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DESCRIPTION

[0001] The present invention concerns a process for the expression of recombinant polypeptides, and in particular the secretion of recombinant polypeptides.

[0002] It is of significant benefit in recombinant polypeptide production if the polypeptide of interest can be exported from the cell in which it is expressed. Expression systems are therefore advantageously designed to enable such export, or secretion. Secretion of the recombinant polypeptide from the host cell commonly involves use of signal peptides, which are found on the majority of eukaryotic and prokaryotic proteins that are destined for export from the cytoplasm. Secretion leaders employed in such expression systems are typically native to the expression host, for example, the PhoA, MalB and OmpA signal peptides of *Escherichia coli* have been used extensively to secrete polypeptides to the periplasm of that organism.

[0003] US7,071,172 describes the use of fibronectin secretion leaders in AAV-based delivery vectors for use in gene therapy.

[0004] According to a first aspect of the present invention, there is provided a process for the production of a target polypeptide which comprises:

1. a) expressing an expression vector for expressing a target polypeptide in a CHO host cell, the expression vector comprising an expression cassette comprising a polynucleotide encoding a recombinant polypeptide operably linked to a fibronectin secretion leader sequence; and
2. b) recovering the target polypeptide.

[0005] Fibronectin secretion leaders that can be employed in the present invention include mammalian and reptilian fibronectin secretion leaders. Examples of reptilian fibronectin secretion leaders include *Xenopus laevis* fibronectin secretion leaders. Examples of mammalian fibronectin secretion leaders include human, rat, murine, bovine, porcine, canine, feline and Chinese hamster fibronectin secretion leaders, and functional equivalents thereof, such as human fibronectin secretion leader having the sequence MLRGPGPGLLLLAVQCLGTAVPSTGA (SEQ ID No. 1). In certain embodiments, the Chinese hamster fibronectin secretion leader having the amino acid sequence MLRGPGPGLLLAVLCLGTAVRCTEA (SEQ ID No. 2) and functional equivalents thereof is preferred.

[0006] A functional equivalent to a secretion leader is one that shares 70% or greater identity with an amino acid sequence, preferably 75% or greater identity, more preferably 80% or greater identity and most preferably 90% or greater identity, such as 95% identity or more, and which retains the ability to secrete the recombinant polypeptide. In some embodiments, the

functionally equivalent secretion leader differs by a single amino acid, by any of addition, deletion or replacement.

[0007] In many embodiments, polynucleotide sequences which are operably linked are contiguous and, in the case of a secretion leader, contiguous and in the same reading frame.

[0008] Preferably, the linkage between the polynucleotide encoding the fibronectin secretion leader sequence and the polynucleotide encoding the target polypeptide is such that the secretion leader is attached to the N-terminal of the recombinant polypeptide. In certain embodiments, the recombinant polypeptide comprises an N-terminal tag, the linkage between the secretion leader sequence and the polynucleotide encoding the recombinant polypeptide being such that the secretion leader is attached to the tag, preferably to the N-terminus of the tag.

[0009] The polynucleotide encoding the fibronectin secretion leader sequence is preferably attached at the 5' end of the polynucleotide encoding the target polypeptide and preferably has the sequence ATGCTGAGAGGCCCTGGACCTGGACTGCTGC TGCTGGCTGTGCAGTGTCTGGGAACCGCCGTGCCTTCTACCGGCGCC (SEQ ID No. 3) or ATGCTCAGGGGTCCGGGACCCGGGCTGCTGCTGGCCGTCCTGTGCCTGGG GACAGCGGTGCGCTGTACCGAAGCC (SEQ ID No. 4).

[0010] The vectors of the present disclosure comprise a promoter operably linked to the expression cassette for the secretion leader and recombinant polypeptides.

