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(54) **CONSTRUCT AND SEQUENCE FOR ENHANCED GENE EXPRESSION**

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See application file for complete search history.

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(57) **ABSTRACT**

The invention relates to a method for transcription and expression using a nucleic acid construct which is characterized by the presence of a promoter followed by an intronic promoter. The invention further relates to a nucleic acid construct, an expression vector and a cell comprising the construct, and its use. The invention also relates to methods for transcription and optionally expression using a nucleotide sequence. The invention further relates to a nucleotide sequence and a construct, expression vector and cell comprising said nucleotide sequence, and its use.

14 Claims, 11 Drawing Sheets

Specification includes a Sequence Listing.

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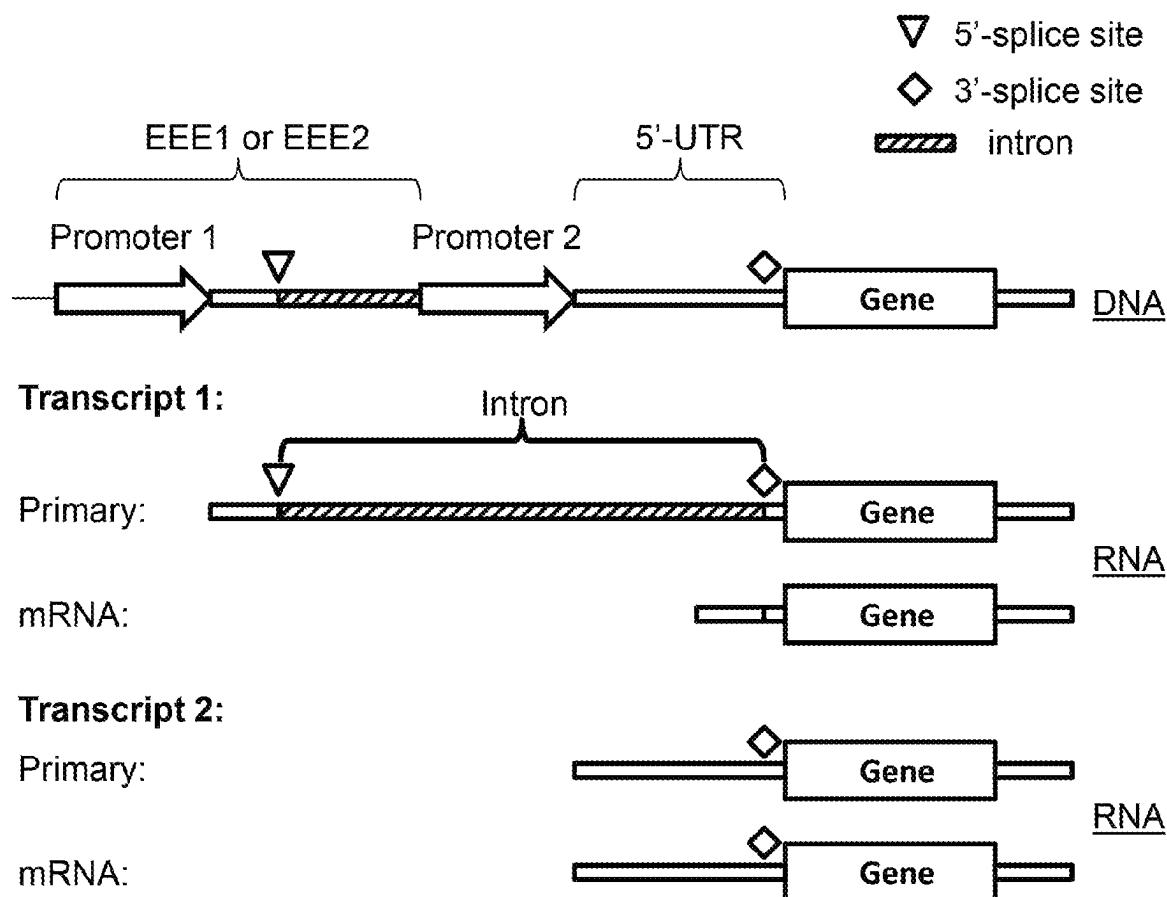
Fig. 1

