

line generation for clinical hetero-mAb therapeutics (Biotechnol. Prog. 33 (2017) 469-477).

5 WO 2017/184831 allegedly discloses site-specific integration and expression of recombinant proteins in eukaryotic cells, especially methods for improved expression of antibodies including bispecific antibodies in eukaryotic cells, particularly Chinese hamster (*Cricetulus griseus*) cell lines, by employing an expression-enhancing locus. The data in this document is presented in an anonymized way, thus, not allowing a conclusion what has actually been shown.

Summary

10 Herein is reported a recombinant mammalian cell expressing a bivalent, bispecific antibody, especially a bivalent, bispecific antibody with a domain exchange. A bivalent, bispecific antibody is a heteromultimeric polypeptide not naturally expressed by said mammalian cell. More specifically, a bivalent, bispecific antibody is a heteromultimeric protein consisting of four polypeptides or polypeptide chains:
15 one light chain, which is a full length light chain; a further light chain, which is a domain exchanged light chain; one heavy chain, which is a full length heavy chain; and a further heavy chain, which is a domain exchanged heavy chain. To achieve expression of a bivalent, bispecific antibody a recombinant nucleic acid comprising multiple different expression cassettes in a specific and defined sequence has been
20 integrated into the genome of a mammalian cell.

Herein is also reported a method for generating a recombinant mammalian cell expressing bivalent, bispecific antibody and a method for producing bivalent, bispecific antibody using said recombinant mammalian cell.

In one preferred example the bivalent, bispecific antibody comprises

- 25
- a first heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
 - a second heavy chain comprising from N- to C-terminus the first light chain variable domain, a CH1 domain, a hinge region, a CH2 domain
30 and a CH3 domain,
 - a first light chain comprising from N- to C-terminus a second heavy chain variable domain and a CL domain, and

- a second light chain comprising from N- to C- terminus a second light chain variable domain and a CL domain,

5 wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

In one preferred example the bivalent, bispecific antibody comprises

- a first heavy chain comprising from N- to C-terminus a first heavy chain variable domain, a CH1 domain, a hinge region, a CH2 domain and a CH3 domain,
- 10 - a second heavy chain comprising from N- to C-terminus a second heavy chain variable domain, a CL domain, a hinge region, a CH2 domain and a CH3 domain,
- a first light chain comprising from N- to C-terminus a first light chain variable domain and a CH1 domain, and
- 15 - a second light chain comprising from N- to C- terminus a second light chain variable domain and a CL domain,

wherein the first heavy chain variable domain and the second light chain variable domain form a first binding site and the second heavy chain variable domain and the first light chain variable domain form a second binding site.

20 The current disclosure is based, at least in part, on the finding that the sequence of the different expression cassettes required for the expression of the heteromultimeric bivalent, bispecific antibody, i.e. the expression cassette organization, as integrated into the genome of a mammalian cell influences the expression yield of bivalent, bispecific antibody.

25 The current disclosure is based, at least in part, on the finding that by integrating a nucleic acid encoding the heteromultimeric bivalent, bispecific antibody that has a specific expression cassette organization into the genome of a mammalian cell efficient recombinant expression and production of the bivalent, bispecific antibody can be achieved.

It has been found that the defined expression cassette sequence can advantageously be integrated into the genome of a mammalian cell by a double recombinase mediated cassette exchange reaction.

The current disclosure provides a method for producing bivalent, bispecific antibody comprising the steps of

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- a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding bivalent, bispecific antibody optionally under conditions suitable for the expression of bivalent, bispecific antibody, and
- b) recovering bivalent, bispecific antibody from the cell or the cultivation medium,

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wherein the deoxyribonucleic acid encoding bivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'- direction

either (1)

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- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

or (2)

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- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the first heavy chain.

One aspect according to the current disclosure is a method for producing a bivalent, bispecific antibody comprising the steps of

25

- a) cultivating a mammalian cell comprising a deoxyribonucleic acid encoding the bivalent, bispecific antibody, and
- b) recovering the bivalent, bispecific antibody from the cell or the cultivation medium,

wherein the deoxyribonucleic acid encoding the bivalent, bispecific antibody is stably integrated into the genome of the mammalian cell and comprises in 5'- to 3'-direction

- 5
- a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the first heavy chain,
 - a third expression cassette encoding the second light chain, and
 - a fourth expression cassette encoding the second heavy chain,

10 wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

In one example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

The current disclosure provides a deoxyribonucleic acid encoding bivalent, bispecific antibody comprising in 5'- to 3'-direction

- 15 either (1)
- a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the first heavy chain,
 - a third expression cassette encoding the second light chain, and
 - a fourth expression cassette encoding the second heavy chain,
- 20 or (2)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the first heavy chain.