[0011] Promoters that can be employed in CHO host cells may be endogenous or exogenous to the CHO cells. Suitable promoters include viral promoters such as CMV, SV40 promoter, and RSR-LTR. Promoters from housekeeping genes such as hEF1a and murine phosphoglycerate kinase (mPGK) may also be utilised. In some embodiments, preferred promoters are human CMV and rat CMV. The promoters may be the same or different if more than one polypeptide is being expressed (eg MAb HC and LC polypeptides). The promoter may be employed in combination with an enhancer sequence, such as the major immediate early enhancer of a cytomegalovirus, especially human cytomegalovirus.

[0012] The expression vector may be integrated into the host cell genome or comprised within an extrachromosomal element such as a plasmid.

[0013] The expression vector typically also comprises a selectable marker. Selectable markers for mammalian cells, and especially for Chinese hamster ovary cells include glutamine synthetase and dihydrofolate reductase marker systems.

[0014] The vectors employed comprise features conventional in the art appropriate for expression in the appropriate CHO host cell. CHO expression vectors typically comprise a polyadenylation sequence, such as human betaglobin polyA sequence, bovine growth hormone polyA sequence and SV40 early or late poly A sequences.

[0015] The expression vector can be employed to express recombinant polypeptides, especially proteins in CHO host cells.

[0016] Preferred host cells are Chinese hamster ovary (CHO) cells, and in particular CHOK1, DG44, DUXKB11 and CHO pro3-cells.

[0017] The expression vector is commonly employed in the form of a plasmid. The plasmids may be autonomously replicating plasmids or integrative plasmids.

[0018] In certain highly preferred embodiments of the present invention, the fibronectin secretion leader is Chinese hamster fibronectin.

[0019] Polypeptides which can be expressed by the process of the present invention include therapeutic proteins and peptides, including cytokines, growth factors, antibodies, antibody fragments, immunoglobulin like polypeptides, enzyme, vaccines, peptide hormones, chemokines, receptors, receptor fragments, kinases, phosphatases, isomerases, hydrolyases, transcription factors and fusion polypeptides.

[0020] Antibodies which can be expressed include monoclonal antibodies, polyclonal antibodies and antibody fragments having biological activity, including multivalent and/or multispecific forms of any of the foregoing.

[0021] Naturally occurring antibodies typically comprise four polypeptide chains, two identical heavy (H) chains and two identical light (L) chains inter-connected by disulfide bonds. Each heavy chain comprises a variable region (V_H) and a constant region (C_H), the C_H region comprising in its native form three domains, C_H1, C_H2 and C_H3. Each light chain comprises a variable region (V_L) and a constant region comprising one domain, C_L.

[0022] The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

[0023] Antibody fragments which can be expressed comprise a portion of an intact antibody, said portion having a desired biological activity. Antibody fragments generally include at least one antigen binding site. Examples of antibody fragments include: (i) Fab fragments having V_L, C_L, V_H and C_H1 domains; (ii) Fab derivatives, such as a Fab' fragment having one or more cysteine residues at the C-terminus of the C_H1 domain, that can form bivalent fragments by disulfide bridging between two Fab derivatives; (iii) Fd fragment having V_H and C_H1 domains; (iv) Fd derivatives, such as Fd derivatives having one or more cysteine residues at the C-terminus of the C_H1 domain; (v) Fv fragments having the V_L and V_H domains of a single arm of

an antibody; (vi) single chain antibody molecules such as single chain Fv (scFv) antibodies in which the V_L and V_H domains are covalently linked; (vii) V_H or V_L domain polypeptide without constant region domains linked to another variable domain (a V_H or V_L domain polypeptide) that is with or without constant region domains, (e.g., V_H-V_H, V_H-V_L, or V_L-V_L) (viii) domain antibody fragments, such as fragments consisting of a V_H domain, or a V_L domain, and antigen-binding fragments of either V_H or V_L domains, such as isolated CDR regions; (ix) so-called "diabodies" comprising two antigen binding sites, for example a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L), in the same polypeptide chain; and (x) so-called linear antibodies comprising a pair of tandem Fd segments which, together with complementary light chain polypeptides, form a pair of antigen binding regions.