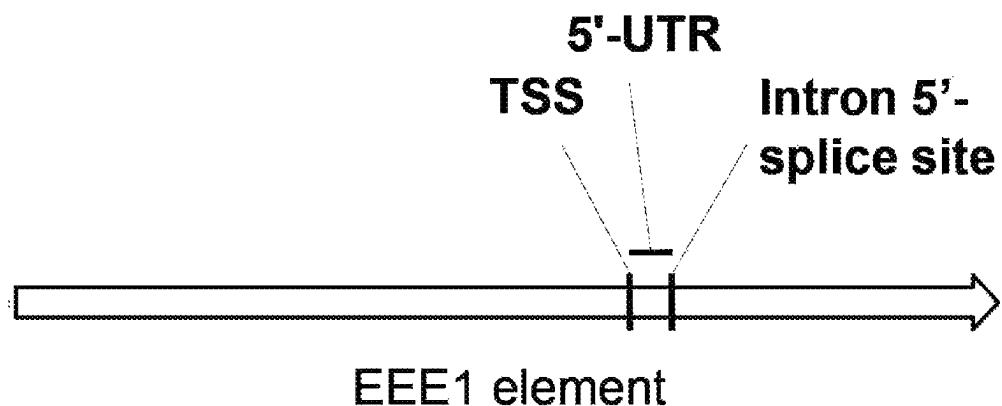
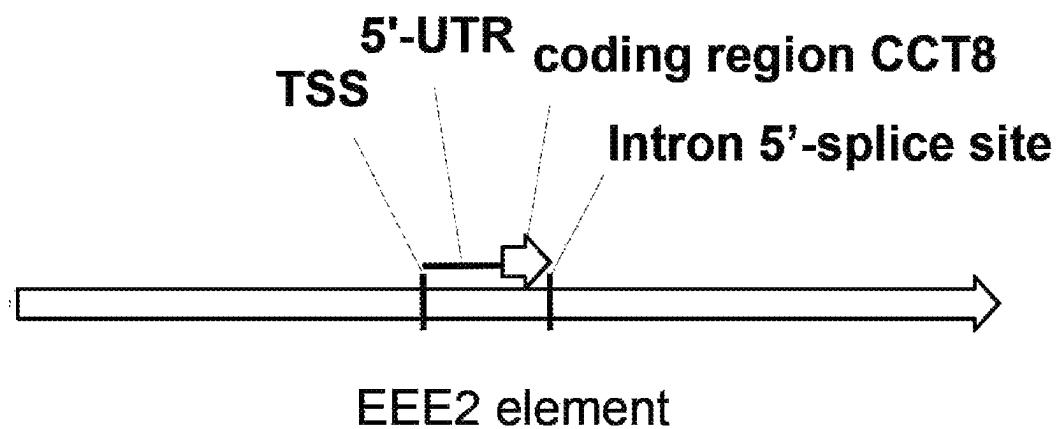
Fig. 2a*Fig. 2b*

