, open state, allowing for high transcriptional activity of native immunoglobulin promoters or recombinant expression constructs integrated into those gene loci.

As a disadvantage, due to the principle of homologous recombination, the targetting sequence will target efficiently in murine cell lines only matching the sequence of the gamma 2 A targetting sequence harboring a recombinatorial hot spot; for high level expression, the gamma 2A locus region must be a transcriptionally active genomic region, limiting its effectiveness for homologous recombination to B-cell types.

It is an object of the present invention to devise another expression system for CHO protein expression in biotechnology which allows for enhanced expression from a standard promoter. According to the present invention, this aim is surprisingly achieved by equipping a gene expression vector for CHO cells with a gene targetting sequence having been originally devised for homologous recombination in murine B-cells.

Possible embodiments of the invention are shown in the figures. What is shown is:

Fig. 1 Relative expression levels of green fluorescent protein (GFP) from hCMV promoter and hCMV promoter in the presence of the IgG 2A hot spot sequence in transient transfection of CHO-K1 cells

Fig. 2 Relative GFP expression levels from hCMV promoter and hCMV promoter in the presence of the IgG 2A hot spot sequence in stably transfected CHO-K1 cells.

Fig. 3 Plasmid map of hCMV-MIE expression vector carrying IgG 2A targetting sequence

According to the present invention, a DNA sequence for expression of a recombinant gene in a mammalian cell comprises a recombinant product gene and a promoter for expressing the recombinant product gene, preferably a CMV promoter, and further comprises a

murine immunoglobulin gamma 2A locus DNA sequence or fragments or sequence variants thereof capable of enhancing expression from the promoter. According to the present invention, such a DNA sequence is useful expression vector construct for expression of recombinant product gene in CHO cells.

According to the present invention, the method of expressing a recombinant protein comprises the steps of

a. culturing a CHO cell transfected with an expression vector comprising a promoter active in CHO cells driving expression of a recombinant product protein and further comprising the murine IgG 2 A gene locus DNA or a DNA sequence variant or DNA fragment thereof which is enhancing activity of said promoter, and
b. harvesting the product protein

A recombinant product gene according to the present invention is the product protein that is sought to be expressed and harvested in high amount. It may be any protein of interest, e.g. therapeutic proteins such as interleukins or enzymes or subunits of multimeric proteins such as antibodies or fragments thereof. The recombinant product gene may include a signal sequence coding sequence portion allowing secretion of the once expressed polypeptide from the host producer cell. In a further prefered embodiment of the present invention, the product protein is a secreted protein. More preferably, the first or product protein is an antibody or engineered antibody or a fragment thereof, most preferably it is an Immunoglobulin G (IgG) antibody.

The DNA sequence of the murine immunoglobulin gamma 2A gene locus (IgG 2A) has originally been devised in WO 95/17516 for use as a genomic targetting sequence for generating stably recombinant lymphoid B-cell lines that show high expression of the recombinant gene product. B lymphocytes or plasma cells normally express extremely high levels of immunoglobulin RNA from the the Ig heavy chain locus, probably due to cell-type specific enhancer/transcription factor activity and open chromatin structure. The preferred murine immunoglobuline gamma 2A gene sequence of the present invention is the same as the targetting sequence used in WO 95/17516. It is a 5.1 kb BamHI genomic

fragment which includes all of the coding region of murine Ig gamma 2A except the most 5' part of the CH1 exon (Yamawaki-Kataoka, Y. et al., Proc. Natl. Acad. Sci. U.S.A. (1982) 79: 2623-2627; Hall, B. et al., Molecular Immunology (1989) 26:819-826; Yamawaki-Kataoka, Y. et al., Nucleic Acid Research (1981) 9: 1365-1381). According to the present invention, promotion of site-directed, homologous recombination is not the relevant property of the immunoglobulin gamma 2A gene sequence (IgG 2A). Accordingly, any sequence variant of said IgG 2A gene sequence or sequence fragment or variant sequence fragment that is functional in or capable of enhancing recombinant product gene expression from the promoter, preferably from a hCMV promoter as set forth below, both under condition of transient or stable expression in CHO cells is also encompassed by the present invention.

Such ‚functional' variants encompass e.g. base insertions, deletions or point mutations and be generated by methods well-known in the art, e.g. by primer-directed PCR, 'error-prone' PCR, 'gene-shuffling' termed PCR-reassembly of overlapping DNA fragments or by in-vivo random mutagenesis of bacterial clones followed by library transfection and functional selection in CHO cells. For instance, random mutagenesis can be achieved by alkylating chemicals or UV-irradiations as described in Miller, J., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory 1972). Optionally, a natural mutator-strain of a host bacterium may be used.

Preferably, such variant sequence or sequence fragment is at least 65%, more preferably 75%, most preferably 90% homologous in DNA sequence to the corresponding part of the natural murine immunoglobuline gamma 2A gene locus. For instance, it is possible to insert a Sal I restriction site at the naturally occurring Stu I site present 39 bp upstream of membrane exon 2 (M2) to provide a unique site for linearization within the murine immunoglobulin gamma 2A sequence; such sequence variant was originally devised for site-specific recombination targetting, but can as well be employed in the context of the present invention.