[0024] Preferred antibody fragments that can be prepared are mammalian single variable domain antibodies, being an antibody fragment comprising a folded polypeptide domain which comprises sequences characteristic of immunoglobulin variable domains and which specifically binds an antigen (i.e., dissociation constant of 500 nM or less, such as 400 nM or less, preferably 250 nM or less, and most preferably 100 nM or less), and which binds antigen as a single variable domain; that is, without any complementary variable domain. Single variable domain antibodies include complete antibody variable domains as well as modified variable domains, for example in which one or more loops have been replaced by sequences which are not characteristic of antibody variable domains or antibody variable domains which have been truncated or comprise N- or C-terminal extensions, as well as folded fragments of variable domains. Preferred single variable domains which can be prepared are selected from the group of V_H and V_L, including V_{kappa} and V_{lambda}. Most preferably the single variable domains are human or camelid domains, including humanised camelid domains.

[0025] Where the target polypeptide comprises two or more chains to be secreted, particularly where the target polypeptide is an antibody or a fragment antibody comprising two or more chains, at least one, and preferably each, of the chains is attached to a fibronectin secretion leader, and polynucleotides encoding such polypeptides are designed accordingly. The fibronectin secretion leaders employed may be the same or different. The polynucleotides encoding the two or more chains may be comprised within the same expression cassette, but are preferably comprised in different expression cassettes. Where different expression cassettes are employed, the expression cassettes may be located on different vectors, but are preferably on the same vector. Promoters employed may be the same or different.

[0026] The expression system is expressed by methods well known in the art for the cells employed. Preferred expression methods include culturing the host cells in growth medium, and then recovering the expressed polypeptide. The term "growth medium" refers to a nutrient medium used for growing the host cells. In many embodiments, a nutrient solution is employed. Suitable growth media for given host cells and methods of recovering polypeptides are well known in the art.

[0027] In many embodiments, the polypeptide recovery comprises one or more of filtration,

centrifugation, diafiltration, ion-exchange chromatography, affinity chromatography, such as Protein A affinity chromatography, Hydrophobic Interaction Chromatography (HIC), Gel Filtration and HPLC.

[0028] According to a preferred aspect of the present invention there is provided a process for the production of a target polypeptide which comprises:

1. (a) transfection or transformation of a host cell with an expression vector for expressing a target polypeptide in a CHO host cell, the expression vector comprising an expression cassette comprising a polynucleotide encoding the target polypeptide operably linked to a fibronectin secretion leader sequence;
2. (b) culturing the host cell under conditions which allow proliferation of the host cell and expression and secretion of the target polypeptide from the host cell
3. (c) and recovering the target polypeptide.

[0029] According to a further aspect of the present invention, there is provided a Chinese hamster ovary cell, preferably a CHOK1, DG44, DUXKB11 or CHO pro3- cell, transfected with an expression vector comprising an expression cassette comprising a polynucleotide encoding the target polypeptide operably linked to a fibronectin secretion leader sequence.

[0030] The target polypeptide encoded preferably comprises a monoclonal antibody. An expression cassette comprising polynucleotides encoding both heavy and light chains of a monoclonal antibody, preferably each operably linked to a fibronectin secretion leader, may be employed. In some embodiments, separate expression cassettes comprising heavy and light chains are employed, which may be located on separate vectors, but are often located on the same vector. The fibronectin secretion leaders employed may be the same or different, but are preferably the same.

[0031] In many preferred embodiments, the expression cassette comprises a housekeeping gene promoter, especially an hEF1a promoter operably linked to the polynucleotide encoding the target polypeptide, and when two or more expression cassettes are employed, each expression cassette comprises a housekeeping gene promoter, preferably the same promoter, and most preferably an hEF1a promoter.

[0032] The, or each, expression cassette for the target polypeptide preferably comprises a bovine growth hormone polyA sequence.