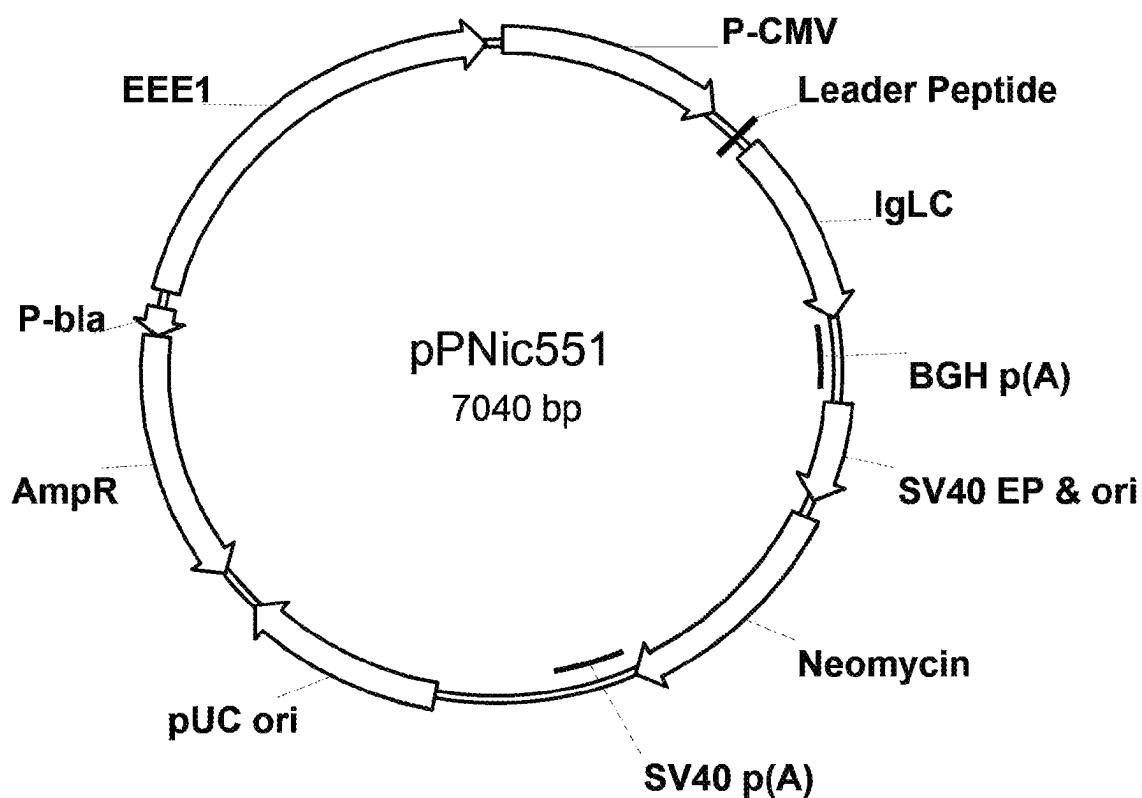
Fig. 3

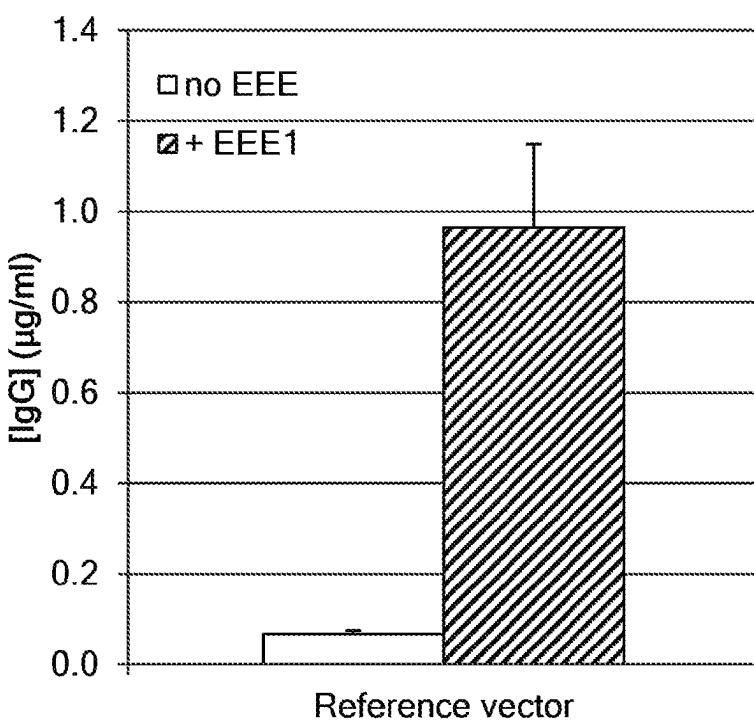
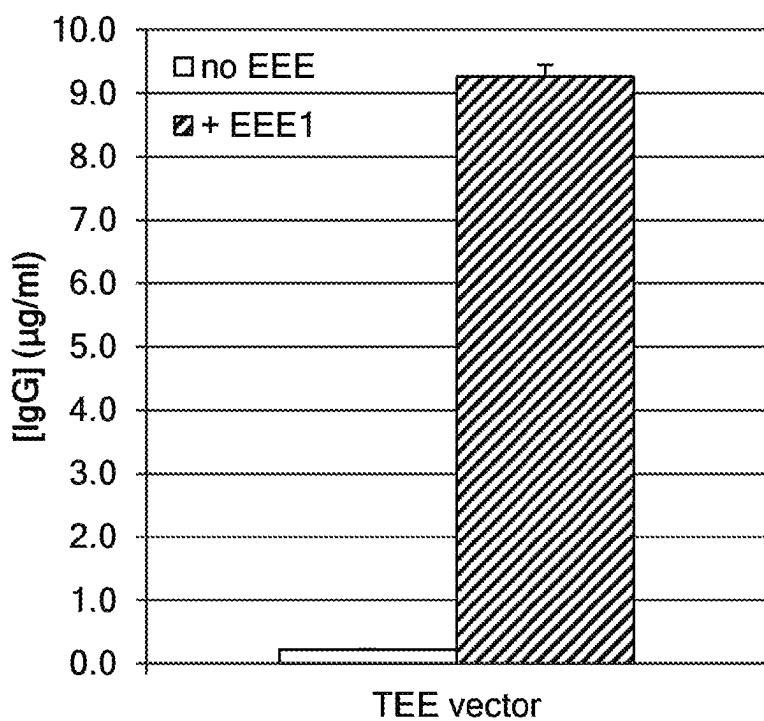
Fig. 4a*Fig. 4b*