A 'promoter' is defined as a DNA sequence that directs RNA polymerase to bind to DNA and intiates RNA synthesis. According to the present invention, it is a promoter that is active in CHO cells. Such a promoter preferably is a strong promoter. A strong promoter is one which causes mRNAs to be initated at high frequency equal to or higher than that of

hCMV core promoter/enhancer fragment (described in US5168062) in CHO-KI cells. Such promoter may be a cell-type dependent strong promoter, as are cited in US5589392, or preferably is a ubiquitously active strong promoter, more preferably a constitutively active viral promoter such as e.g. early and late promoters of the SV40 virus, the immediate early promoter of the human cytomegalovirus (hCMV) or of murine cytomegalovirus (mCMV), the thymidine kinase promoter (TK) of Herpes Simplex virus or the Rous Sarcoma Virus long terminal repeat promoter (RS-LTR), more preferably it is the hCMV-MIE promoter as defined by the 2.1 kb Pst I fragment described in US 5,385,839 and/or EP-323 997-A1 or a functional part thereof having promoter activity. The hCMV promoter construct harboring the complete first functional intron of the major immediate early (MIE) gene of hCMV, as set forth in EP-323 997-A1 , is a particularly preferred embodiment of the present invention.

Preferably a hCMV promoter employed in the present invention lacks the 'modulator' sequence part in the upstream/enhancer portion of the promoter. The ' modulator' sequence has been found to be detrimental to hCMV promoter activity in CHO cells and stretches from position -750 to position -1150 relative to the MIE transcription start site (Meier et al ., 1996, Intervirology 39: 331-342, Regulation of hCMV immediate-early gene expression), in particular in transient transfection. Without the modulator sequence, the enhancing effect of the presence of the IgG 2A host spot sequence on (modulator negative or mod- for short) hCMV promoter is even more pronounced.

A transient transfection is characterised by non-appliance of any selection pressure for a vector borne selection marker. A pool or batch of cells originating from a transient transfection is a pooled cell population that comprises cells which have taken up and do express and cells that have not taken up the foreign DNA. Cells that express the foreign expression cassette do usually not have integrated the transfected DNA into their genome yet and tend to lose the foreign DNA and to overgrow transfected cells in the population upon culture of the transiently transfected cell pool. Therefore expression is strongest in the period immediately following transfection and decreases with time. Preferably, a transient transfectant according to the present invention is understood as a cell that is maintained in cell culture in the absence of selection pressure up to a time of 90 hours post transfection.

Preferably, a transfected CHO host cell according to the present invention is a stably transfected host cell, in particular in combination with a hCMV promoter as set forth above. Stable transfection means that newly introduced foreign DNA is becoming incorporated into genomic DNA, usually by random, non-homologous recombination events; in case of a vector sequence, stable transfection according to the present invention may result in loss of vector sequence parts not directly related to expression of the recombinant product gene, such as e.g. bacterial copy number control regions rendered superfluous upon genomic integration. A transfected host cell has integrated at least part or different parts of the expression vector into the genome. Likewise, transfection of CHO cells with two or several DNA fragments giving rise at least in vivo to functional equivalents of the essential elements of the expression vector of the invention, namely the product gene under control of a suitable promoter and the hot spot IgG 2A sequence, is contained in the definition of such transfected host cells. In vivo assembly of functional DNA sequences after transfection of fragmented DNA is described e.g. in WO 99/53046. It is possible that such stable integration gives rise, upon exposure to further selection pressure for gene amplification, to double minute chromosomes in CHO cells. This is comprised in the present meaning of 'stable'. Upon random genomic integration of the expression vector of the present invention in CHO, the presence of the targetting sequence enhances promoter activity for expression of the recombinant product protein. Such effect has not been observed nor could it have been anticipated upon homologous gene targetting in mature murine B-cell lines including plasmacytoma/myeloma cell lines; there, the IgG 2A targetting sequence served solely to increase the frequency of high-yielding homologous integrants since the IgG 2 A locus proved to be a recombinatorial 'hot spot'. As said before, the chromatin of the immunglobuline genomic region is in an open, highly active state in suitably targetted B-cell lines.

'Expression vectors' are defined herein as DNA sequences that are required for transcription and the translation of their mRNAs in an appropriate mammalian host cell line after transfection with vector. An appropriately constructed expression vector should usually contain: at least one expressable marker selectable in animal cells, a limited number of useful restriction sites for insertion of the expression cassette for the recombinant product gene under control of an upstream promoter region. Where used in