5 Another aspect of the current disclosure is a deoxyribonucleic acid encoding a bivalent, bispecific antibody comprising in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- 10 - a fourth expression cassette encoding the second heavy chain,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat). The current disclosure provides the use of a deoxyribonucleic acid comprising in 5'-
15 to 3'-direction

either (1)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- 20 - a fourth expression cassette encoding the second heavy chain,

or (2)

- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the second heavy chain,
- a third expression cassette encoding the second light chain, and
- 25 - a fourth expression cassette encoding the first heavy chain,

for the expression of bivalent, bispecific antibody in a mammalian cell.

Another aspect of the current disclosure is the use of a deoxyribonucleic acid comprising in 5'- to 3'-direction

- a first expression cassette encoding the first light chain,
- 30 - a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

for the expression of the bivalent, bispecific antibody in a mammalian cell,

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

5 In one example of the use the deoxyribonucleic acid is integrated into the genome of the mammalian cell.

In one example exactly one copy of the use the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

10 The current disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding bivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding bivalent, bispecific antibody comprises in 5'- to 3'-direction

either (1)

- 15
- a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the first heavy chain,
 - a third expression cassette encoding the second light chain, and
 - a fourth expression cassette encoding the second heavy chain,

or (2)

- 20
- a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the second heavy chain,

- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the first heavy chain.

Another aspect of the current disclosure is a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a bivalent, bispecific antibody integrated in the genome of the cell, wherein the deoxyribonucleic acid encoding the bivalent, bispecific antibody comprises in 5' - to 3'-direction

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- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain,
- a third expression cassette encoding the second light chain, and
- a fourth expression cassette encoding the second heavy chain,

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wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

In one example exactly one copy of the deoxyribonucleic acid is stably integrated into the genome of the mammalian cell at a single site or locus.

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In one example of the above the deoxyribonucleic acid encoding bivalent, bispecific antibody further comprises

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- a first recombination recognition sequence located 5' to the first (most 5') expression cassette,
- a second recombination recognition sequence located 3' to the fourth (most 3') expression cassette, and
- a third recombination recognition sequence located
 - between the first and the second recombination recognition sequence, and
 - between two of the expression cassettes,

25

and

wherein all recombination recognition sequences are different.

In one example the third recombination recognition sequence is located between the second and the third expression cassette.

5 In one example the deoxyribonucleic acid encoding bivalent, bispecific antibody comprises a further expression cassette encoding for a selection marker and the expression cassette encoding for the selection marker is located partly 5' and partly 3' to the third recombination recognition sequence, wherein the 5'-located part of said expression cassette comprises the promoter and the start-codon and the 3'-located part of said expression cassette comprises the coding sequence without a
10 start-codon and a polyA signal, wherein the start-codon is operably linked to the coding sequence.

The current disclosure provides a composition comprising two deoxyribonucleic acids, which comprise in turn three different recombination recognition sequences and eight expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,
either (1)

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

or (2)

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the second heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction
either (1)

- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the first heavy chain, and
- a second recombination recognition sequence.

Another aspect of the current disclosure is a composition comprising two
deoxyribonucleic acids, which comprise in turn three different recombination
recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5' - to 3'-direction
 - a second copy of the third recombination recognition sequence,
 - a third expression cassette encoding the second light chain,
 - a fourth expression cassette encoding the second heavy chain, and
 - a second recombination recognition sequence,

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wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat).

10 In one example the first and the second deoxyribonucleic acid both comprises the organization according to (1); or the first and the second deoxyribonucleic acid both comprises the organization according to (2).

In one example of the above the deoxyribonucleic acid encoding bivalent, bispecific antibody further comprises a further expression cassette encoding for a selection marker.

15 In one example the expression cassette encoding for a selection marker is located either

i) 5', or

ii) 3', or

iii) partly 5' and partly 3'

to the third recombination recognition sequence.

5 In one example the expression cassette encoding for a selection marker is located partly 5' and partly 3' to the third recombination recognition sequences, wherein the 5'-located part of said expression cassette comprises the promoter and a start-codon and the 3'-located part of said expression cassette comprises the coding sequence without a start-codon and a polyA signal.

10 In one example the 5'-located part of the expression cassette encoding the selection marker comprises a promoter sequence operably linked to a start-codon, whereby the promoter sequence is flanked upstream by (i.e. is positioned downstream to) the second expression cassette and the start-codon is flanked downstream by (i.e. is positioned upstream of) the third recombination recognition sequence; and the 3'-located part of the expression cassette encoding the selection marker comprises a nucleic acid encoding the selection marker lacking a start-codon and is flanked
15 upstream by the third recombination recognition sequence and downstream by the third expression cassette.