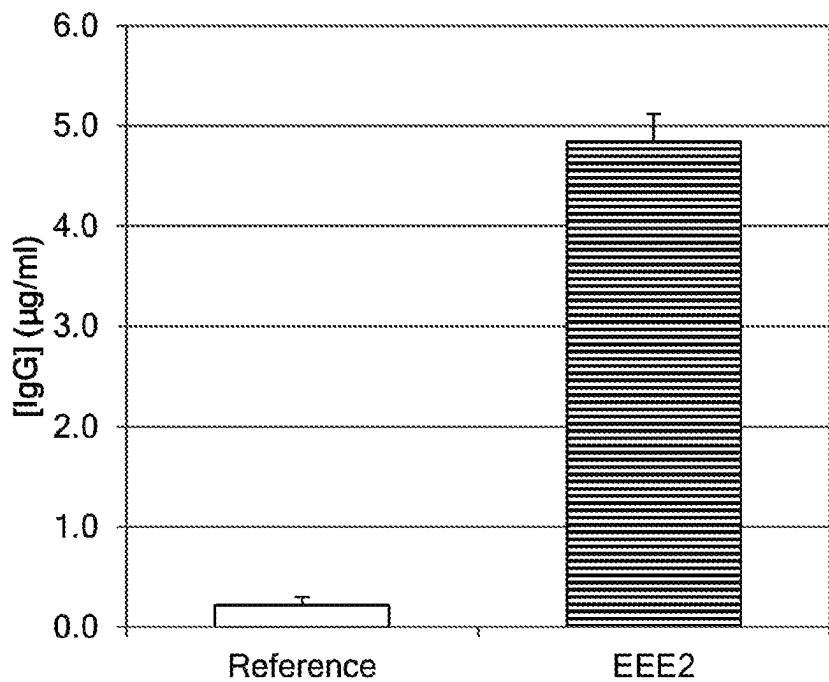
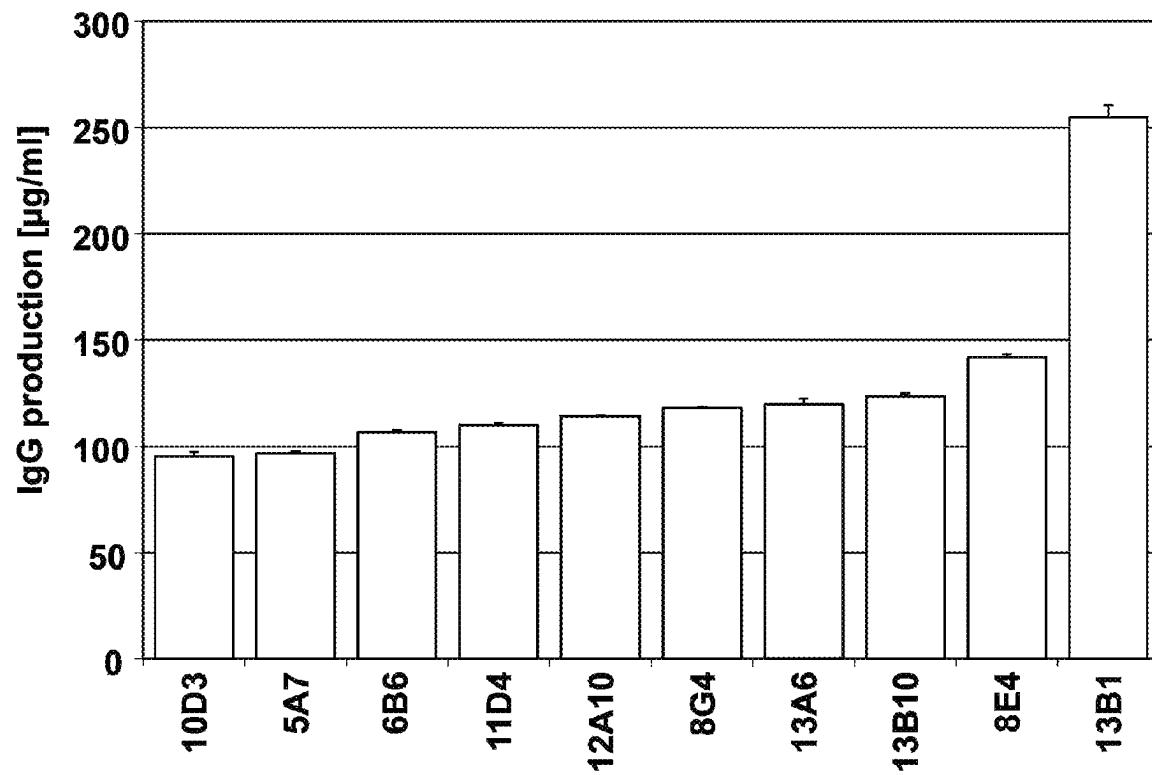
Fig. 5*Fig. 6*