[0033] The expression vector preferably comprises a selection marker, most preferably a dihydrofolate reductase marker system. In certain instances, the dihydrofolate reductase marker system comprises an expression cassette further comprising a murine phosphoglycerate kinase promoter.

[0034] In certain instances, the dihydrofolate reductase marker system comprises an

expression cassette further comprising a murine phosphoglycerate kinase promoter.

[0035] The present invention is illustrated without limitation by the following examples.

Example 1

[0036] For each secretion leader (SL) being assessed, two single gene vectors were constructed which contained either the hγ1 FL heavy chain (HC) of an anti-MUC-1 MAb or the lambda light chain (LC) of an anti-MUC-1 MAb. Each expression cassette consisted of a rat CMV promoter, functionally linked to a polynucleotide sequence encoding the secretion leader which was linked in frame to a polynucleotide sequence encoding the HC or LC mature polypeptide and a human betaglobin polyA sequence. The structure of the expression cassettes is illustrated in Figure 1.

[0037] Secretion leaders employed were as follows:

Secretion leader A: human collagen, sequence MLSFVDTRTLLLLAVTLCLATCQS (SEQ ID No. 5)

Secretion leader B: human fibronectin, sequence MLRGPGPGLLLLAVQCLGTAVPSTGA (SEQ ID No. 1)

Secretion leader C: Chinese hamster fibronectin, sequence MLRGPGPGLLLAVLCLGTAVRCTEA (SEQ ID No. 2)

Secretion leader D: Chinese hamster albumin, sequence MKWVTFLLLLFVSDSAFS (SEQ ID No. 6)

[0038] CHO DG44 host cells were counted and seeded onto wells of a 6 well plate at 1.2 x 10^6 cells/well in MEM-α medium supplemented with 10% serum, 2mM Glutamine and 0.45% glucose, and incubated overnight at 36.5°C, 7.5% CO_2.

[0039] For each transfection 4µg of the HC and LC single gene vectors (2µg) were mixed together and diluted in 250µL serum free MEM-α medium (Life Technologies). A mock transection (PBS only) was also included. For each transfection 12.5µL Lipofectamine 2000 (Life Technologies) was diluted in 250µL serum free MEM-α medium and mixed. The mixture was incubated at room temperature (15-25°C) for 5 minutes. The diluted DNA and Lipofectamine 2000™ reagent were combined, mixed and incubated for 20 minutes at room temperature. A further 500µL MEM-α medium was added to each transfection mix, growth medium was removed from the well and the complex was then added to a well of the 6-well plate containing the cells. After 5 hours the medium was removed and fresh growth medium was added. Cells were incubated for 5 days at 36.5°C, 7.5% CO_2 Supernatant was harvested

and clarified by centrifugation. Antibody titre was determined using an Octet (Forte Bio) protein A assay.

[0040] The results are given in Table 1 below.

Table 1

Secretion leader used	Mean antibody titre (mg/l)
A	2.91
B	7.79
C	8.85
D	1.89

[0041] The antibody produced is recovered from the supernatant by Protein A capture, elution at low pH and purified by cation exchange chromatography followed by anion exchange chromatography. Eluent from the anion exchange chromatography is subject to viral nanofiltration, followed by buffer exchange and concentration.

Example 2

Vector construction

[0042] Double gene vectors were constructed which contained a hEF1a promoter driving expression of both the hy1 FL heavy chain of an anti-MUC-1 MAb and the human lambda light chain of an anti-MUC-1 MAb.

[0043] Further double gene vectors were constructed where the hEF1a promoter was exchanged for either a hCMV-MIE promoter or rat CMV promoter.

[0044] Each expression cassette within the double gene vectors consisted of the promoter functionally linked to a polynucleotide sequence encoding the CHO fibronectin signal peptide of Example 1, which was linked in frame to a polynucleotide sequence encoding the HC or LC mature polypeptide. Correct mRNA processing was ensured by the presence of a bovine growth hormone poly A sequence.