particular for transient/episomal expression only, it may further comprise an origin of replication such as origin of Eppstein Barr Virus (EBV) or SV40 virus for autonomous replication/episomal maintenance in eukaryotic host cells but may be devoid of a selectable marker. Expression vectors are e.g., but are not limited to, linear DNA fragments, DNA fragments encompassing nuclear targetting sequences or are specially optimized for interaction with transfection reagents, animal viruses or suitable plasmids that can be shuttled and produced in bacteria. Any selection marker commonly employed such as thymidine kinase (tk), dihydrofolate reductase (DHFR) or glutamine synthetase (GS) may be used. In a preferred embodiment, an expressable GS selection marker is employed (Bebbington et al., 1992, High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker, Bio/Technology 10:169-175; Cockett et al., 1990, High level expression of tissue inhibitor of metalloproteinases in Chinese Hamster Ovary (CHO) cells using Glutamine synthetase gene amplification, Bio/Technology 8: 662-667). - The GS-system is one of only two systems that are of particular importance for the production of therapeutic proteins. In comparison to the dihydrofolate reductase (DHFR) system, the GS system offers a large time advantage during development because highly productive cell lines can often be created from the initial tranfectant thus avoiding the need for multiple rounds of selection in the presence of increasing concentrations of selective agent in order to achieve gene amplification (Brown et al., 1992, Process development for the production of recombinant antibodies using the glutamine synthetase (GS) system, Cytotechnology 9:231-236). It goes without saying that equivalent to a second transcription unit for expression of the marker gene, an expression unit could use a monocistronic expression cassette both for the product gene and the marker gene by employing e.g. internal ribosome entry sites as is routinely employed in the art. Vice versa, it goes without saying that the hot spot IgG 2 A sequence of the present invention and the expression cassette for the product protein comprising a promoter and/or marker cassette are not required to work in cis on a single expression vector; the elements can be well carried on separate co-transfected vectors or DNA fragments which may then be chromosomally integrated at a single, concatemeric integration site.

A further object of the present invention are CHO host cells transfected with the DNA sequences of the present invention. Further objects are a method for transfection of such

host cells and a method for expression of the recombinant product gene in such host cells. The explanations and references made to preferred embodiments in the present specification of the invention relate likewise to all these further objects of the present invention. It is to be noted that a host cell transfected with the DNA sequence or vector of the present invention is to be construed as being a transiently or stably transfected cell line. Any transfection technique such as those well-known in the art, e.g. electoporation, Ca-phosphate precipitation, DEAE-dextrane transfection, lipofection, can be employed according to the present invention if appropriate for a given host cell type.

A suitable host cell line can be any chinese hamster ovary (CHO) cell line (Puck et al., 1958, J. Exp. Med. 108: 945-955). The term 'host cell' refers to cells capable of growth in culture and expressing a desired protein recombinant product protein. Suitable cell lines can be e.g. CHO K1 (ATCC CCL-61), CHO pro3-, CHO DG44, CHO P12 or the dhfr-CHO cell line DUK-BII (Chassin et al., PNAS 77, 1980, 4216-4220) or DUXB11 (Simonsen et al., PNAS 80, 1983, 2495-2499). In CHO cells, the immunoglobuline gene loci are inactive and the chromatin is therefore in a densely packaged or closed state. Thus, any gene construct integrated in the immunoglobuline loci could not give rise to high-level expression of recombinant protein due to the specific state of chromatin, unless it would itself comprise flanking locus control regions promoting opening of the chromatin on both sides of the expression cassette. Further, immunoglobuline gene sequence, and in particular the intron portions of it, show considerably divergence amongst species, e.g. from mouse to hamster. The promoter or enhancer elements of immunoglobline loci are both species and tissue specific and should be active in B-cells only. The murine IgG 2A sequence of the present invention enhances gene expression in CHO cells also in the absence of any natural immunoglobuline promoter that is giving rise to full-length transcripts coding for complete IgG heavy chain. Preferably, the IgG 2A sequence of the present invention is devoid of such promoter. Surprisingly, the murine IgG 2 A targetting sequence even improved gene expression in CHO cells upon transient transfection of CHO cells with expression vectors according to the present invention (Fig. 1); such transient expression is a further preferred embodiment of a method according to the present invention. In transient expression assays which are commonly taking place about 20-50 hours post transfection, the transfected vectors are maintained as episomal elements and are not yet integrated into the genome.

Suitable media and culture methods for mammalian cell lines are well-known in the art, as described in US 5633162 for instance. Examples of standard cell culture media for laboratory flask or low density cell culture and being adapted to the needs of particular cell types are for instance: Roswell Park Memorial Institute (RPMI) 1640 medium (Morre, G., The Journal of the American Medical Association, 199, p.519 f. 1967), L-15 medium (Leibovitz, A. et al., Amer. J. of Hygiene, 78, 1p.173 ff, 1963), Dulbecco's modified Eagle's medium (DMEM), Eagle's minimal essential medium (MEM), Ham's F12 medium (Ham, R. et al., Proc. Natl. Acad. Sc.53, p288 ff. 1965) or Iscoves' modified DMEM lacking albumin, transferrin and lecithin (Iscoves et al., J. Exp. med. 1, p. 923 ff., 1978). For instance, Ham's F10 or F12 media were specially designed for CHO cell culture. Other media specially adapted to CHO cell culture are described in EP-481 791. It is known that such culture media can be supplemented with fetal bovine serum (FBS, also called fetal calf serum FCS), the latter providing a natural source of a plethora of hormones and growth factors. The cell culture of mammalian cells is nowadays a routine operation well-described in scientific textbooks and manuals, it is covered in detail e.g. in R. Ian Fresney, Culture of Animal cells, a manual, 4$^{th}$ edition, Wiley-Liss/N.Y., 2000.