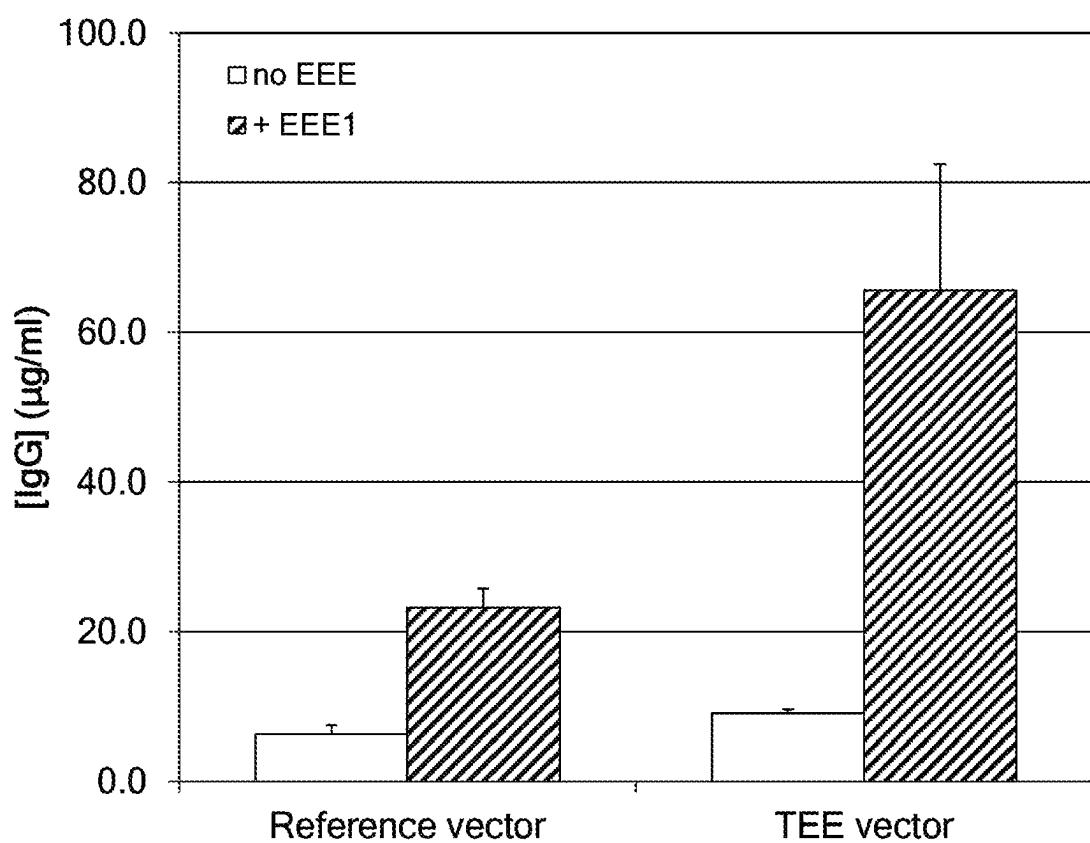
Fig. 7a

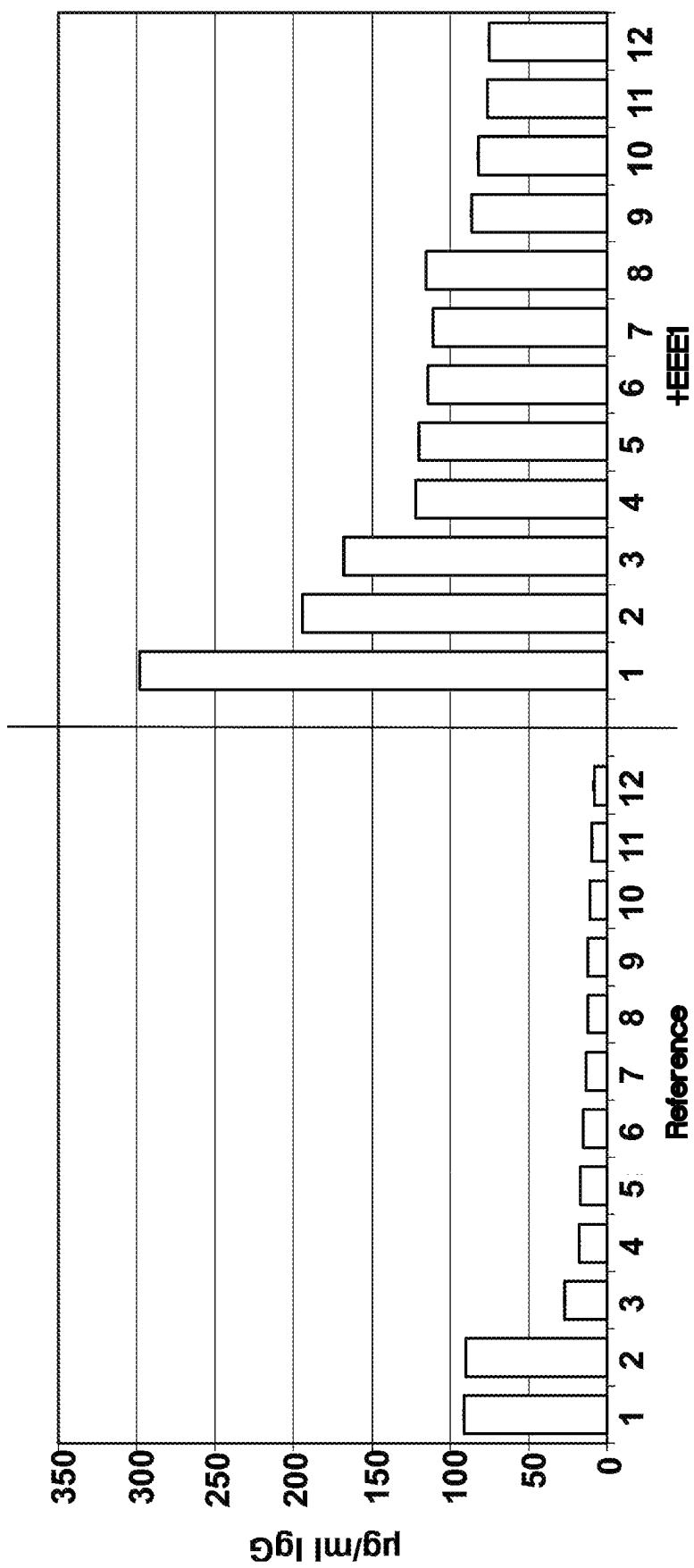
Fig. 7b