In one example the start-codon is a translation start-codon. In one example the start-codon is ATG.

20 The current disclosure provides a recombinant mammalian cell comprising a deoxyribonucleic acid encoding bivalent, bispecific antibody integrated in the genome of the cell,

wherein the deoxyribonucleic acid encoding bivalent, bispecific antibody comprises the following elements:
a first, a second and a third recombination recognition sequence,
25 a first and a second selection marker, and
a first to fourth expression cassette,

wherein the sequences of said elements in 5'-to-3' direction is

RRS1-1st EC-2nd EC-RRS3-SM1-3rd EC-4th EC-RRS2

with

RRS = recombination recognition sequence,

EC = expression cassette,

SM = selection marker.

The current disclosure provides a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding bivalent, bispecific antibody and secreting bivalent, bispecific antibody comprising the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction, either (1)

- a first recombination recognition sequence,
 - a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the first heavy chain, and
 - a first copy of a third recombination recognition sequence,
- or (2)

- a first recombination recognition sequence,
 - a first expression cassette encoding the first light chain,
 - a second expression cassette encoding the second heavy chain, and
 - a first copy of a third recombination recognition sequence,
- and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction either (1)

- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a second recombination recognition sequence,

or (2)

- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the first heavy chain, and
- a second recombination recognition sequence,

5 wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

10 wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b); or

15 ii) sequentially thereafter

one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid;

20 (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting bivalent, bispecific antibody,

25 thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding bivalent, bispecific antibody and secreting bivalent, bispecific antibody.

Another aspect of the current disclosure is a method for producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding a bivalent, bispecific antibody and secreting the bivalent, bispecific antibody, comprising

30 the following steps:

a) providing a mammalian cell comprising an exogenous nucleotide sequence integrated at a single site within a locus of the genome of the mammalian cell, wherein the exogenous nucleotide sequence comprises a first and a second recombination recognition sequence flanking at least one first selection marker, and a third recombination recognition sequence located between the first and the second recombination recognition sequence, and all the recombination recognition sequences are different;

b) introducing into the cell provided in a) a composition of two deoxyribonucleic acids comprising three different recombination recognition sequences and four expression cassettes, wherein

- the first deoxyribonucleic acid comprises in 5'- to 3'-direction,

- a first recombination recognition sequence,
- a first expression cassette encoding the first light chain,
- a second expression cassette encoding the first heavy chain, and
- a first copy of a third recombination recognition sequence,

and

- the second deoxyribonucleic acid comprises in 5'- to 3'-direction

- a second copy of the third recombination recognition sequence,
- a third expression cassette encoding the second light chain,
- a fourth expression cassette encoding the second heavy chain, and
- a second recombination recognition sequence,

wherein the first to third recombination recognition sequences of the first and second deoxyribonucleic acids are matching the first to third recombination recognition sequence on the integrated exogenous nucleotide sequence,

wherein the 5'-terminal part and the 3'-terminal part of the expression cassette encoding the one second selection marker when taken together form a functional expression cassette of the one second selection marker;

wherein the first heavy chain comprises in the CH3 domain the mutation T366W (numbering according to Kabat) and the second heavy chain comprises in the CH3 domain the mutations T366S, L368A, and Y407V (numbering according to Kabat);

c) introducing

i) either simultaneously with the first and second deoxyribonucleic acid of b);

or

ii) sequentially thereafter

one or more recombinases,

wherein the one or more recombinases recognize the recombination recognition sequences of the first and the second deoxyribonucleic acid; (and optionally wherein the one or more recombinases perform two recombinase mediated cassette exchanges;)

and

d) selecting for cells expressing the second selection marker and secreting the bivalent, bispecific antibody;

thereby producing a recombinant mammalian cell comprising a deoxyribonucleic acid encoding the bivalent, bispecific antibody and secreting the bivalent, bispecific antibody.

In one example the first and the second deoxyribonucleic acid both comprises the organization according to (1); or the first and the second deoxyribonucleic acid both comprises the organization according to (2).

In one example the expression cassette encoding the one second selection marker is located partly 5' and partly 3' to the third recombination recognition sequences, wherein the 5'-located part of said expression cassette comprises the promoter and the start-codon and said 3'-located part of the expression cassette comprises the

coding sequence of the one second selection marker without a start-codon and a polyA signal.

5 In one example the 5'-terminal part of the expression cassette encoding the one second selection marker comprises a promoter sequence operably linked to the start-codon, whereby the promoter sequence is flanked upstream by (i.e. is positioned downstream to) the second expression cassette and the start-codon is flanked downstream by (i.e. is positioned upstream of) the third recombination recognition sequence; and the 3'-terminal part of the expression cassette encoding the one second selection marker comprises the coding sequence of the one second selection marker
10 lacking a start-codon flanked upstream by the third recombination recognition sequence and downstream by the third expression cassette.