Preferably, the cell culture medium according to the present invention is devoid of fetal calf serum (FCS or FBS), which then is being termed 'serum-free'. Cells in serum-free medium generally require insulin and transferrin in a serum-free medium for optimal growth. Transferrin may at least partially be substituted by non-peptide chelating agents or siderophores such as tropolone as described in WO 94/02592 or increased levels of a source of anorganic iron favorably in conjunction with antioxidants such as vitamin C. Most cell lines require one or more of synthetic growth factors (comprising recombinant polypeptides), including e.g. epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin like growth factors I and II (IGFI, IGFII), etc.. Other classes of factors which may be necessary include: prostaglandins, transport and binding proteins (e.g. ceruloplasmin, high and low density lipoproteins, bovine serum albumin (BSA)), hormones, including steroid-hormones, and fatty acids. Polypeptide factor testing is best done in a stepwise fashion testing new polypeptide factors in the presence of those found to be growth stimulatory. Those growth factors are synthetic or recombinant. There a several methodological approaches well-known in animal cell culture, an exemplary being

described in the following. The initial step is to obtain conditions where the cells will survive and/or grow slowly for 3-6 days after transfer from serum-supplemented culture medium. In most cell types, this is at least in part a function of inoculum density. Once the optimal hormone/growth factor/polypeptide supplement is found, the inoculum density required for survival will decrease. In a more preferred embodiment, the cell culture medium is protein-free, that is free both of fetal serum and individual protein growth factor supplements or other protein such as recombinant transferrin.

A possible embodiment of one method of the present invention, namely expression and harvest of the recombinant product protein, is high-density growth of the animal host cells e.g. in an industrial fed-batch bioreactor. Conventional downstream processing may then be applied. Consequently, a high-density growth culture medium has to be employed. Such high-density growth media can usually be supplemented with nutrients such as all amino acids, energy sources such as glucose in the range given above, inorganic salts, vitamins, trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), buffers, the four nucleosides or their corresponding nucleotides, antioxidants such as Glutathione (reduced), Vitamine C and other components such as important membrane lipids, e.g. cholesterol or phosphatidylcholine or lipid precursors, e.g. choline or inositol. A high-density medium will be enriched in most or all of these compounds, and will, except for the inorganic salts based on which the osmolarity of the essentially isotonic medium is regulated, comprise them in higher amounts (fortified) than the afore mentioned standard media as can be incurred from GB2251 249 in comparison with RPMI 1640. Preferably, a high-density culture medium according to the present invention is balancedly fortified in that all amino acids except for Tryptophane are in excess of 75 mg/l culture medium. Preferably, in conjunction with the general amino acid requirement, Glutamine and/or Asparagine are in excess of 1 g/l, more preferably of 2 g/l of high-density culture medium. In the context of the present invention, high-density cell culture is defined as a population of animal cells having temporarily a density of viable cells of at least or in excess of $10^5$ cells/ml, preferably of at least or in excess of $10^6$ cells/ml, and which population has been continously grown from a single cell or inoculum of lower viable cell density in a cell culture medium in a constant or increasing culture volume.

In a further prefered embodiment, the fed-batch culture is a culture system wherein at least Glutamine, optionally with one or several other amino acids, preferably glycine, is fed to the cell culture as described in GB2251249 for maintaing their concentration in the medium, apart from controlling glucose concentration by spearate feed. More preferably, the feed of glutamine and optionally one or several other amino acids is combined with feeding one or more energy sources such as glucose to the cell culture as described in EP-229 809-A. Feed is usually initiated at 25-60 hours after start of the culture; for instance, it is useful to start feed when cells have reached a density of about $10^6$ cells/ml. It is well known in the art that in cultured animal cells, 'glutaminolysis' (McKeehan et al., 1984, Glutaminolysis in animal cells,in: Carbohydrate Metabolism in Cultured Cells, ed. M.J. Morgan, Plenum Press, New York, pp. 11-150) may become an important source of energy during growth phase. The total glutamine and/or asparagine feed (for substitution of glutamine by asparagine, see Kurano, N. et al., 1990, J. Biotechnology 15, 113-128) is usually in the range from 0.5 to 10 g per l, preferably from 1 to 2 g per l culture volume; other amino acids that can be present in the feed are from 10 to 300 mg total feed per litre of culture, in particular glycine, lysine, arginine, valine, isoleucine and leucine are usually fed at higher amounts of at least 150 to 200 mg as compared to the other amino acids. The feed can be added as shot-addition or as contionusly pumped feed, preferably the feed is almost continously pumped into the bioreactor. It goes without saying that the pH is carefully controlled during fed-batch cultivation in a bioreactor at an approximately physiological pH optimal for a given cell line by addition of base or buffer. When glucose is used as an energy source the total glucose feed is usually from 1 to 10, preferably from 3 to 6 grams per litre of the culture. Apart from inclusion of amino acids, the feed preferably comprises a low amount of choline in the range of 5 to 20 mg per litre of culture. More preferably, such feed of choline is combined with supplementation of ethanolamine essentially as described in US 6048728, in particular in combination with feeding glutamine. It goes without saying that upon use of the GS-marker system, lower amounts of glutamine will be required as compared to a non-GS expression system since accumulation of excessive glutamine in addition to the endogenously produced would give rise to ammonia production and concomittant toxicity. For GS, glutamine in the medium or feed is mostly substituted by its equivalents and/or precursors, that is asparagine and/or glutamate.