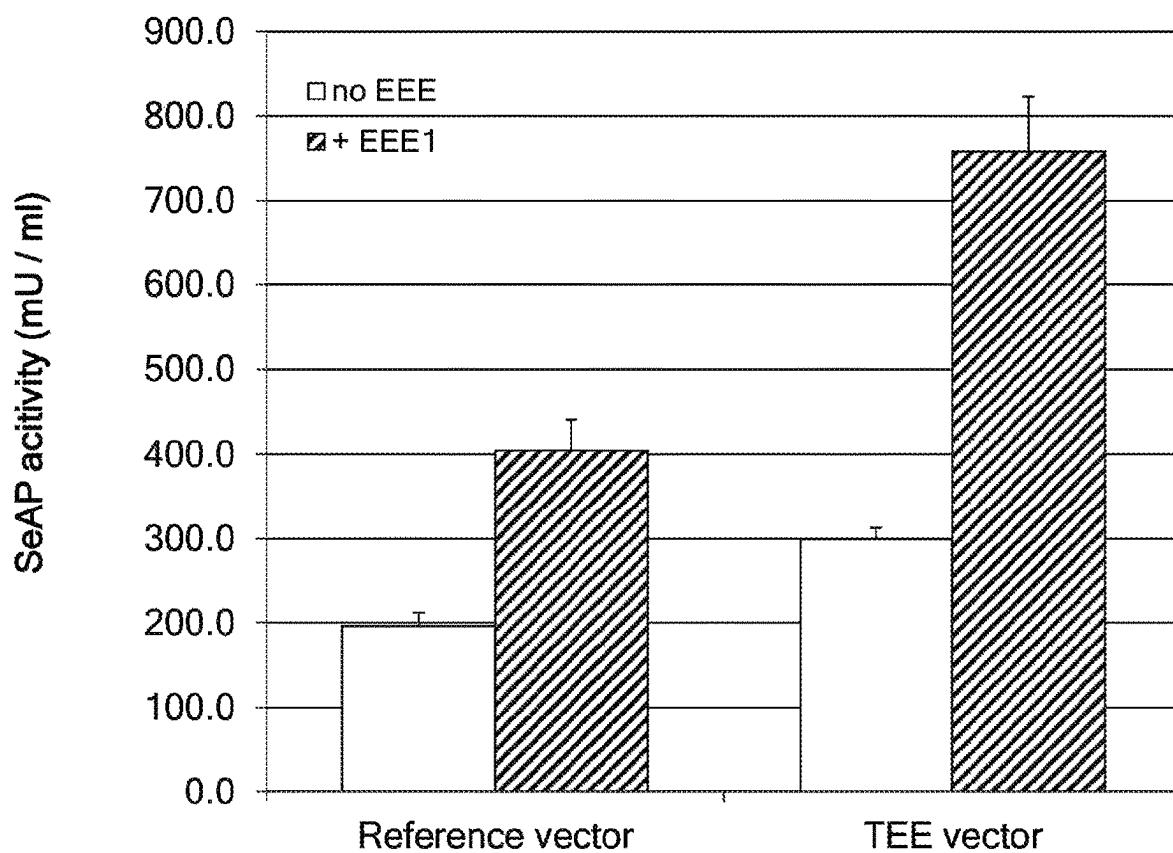
Fig. 8

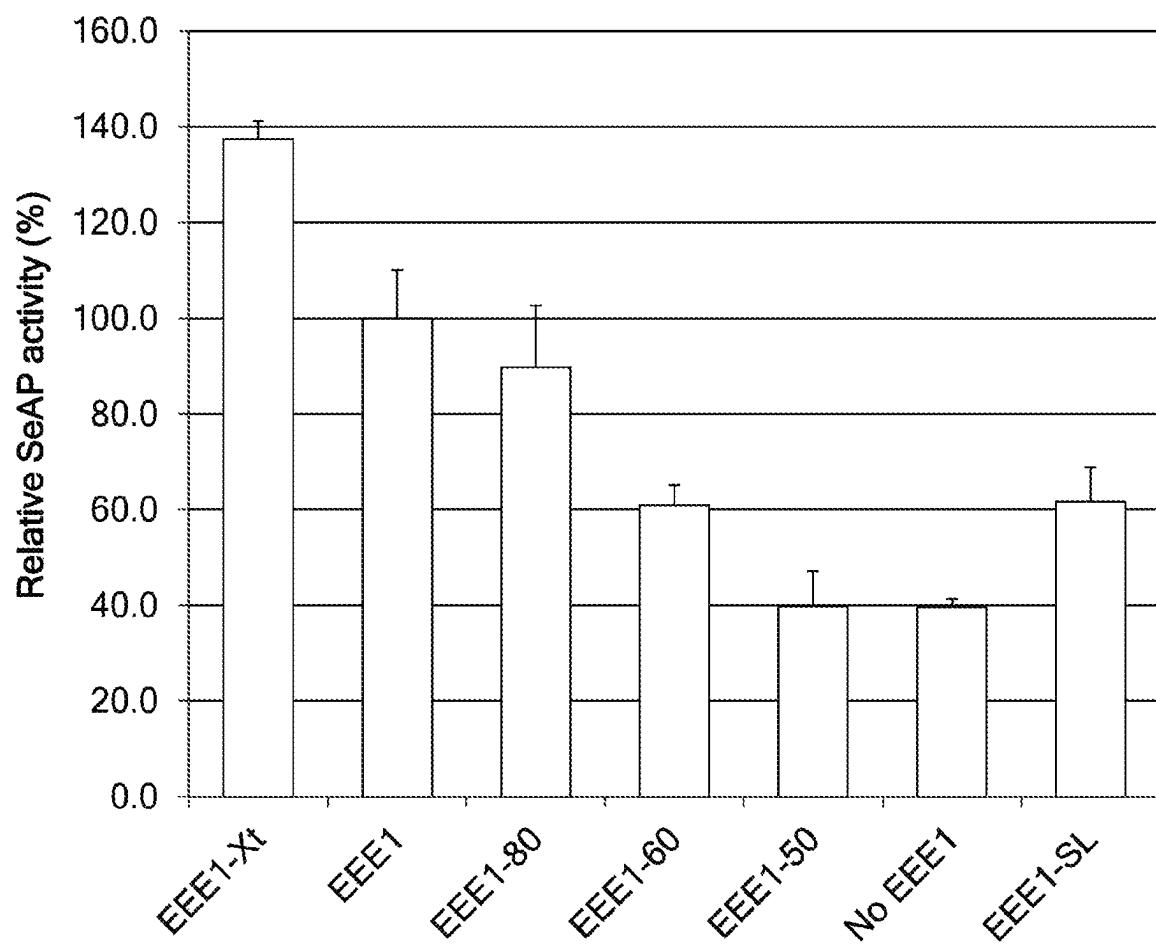
Fig. 9

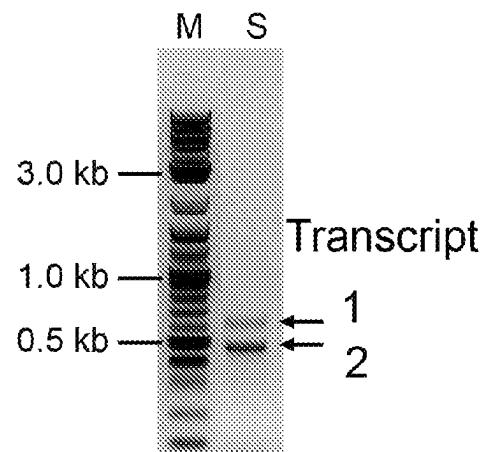