[0045] To allow selection of stable cell lines the vectors also contained a copy of the mouse dyhydrofolate reductase (dhfr) gene under control of the murine phosphoglycerate (mPGK) promoter and the hygromycin resistance gene under the control of the thymidine kinase (TK) promoter.

Routine subculture of CHO DG44 cells:

[0046] CHO DG44 cells were routinely cultured in suspension shaker flasks in EX-CELL ACF CHO medium (Sigma) supplemented with 8 mM L-glutamine and 1 x HT supplement (Life Technologies). Cells were seeded at a concentration of 2 x 10^5 cells/ml, and cells were split every 3 days. Flasks were cultured at 37°C, 7.5% CO_2 in an orbital shaking incubator at 140 rpm.

Transfections for generation of stable cell lines

[0047] Cells used for transfections were grown in cell suspension culture, as detailed above. Cells from growing cultures were centrifuged and re-suspended to a concentration of 2x10^7 cells/mL. A 0.1 mL volume of the cell suspension and 4 µg of linearised plasmid DNA were added to an electroporation cuvette. The cuvette was then placed in the Amaxa nucleofector (Lonza) and nucleofected. Following transfection, the cells were added to 20ml pre-warmed EX-CELL ACF CHO medium (Sigma) supplemented with 8mM Glutamine and 1 x HT supplement in a T75 flask. Transfected cells were incubated at 37°C, 7.5% CO_2. Following the removal of hypoxanthine and thymidine (HT) (48hrs post transfection) from the medium and addition of 400µg/ml Hygromycin B (Invitrogen) and 25nM MTX (144hrs post transfection) cells were plated out into 96 well plates at 5000 cells/well (2.5 x 10^4/mL). The plates were incubated at 37°C in an atmosphere of 7.5% CO_2 in air. The plates were monitored for colony growth up to approximately three weeks after transfection. Supernatant from up to 100 wells containing cell growth were harvested and analysed for the Antibody using an Octet (Forte Bio) protein A assay. The top 24 expressing colonies were expanded into 24 well plates and cultured for 10 days. Supernatant was then assayed for the Antibody using an Octet (Forte Bio) protein A assay. The results are given in Table 2 below.

Table 2

	hEF1α		hCMV-MIE		Rat CMV	
	96 wp	24 wp	96 wp	24 wp	96 wp	24 wp
Max Exp Level (µg/mL)	7.3	18.0	6.1	3.2	6.1	nd
Mean Exp Level (µg/mL)	3.3	4.2	0.7	1.3	0.6	nd

Example 3

Purification of Antibody from CHO supernatant

[0048] Supernatant from recombinant CHO DG44 cell lines generated using the hEF1a

promoter double gene vector described in Example 2 was purified using protein A resin. 350mL of clarified harvest was loaded onto a pre-packed column containing MabSelect SuRe resin (GE Healthcare). Resin was washed first with 20mM Sodium Phosphate, 1M NaCl (pH7.0) and then with 20mM Sodium Phosphate (pH7.0). Antibody was then eluted with 100mM Acetic acid. Recovered product was quantified using an Octet (Forte Bio) protein A assay and is shown in Table 3.

Table 3

	Volume (mL)	Concentration (mg/mL)
Clarified Harvest	350	1.3
Eluted Antibody	50.55	7.7

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- US7071172B [0003]

Patentkrav

1. Fremgangsmåde til produktion af et målpolypeptid, som omfatter:
(a) ekspression af en ekspressionsvektor til ekspression af et målpolypeptid i en CHO-værtscelle, hvilken ekspressionsvektor omfatter en ekspressionskassette, der omfatter et polynukleotid, der koder for det rekombinante polypeptid, der er operabelt koblet til en fibronectinsekretionsledersekvens; og
(b) indvinding af målpolypeptidet.