It is a further, independent object of the present invention to devise an expression vector comprising at least a (first) transcription unit for a product gene, giving rise to product protein upon expression in a host cell, and which transcription unit is under the control of the mouse Cytomegalovirus promoter (mCMV promoter), and further comprising a second transcription unit comprising a glutamine synthetase (GS) marker gene. Such a product gene, or gene of interest (GOI) as it may be termed, can be e.g. an immunoglobulin coding sequence. A glutamine synthetase marker gene is any enzymatically active GS coding sequence, be it a natural gene sequence or a variant thereof. The above definitions of 'functional variant' as set forth above apply here as well including the preferred ranges of sequence homology. Preferably, the GS marker gene is a mammalian GS marker gene or derived thereof. Surprisingly, such expression vector allows for much higher transfection rates upon transfection in CHO cells than does e.g. an expression vector in which the first transcription unit harboring the gene of interest is under control of the hCMV promoter. This despite the fact that in CHO cells, transcriptional activity of the mCMV promoter is much higher than that of hCMV promoter; usually it is believed that upon transfection, higher metabolic load reduces clonal survival upon transfection, resulting in lower numbers of transfectants . Thus the effect can not be correlated in an obvious manner with the amount or unexpected toxicity of product protein expressed, the latter possibly adversely affecting growth of transfectants. Indeed, the finding is the very opposite of any expectation of a skilled person.

Further objects according to the present invention are animal host cells, in particular CHO cells, transfected with such an expression vector which vector can be maintained episomally or can be stably integrated in the genome and a respective transfection method. Likewise, transfection of animal cells, in particular CHO cells, with two or more gene fragments giving rise *in-vivo* to functional equivalents of the transcription units of the present object of the invention, is within the definition of such transfected host cells. Preferably, said host cells are stably transfected cells, meaning that the first and second transcription unit are chromosomally integrated.

A further object is the use of mCMV promoter to enhance transfection rate in CHO cells, preferably when using an expression vector comprising at least a first transcription unit for a product gene which first unit is giving rise to product protein upon expression in a host

cell and which first transcription unit is further under the control of the mouse Cytomegalovirus promoter (mCMV promoter), and further comprising a second transcription unit comprising a glutamine synthetase (GS) marker gene. It may also be possible to transfect the first and second expression borne on different vectors, or as isolated gene fragments harboring individual expression units. Further, it may be possible to transfect a CHO cell that is already recombinant for and expresses GS with a first transcription unit harboring mCMV. According to the present invention, 'enhancing transfection rate' is defining by comparing transfection rate in the presence of the mCMV promoter and expression vector according to the present invention with the transfection rate of the same expression vector and host cell under identical transfection and cell culture conditions except that in the expression vector, the mCMV promoter is substituted to the hCMV-first intron enhancer/promoter construct as defined in US 5658 759 and as set forth e.g. in sequence ID. No. 3 of the present invention. This hCMV-intron MIE-promoter construct, for a given identical product gene, serves as a standard for determining the claimed effect of enhanced transfection rates. Preferably, use of mCMV promoter results in at least 10-times enhanced transfection rate.

All relevant definitions given further above apply likewise to the present, independent objects of the invention. It must be stressed that the present object of the invention does not require the presence of the murine IgG 2 A targetting sequence as a prerequisite.

Murine cytomegalovirus (mCMV) is a member of the highly diverse group of herpesviridae. Even amongst cytomegaloviruses of different host species there can be wide variation. For example, mCMV differs considerably from the human cytomegalovirus (hCMV) with respect to biological properties, immediate early (IE) gene organization, and overall nucleotide sequence. The 235-kbp genome of mCMV also lacks large internal and terminal repeat characteristics of hCMV. Accordingly, no isomeric forms of the MCMV genome exist (Ebeling, A. et al., (1983), J. Virol. 47, 421-433; Mercer, J. A. et al., (1983), Virology 129, 94-106). According to the present invention, it is possible to employ the promoter region essentially corresponding to a large approx. 2.1 kb PstI fragment described in US 4968615 or any functional fragment thereof. In a more preferred embodiment, the mCMV promoter fragment employed comprises the transcription start site (+0) and extends upstream to about position –500. Surprisingly, such fragment has

been found to promote stronger expression than a promoter cassette extending 800 bp further upstream beyond position –500. In a most preferred embodiment, a core promoter region is employed that extends from the transcription start site upstream but to the Xho I restriction site at about position –150 from the natural transcription start site or even extending but to position –100 upstream from the natural transcriptions start site. It goes without saying that the transcription start site might be engineered in order to comprise a suitable restriction site for insertion of the recombinant product gene.