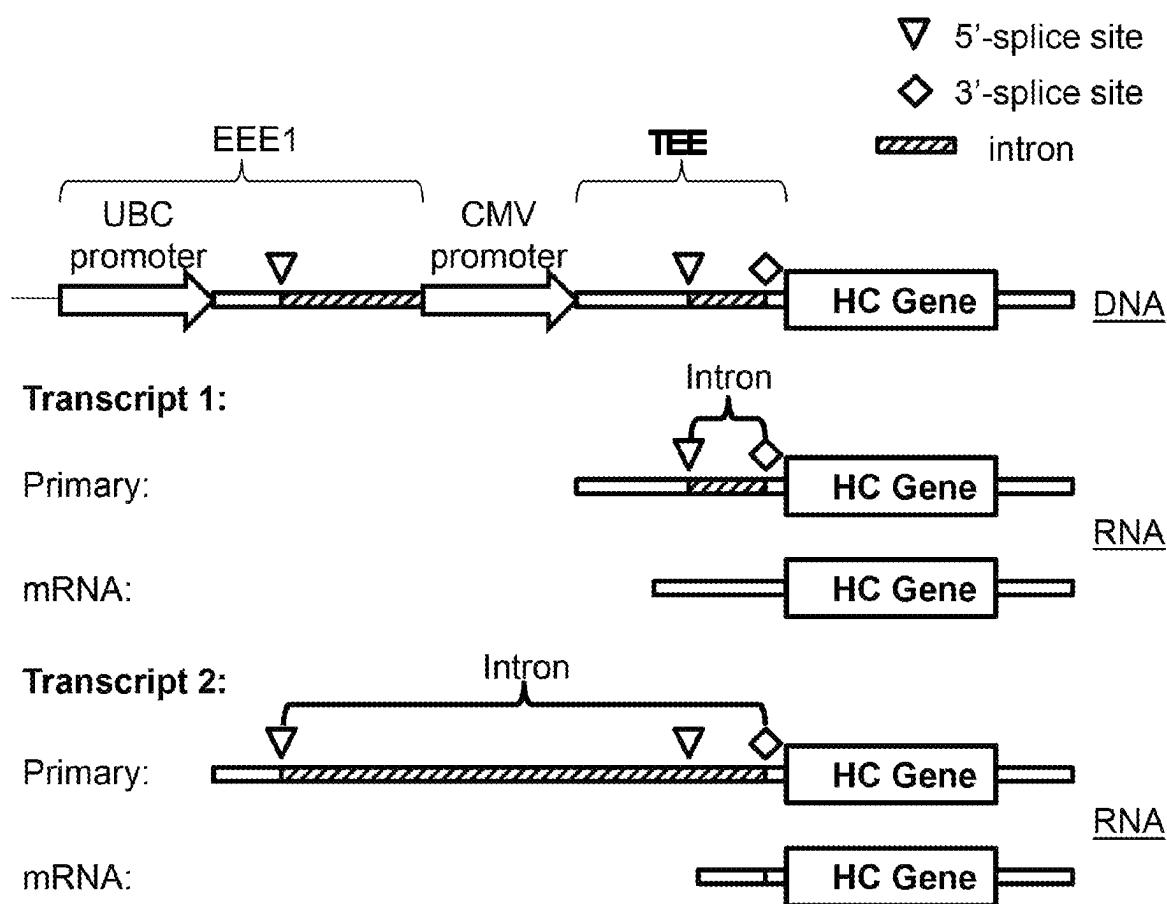
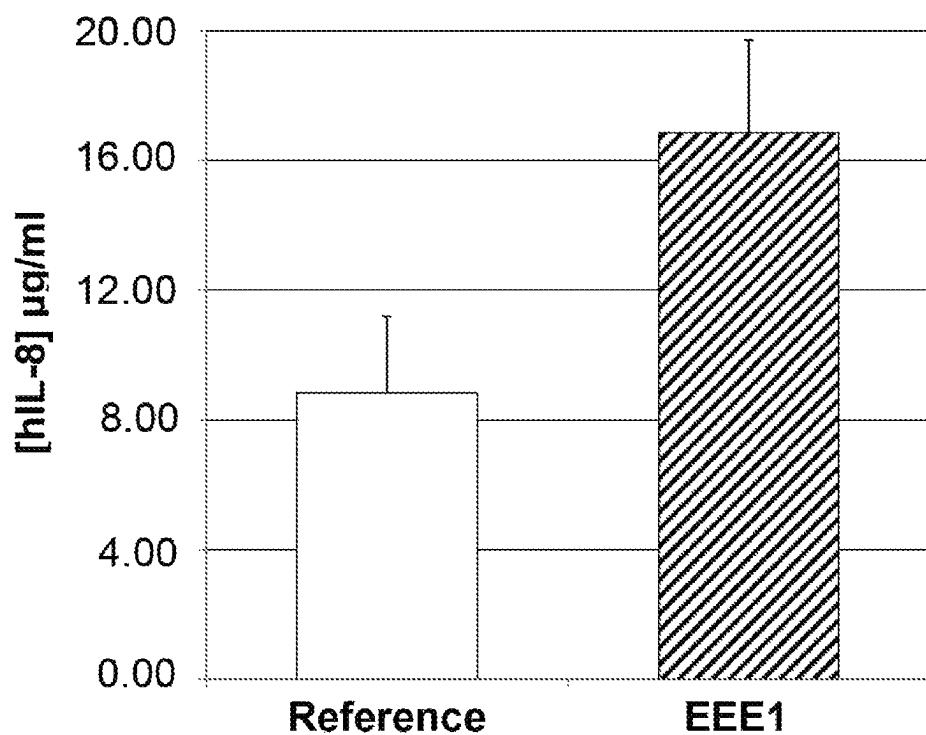
Fig. 10a*Fig. 10b*