2. Fremgangsmåde til produktion af et målpolypeptid, som omfatter:
(a) transfektion eller transformation af en CHO-værtscelle med en ekspressionsvektor til ekspression af et målpolypeptid i værtscellen, hvilken ekspressionsvektor omfatter en ekspressionskassette, der omfatter et polynukleotid, der koder for målpolypeptidet, der er operabelt koblet til en fibronectinsekretionsledersekvens;
(b) dyrkning af værtscellen under betingelser, der åbner mulighed for proliferation af værtscellen og ekspression og sekretion af målpolypeptidet fra værtscellen; og
(c) indvinding af målpolypeptidet.

3. Fremgangsmåde ifølge det ene eller det andet af ovennævnte krav, hvor fibronectinsekretionslederen er valgt, så den svarer til værtscellen.

4. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor ekspressionskassetten omfatter en hEF1a-promotor.

5. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor ekspressionskassetten omfatter en polyA-sekvens.

6. Fremgangsmåde ifølge krav 5, hvor polyA-sekvensen er valgt blandt human betaglobin-polyA-, bovin væksthormon-polyA- SV40-tidlig eller sen polyA-sekvens.

7. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor fibronectinsekretionslederen har sekvensen MLRGPGPGLLLAVLCLGTAVRCTEA eller en sekvens, der har 90 % sekvensidentitet dermed og bevarer evnen til at secernere målpolypeptidet.

8. Fremgangsmåde ifølge et hvilket som helst af ovennævnte krav, hvor der anvendes to ekspressionskassetter, én ekspressionskassette, der omfatter et polynukleotid, der koder for en let kæde af et monoklonalt antistof, og en anden ekspressionskassette, der omfatter et polynukleotid, der koder for en tung kæde af et monoklonalt antistof.

9. Fremgangsmåde ifølge krav 8, hvor de to ekspressionskassetter omfatter den samme promotor, sekretionsleder og polyA-sekvens.

10. Fremgangsmåde ifølge krav 9, hvor promotoren er en hEF1a-promotor, fibronectinsekretionslederen er fibronectinsekretionsleder fra kinesisk hamster, og polyA-sekvensen er bovin betaglobin-polyA-sekvens.

11. Ovariecelle fra kinesisk hamster, fortrinsvis en CHOK1-, DG44-, DUXKB11- eller CHO-pro3÷-celle, der er transficeret med en ekspressionsvektor, der omfatter en ekspressionskassette, der omfatter et polynukleotid, der koder for et målpolypeptid, der er operabelt koblet til en fibronectinsekretionsledersekvens.

12. Ovariecelle fra kinesisk hamster ifølge krav 11, hvor målpolypeptidet omfatter et monoklonalt antistof.

13. Ovariecelle fra kinesisk hamster ifølge krav 12, hvor der anvendes separate ekspressionskassetter, der omfatter tunge og lette kæder.

14. Ovariecelle fra kinesisk hamster ifølge krav 13, hvor

ekspressionskassetterne er placeret på den samme vektor.

15. Ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 14, hvor hver ekspressionskassette omfatter en promotor for et husholdningsgen, især en hEF1a-promotor, der er operabelt koblet til polynukleotidet, der koder for målpolypeptidet.

16. Ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 15, hvor hver ekspressionskassette omfatter en bovin væksthormon-polyA-sekvens.

17. Ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 16, der yderligere omfatter et dihydrofolatreductasemarkørsystem.

18. Ovariecelle fra kinesisk hamster ifølge krav 17, hvor dihydrofolatreductasemarkørsystemet omfatter en promotor for murin phosphoglyceratkinase.

19. Ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 18, hvor fibronectinsekretionslederen er CHO-fibronectin.

20. Ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 19, hvor fibronectinsekretionslederen har sekvensen MLRGPGPGLLLAVLCLGTAVRCTEA eller en sekvens, der har 90 % sekvensidentitet dermed og bevarer evnen til at secernere målpolypeptidet.

21. Fremgangsmåde til produktion af et polypeptid, som omfatter dyrkning af en ovariecelle fra kinesisk hamster ifølge et hvilket som helst af kravene 11 til 20.

DRAWINGS

Figure 1 Structure of Expression Cassettes