According to the present invention, it is also possible that the first transcription unit that is under control of the mCMV promoter harbors at least one intron sequence. Such measure is well-known in the art for stabilising RNA transcripts and for promoting efficient protein synthesis from the corresponding mRNA. For efficient protein synthesis without having regard to the claimed effect on transfection rate, it is however not advisable to include the first, natural intron of mCMV in the mCMV promoter construct. In contrast to the situation with hCMV promoter (cf. US 5591639), such natural first intron of mCMV was found to decrease expression of a recombinant gene from the mCMV promoter and is therefore excluded in a further preferred embodiment.

Examples of preferred, possible embodiments of GS marker gene cassettes are given in the sequence listings. Seq IDs No. 1 (pEE 15.1 hCMV/GFP + hot spot) +2 (pEE 14.4 hCMV/GFP) give examples of suitable GS-gene cassettes that are expressed from the SV40 (early and late, respectively) promoter, a weak to medium level promoter, further comprising an expression cassette for GFP (Green fluorescent protein) that is under control of the hCMV promoter. Seq. ID No. 1 describes a GS cDNA sequence described in more detail in the figure legend of Fig. 3, under control of the SV40 early promoter. Seq. ID No. 2 specifies an artifical GS-minigene cassette comprising an intron that is under control of the SV40 late promoter. CHO cells are not naturally glutamine auxothropic, therefore selection schemes as e.g. described in Cockett et al., 1990, High level expression of tissue inhibitor of metalloproteinases in Chinese Hamster Ovary (CHO) cells using Glutamine synthetase gene amplification, Bio/Technology 8: 662-667, can be applied. Examples of suitable transfection methods for CHO cells are equally given therein; it is possible to employ e.g. classic calcium phosphate precipitation or more modern lipofection

techniques. Transfection rate is routinely defined as the number of positively transfected cells (transient transfection) or clones (stable transfection after selection period) obtained from a pool of cells subjected to transfection. The purported effect of the present object of invention can be seen e.g. by transfecting CHO-K1 cells by lipofection (any commercial reagent and manufacturers protocol) with the plasmids of either Seq. ID No. 3 (pEE 12.4 hCMV-GFP + SV40 early promoter/GS cDNA) or Seq. ID No. 4 (pEE 12.4 mCMV-GFP + SV40 early promoter/GS cDNA). Transfected cells may be grown in any conventional culture medium. The culture medium may be a fetal serum-supplemented or serum-free medium as has been defined above. Preferably, the cell culture medium is a serum-supplemented medium, more preferably a cell culture medium that has been supplemented with at least 1% (v/v) fetal serum, most preferably with at least 5% (v/v) fetal serum such as fetal calf serum or fetal bovine serum. In another preferred embodiment, the transfection method carried out is electroporation.

**Experiments**

Experiment 1

Transient and stable expression of GFP vector comprising hot spot sequence in CHO-K1 cells

CHO-K1 cells (ATCC CCL-61) were adapted and cultured in normal cell culture medium GMEM –S (Gibco, UK) with 10% FCS. - For GS selection, the medium must be completely free of glutamine as set forth in table 1 below; this necessitates use of dialysed FCS. - All culturing was carried out in shake flask at 36.5 °C with orbital shaking at 125 rpm. Lipofectin (Superfectin™ ,Gibco, UK) was used for transfection and green fluorescence of transfectant pool was measured in a FACS with excitation at 488 nm. For every GS/GFP vector construct, transfection was carried out independently five times, all data being the average from five independently analyzed pools. Starting with transient transfectants 48 h post-transfection, the top scoring 10% highly expressing cells of the viable cell pool in the cell count vs. fluorescence diagram were selected to determine mean fluorescence (Fig.1). Viable cell population has been preselected by gating in the Forward vs. sideward scatter diagram.

For generating stable transfectants, GS marker was selected 24 hours post-transfection by supplementing the glutamine-free medium with 25 µM MSX (methionine sulphoximine, Crockett et al., ibd.) and continuing cell culture with regular splitting of cultures for 26 days. Note the impact of medium levels of other amino acids on the potency of MSX for selection, see Bebbington et al., US 5827 739. Flurorescence analysis was then performed again as outlined above (Fig. 2).