In one example the start-codon is a translation start-codon. In one example the start-codon is ATG.

15 In one example of the above the first deoxyribonucleic acid is integrated into a first vector and the second deoxyribonucleic acid is integrated into a second vector.

In one example of the above each of the expression cassettes comprise in 5'-to-3' direction a promoter, a coding sequence and a polyadenylation signal sequence optionally followed by a terminator sequence.

In one example of the above

20 each expression cassette for an antibody chain comprises in 5'-to-3' direction a promoter, a nucleic acid encoding an antibody chain, and a polyadenylation signal sequence and optionally a terminator sequence
and

25 each expression cassette encoding the selection marker comprises in 5'-to-3' direction a promoter, a nucleic acid encoding the selection marker, and a polyadenylation signal sequence and optionally a terminator sequence.

In one example of the above the promoter is the human CMV promoter with or without intron A, the polyadenylation signal sequence is the bGH polyA site and the terminator is the hGT terminator.

5 A terminator sequence prevents the generation of very long RNA transcripts by RNA polymerase II, i.e. the read-through into the next expression cassette in the deoxyribonucleic acid according to the disclosure and used in the methods according to the disclosure. That is, the expression of one structural gene of interest is controlled by its own promoter.

10 Thus, by the combination of a polyadenylation signal and a terminator sequence efficient transcription termination is achieved. That is, read-through of the RNA polymerase II is prevented by the presence of double termination signals. The terminator sequence initiated complex resolution and promotes dissociation of RNA polymerase from the DNA template.

15 In one example of the above the promoter is the human CMV promoter with intron A, the polyadenylation signal sequence is the bGH polyadenylation signal sequence and the terminator is the hGT terminator except for the expression cassette of the selection marker, wherein the promoter is the SV40 promoter and the polyadenylation signal sequence is the SV40 polyadenylation signal sequence and a terminator is absent.

In one example of the above the mammalian cell is a CHO cell. In one example the CHO cell is a CHO-K1 cell.

20 In one example of the above the bivalent, bispecific antibody is an anti-ANG2/VEGF bispecific antibody. In one example the bispecific anti-ANG2/VEGF antibody is RG7221 or vanucizumab.

In one example of the above the bivalent, bispecific antibody is an anti-ANG2/VEGF bispecific antibody. In one example the bispecific anti-ANG2/VEGF antibody is RG7716 or faricimab.

25 Such an ANG2/VEGF bispecific antibodies are reported in WO 2010/040508, WO 2011/117329, WO 2014/009465, which are incorporated herein by reference in its entirety.

30 In one example of the above the bivalent, bispecific antibody is an anti-PD1/TIM3 bispecific antibody. Such an antibody is reported in WO 2017/055404, which is incorporated herein by reference in its entirety.

In one example of the above the bivalent, bispecific antibody is an anti-PD1/Lag3 bispecific antibody. Such an antibody is reported in WO 2018/185043, which is incorporated herein by reference in its entirety.

5 In one example of the above none of the first light chain and the second light chain of the bivalent, bispecific antibody is a common light chain or a universal light chain.

In one example of the above the second heavy chain variable domain and the first light chain variable domain form a first binding site and the first heavy chain variable domain and the second light chain variable domain form a second binding site.

10 In one preferred example of the above exactly two deoxyribonucleic acids are comprised or introduced.

The individual expression cassettes in the deoxyribonucleic acid according to the disclosure are arranged sequentially. The distance between the end of one expression cassette and the start of the thereafter following expression cassette is only a few nucleotides, which were required for, i.e. result from, the cloning procedure.

15 In one example of the above two directly following expression cassettes are spaced at most 100 bps apart (i.e. from the end of the poly A signal sequence or the terminator sequence, respectively, until the start of the following promoter element are at most 100 base pairs (bps)). In one example two directly following expression cassettes are spaced at most 50 bps apart. In one preferred example two directly
20 following expression cassettes are spaced at most 30 bps apart.

Detailed Description of Embodiments of the Invention

25 The current invention is based, at least in part, on the finding that for the expression of a bivalent, bispecific antibody, which is a complex molecule comprising different polypeptides, i.e. which is a heteromultimer, the use of a defined and specific expression cassette organization results in efficient expression and production of the bivalent, bispecific antibody in mammalian cells, such as CHO cells.

The current invention is based, at least in part, on the finding that double recombinase mediated cassette exchange (RMCE) can be used for producing a recombinant mammalian cell, such as a recombinant CHO cell, in which a defined and specific