Untransfected cells served as negative control. The hot spot vector (pEE 15.1 'hCMV + hot spot') driving expression of GFP under control of the hCMV promoter comprising the first complete intron of CMV is given in Seq. ID No. 1 and essentially is the pEE 15.1 vector shown in Fig. 3 into which the GFP sequence was inserted into the Eco RI restriction site in the polylinker. pEE 12.4 'hCMV' corresponding to Seq. ID No. 3 is identical to pEE 15.1 'hCMV + hot spot' except that it does not comprise the 5.1 kb Bam H1 fragment harboring the IgG 2A sequence. pEE 12.4 served as a vector control. A further vector

control pEE 12.4 'hCMV(Kozak-)' was generated by mutating the Kozak sequence of the cloning site coninciding with the translation start site (GCCGCC*A*CCATG*G*) to a frameshifted functional Kozak sequence that (*A*CCATG*G*TCCATGG) by primer directed mutagensis (Sambrook et al., Molecular cloning, Cold Spring Harbor 1983), attentuating the original Kozak and translation start site. The vector of Seq. ID No. 1 was further engineered to delete the 400 bp modulator region of hCMV enhancer portion, deleting the enhancer elements upstream of -750 from the transcription start site, giving rise to pEE 15.1 'hCMV(mod-)/GS cDNA'. By exchange of the GScDNA cassette of pEE 15.1 (s. Fig. 3) with the GS minigene of pEE 14.4 'hCMV(mod-)' /GFP, corresponding to Seq. ID No. 2, the vector pEE 15.1 'hCMV(mod-)/GS minigene' was created. Thus all transfected cells harbored a plasmid vector comprising the GFP coding sequence. The GS minigene contains a single, first intron of the GS gene and about 1 kb of 3' flanking DNA under the control of the SV40 late promoter; the 3' part of the genomic GS DNA is believed to cause a higher copy-number of vector DNA and thus of GS in transfected cells (see US4770359, Bebbington et al.). Whereas all hCMV vectors employed in the present study express the GS maker gene from its cDNA sequence, use of the GS minigene was included as a further control in order to exclude potential effects of GS copy number and expression level.

For generation and expression analysis of stably transfected CHO cells, transfections were performed with linearized hot spot vector pEE 15.1 'hCMV+ hot spot' vector. Sal I linearized plasmid was cut in the IgG 2A comprising sequence portion, free DNA ends potentially stimulating recombination with genomic regions sharing a certain degree of homology with the flanking DNA portions, testing for potential targetting effects of murine IgG 2A in hamster CHO cells. Pvu I cut in the bacterial lactamase marker gene and therefore could promote but heterologous random recombination. Indeed, the mean fluorescence was higher in the Pvu I linearized transfectants showing both some influence of vector linearization as well as that targetting to immunoglobuline loci in CHO cells may not account for the effect of the present invention. In addition, the effect of enhanced promoter activity was consistingly observed in transiently transfected cell populations, nicely correlating with relative strength of individual vector constructs. Clearly, genomic integration is not involved at this early stage of transfection.

Fig. 3 shows vector pEE 15.1 of approximately 12 830 bp. A detailed description of the GS

marker and the hCMV-p/intron expression cassette can be found in US5827739 and US5591639. pEE 15.1.is a possible embodiment of an expression vector according to the present invention, except that the DNA sequence coding for the recombinant product protein has not yet been inserted in the polylinker site. The complete 13535 bp sequence of the pEE15.1 construct harboring GFP is given in Seq. ID No. 1: Therein, the GFP coding sequence was inserted in-frame in the Eco R I restriction site centered at base position 12 814; the introduction of the unique restriction site harboring the ATG start codon and optimizing the Kozak sequence environment of the start codon is described in detail in US 5591639. Thus, the expression of GFP protein is under control of the hCMV-major immediate early gene promoter (hCMV-MIE or hCMV for short) immediately followed by the first intron of hCMV-MIE gene followed by the Nco I site (s. US 5591 639). Polyadenlyation is ensured by the SV40 poly A site further downstream of the polylinker insertion site. pEE 15.1 further harbors a cDNA sequence coding for glutamine synthetase (GS) from hamster that is under control of the SV 40 early promoter and is followed by an SV40 intron +poly A sequence. The IgG 2A gene locus or 'hot spot' sequence (hatched boxes CH1, Hi, CH2, CH3, M1, M2 standing for Heavy chain constant region, hinge, membrane anchor) is the 5.1 kb BamH I fragment of the murine IgG 2 A locus already described in WO 9517516 and the references cited therein. Unique restriction sites Pvu I and Sal I are shown.

Experiment 2

Electroporation of CHO cells with mCMV p12.4 –GFP construct (Seq. ID No.4)

Attached CHO-K1 cells (ATCC CCL-61) were cultured in Iscoves' DMEM medium essentially as described in EP-481 791 comprising 2 mM Glutamine which was further supplemented with 10% FCS. Optionally, the G-MEM medium stated in table 1 and further comprising 2 mM Glutamine could be used prior to GS marker selection as in experiment 1. The cells were detached, pelleted and resuspended twice in serum-free medium, finally at a density of 5.3 $x10^6$ cells/ml. Per 750 µl electroporation batch, a total of $4x10^6$ cells was electroporated. Electroporation was carried out as described in Methods in Molecular Biology, ed. JA Nickoloff ed, Humana Press 1995, Vol. 48/Chap. 8: Animal cell electroporation and electrofusion protocols. p12.4 mCMV-GFP vector DNA (sequence ID No. 4) was linearized. 50µl (20µg) DNA were added to 750µl cells in electroporation

cuvette and electroporate - 300 Volts / 750 μFd - expecting an electorporation time of around 12 - 14 msec. Following electroporation 800μl volume of cells was transferred into 25 ml of modified Glasgow-MEM (GMEM, Gibco) culture medium for GS selection (comprising 10% fetal serum but no glutamine, for details see table 1 ) in a T75 flask. Divide into 2 x T75 flasks by moving 12.9 mls into a second flask and incubate overnight at 37°C in 10% $CO_2$

On the next day 37.5ml of GS-selection GMEM culture medium supplemented with 10% FBS + 33.3μM MSX (methionine sulphoximine) were added. Thus MSX was finally ~25μM. Transfectants were counted after further incubation for 26 days by colony count per flask. Upon microscopic inspection in a standard inverted microscope for inspection of culture flasks, positive colonies brightly lit up in light green and could be easily counted.

The mCMV construct of Seq. ID No. 4 yielded up to 20 times more foci than did cells that were transfected in parallel with the hCMV construct of Seq. ID No. 3. The vector constructs only differed in the CMV promoter elements driving GFP expression, the remaining vector parts of the vectors were identical (including GS-marker; cDNA GS-marker cassette of p12.4) . If cells were diluted out into 96 well plates immediately following transfection, many more colonies come up from mCMV transfected cells (>400 colonies) than from hCMV transfected cells (about 45 colonies).

**TABLE 1**: Medium for GS selection

A. Stock Solutions

1. Double distilled water autoclaved in 400 ml aliquots
2. 10 x Glasgow MEM (GMEM) without glutamine (GIBCO: 042-2541 in UK). Store at 4°C.
3. 7.5% sodium bicarbonate (GIBCO: 043-05080 in UK; 670-5080 in US). Store at 4°C.
4. 100 x non-essential amino acids (NEAA) (GIBCO: 043-01140 in UK; 320-1140 in US). Store at 4°C.
5. 100 x Glutamate + Asparagine (G + A): add 600 mg glutamic acid and 600 mg asparagines (Sigma). Make up to 100 ml in distilled water and sterilize by passing through a sterile 2μm filter (Nalgene). Store at 4°C.
6. 100mM sodium pyruvate (GIBCO: 043-01360 in UK; 320-1360 in US)

| | | |
|---|---|---|
| 7. | 50 x nucleosides: | 35 mg adenosine |
| | | 35 mg guanosine |
| | | 35 mg cytidine |
| | | 35 mg uridine |
| | | 12 mg thymidine |

(each from Sigma). Make up to 100ml with water, filter sterilise and store at -20°C in 10ml aliquots.

8. Dialysed FCS (GIBCO: 014-06300). Heat inactivate at 56°C for 30 min and store at -20°C. It is essential to use dialysed FCS when using GS selection.

9. Penicilin-streptomycin at 5000 units/ml (P/S: GIBCO: 043-05070 in UK; 600-5070 in US).

10. 100 mM L.MSX (Sigma): prepare 18 mg/ml solution in PBS. Filter sterilise and store at -20°C.

B.Medium Preparation

Add the following in the order given using aseptic technique to make GMEM-S medium

| | |
|---|---|
| 1. Water | 400 ml |
| 2. 10 x GMEM | 50 ml |
| 3. Sodium bicarbonate | 18.1 ml |
| 4. NEAA | 5 ml |
| 5. G + A | 5 ml |
| 6. Sodium pyruvate | 5 ml |
| 7. Nucleosides | 10 ml |
| 8. Dialysed FCS | 50 ml |
| 9. Penicillin-streptomycin | 5 ml |

GMEM-S contains the non-essential amino acids, alanine, aspartate, glycine, proline and serine (100 μM), glutamate and asparagines (500 μM), and adenosine, guanosine, cytidine and uridine (30 μM), and thymidine (10 μM).

## Claims

1. CHO cell transfected with an expression vector comprising a promoter that is active in CHO cells and that is driving expression of a recombinant product protein and further comprising a portion from the murine IgG 2 A gene locus DNA which portion is enhancing activity of said promoter.

2. CHO cell according to claim 1, characterized in that the vector further comprises a transcription unit encoding a selectable marker, preferably a glutamin synthetase (GS) marker.

3. CHO cell according to claim 1 or 2, characterized in the CHO cell is stably transfected.

4. Method of expressing a recombinant protein, comprising the steps of

   c. culturing a CHO cell transfected with an expression vector comprising a promoter active in CHO cells driving expression of a recombinant product protein and further comprising the murine IgG 2 A gene locus DNA or a DNA sequence variant or DNA fragment thereof which is enhancing activity of said promoter, and
   d. harvesting the product protein

5. Method according to claim 4, characterised in that the promoter is a strong viral promoter, preferably the hCMV promoter.

6. Method according to one of claims 4 or 5, characterised in that the IgG 2A gene locus portion does lack the natural immunoglobulin promoter.

7. Mammalian expression vector comprising at least a first transcription unit for a product gene which transcription unit is under the control of the mCMV promoter, and further comprising a second transcription unit comprising a glutamine synthetase (GS) marker gene.
8. CHO cell transfected with the vector of claim 7.