Fig. 11

1**CONSTRUCT AND SEQUENCE FOR
ENHANCED GENE EXPRESSION****CROSS-REFERENCE TO RELATED
APPLICATIONS**

This application is a continuation of U.S. patent application Ser. No. 17/146,170, filed on Jan. 11, 2021, which is a continuation of U.S. patent application Ser. No. 15/109,301, filed on Jun. 30, 2015, now U.S. Pat. No. 10,889,822, which is a national stage application under 35 U.S.C. § 371 of International Patent Application No. PCT/NL2014/050907, filed on Dec. 24, 2014, which claims the benefit of European Patent Application No. 13199873.4, filed on Dec. 31, 2013, and European Patent Application No. 13199875.9, filed on Dec. 31, 2013, all of which are hereby incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention relates to a method for transcription and expression using a nucleic acid construct which is characterized by the presence of a promoter followed by an intronic promoter. The invention further relates to said nucleic acid construct, an expression vector and a cell comprising said construct, and its use.

The invention also relates to methods for transcription and optionally expression using a nucleotide sequence. The invention further relates to said nucleotide sequence and a construct, expression vector and cell comprising said nucleotide sequence, and its use.

BACKGROUND OF THE INVENTION

There is still a need in the art for alternative and preferably improved methods for regulating the transcription of a transcript and optionally regulating the expression of a protein or polypeptide of interest in host cells.

SUMMARY OF THE INVENTION

The present invention relates to a method for transcription and optionally purifying the produced transcript comprising the steps of:

- a) providing a nucleic acid construct comprising a first promoter, a second promoter, and a nucleotide sequence of interest, wherein said first and said second promoters are operably linked to said nucleotide sequence of interest, and wherein said second promoter is flanked by a first intronic sequence located upstream of said promoter and a second intronic sequence located downstream of said promoter; and,
- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- d) purifying said produced transcript.

The present invention further relates to a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- a) providing a nucleic acid construct comprising a first promoter, a second promoter and a nucleotide sequence encoding a protein or polypeptide of interest, wherein said first and said second promoters are operably linked to said nucleotide sequence encoding a protein or polypeptide of interest, and wherein said second promoter is flanked by a first intronic sequence located

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upstream of said promoter and a second intronic sequence located downstream of said promoter; and,

- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to express the protein or polypeptide of interest; and optionally, purifying said protein or polypeptide of interest.

Preferably, said first intronic sequence comprises at least a donor splice site and said second intronic sequence comprises at least an acceptor splice site. Moreover, the nucleic acid construct of step a) of the method of the invention comprises the following nucleotide sequences indicated here in their relative positions in the 5' to 3' direction: (i) a first promoter (ii) a first intronic sequence comprising at least a donor splice site, (iii) a second promoter, (iv) a second intronic sequence comprising at least an acceptor splice site; and (v) a nucleotide sequence encoding a protein or polypeptide of interest, wherein preferably said first promoter, said first intronic sequence comprising at least a donor splice site, said second promoter, and said second intronic sequence comprising at least an acceptor splice site are all operably linked to said nucleotide sequence encoding a protein or polypeptide of interest.

Preferably, said first promoter has at least 50% identity to nucleotides 1-969 of SEQ ID NO: 1 or nucleotides 1-614 of SEQ ID NO: 2 over its whole length. An overview of all SEQ ID NOs is given in Table 1. Preferably, a nucleotide sequence comprising both said first promoter and said first intronic sequence comprising at least a donor splice site has at least 50% identity to SEQ ID NO: 1 or 2 over its whole length. Preferably, said second promoter has at least 50% sequence identity with SEQ ID NO: 57 or SEQ ID NO: 58 over its whole length.

The present invention further relates to a nucleic acid construct comprising a first promoter and a second promoter, wherein said first and said second promoters are configured to be both operably linked to an optional nucleotide sequence of interest, and wherein said second promoter is flanked by a first intronic sequence located upstream of said promoter and a second intronic sequence located downstream of said promoter. Preferably, said first intronic sequence comprises at least a donor splice site and preferably said second intronic sequence comprises at least an acceptor splice site. Moreover, preferably a nucleic acid construct of the invention comprises the following nucleotide sequences indicated here in their relative positions in the 5' to 3' direction: (i) a first promoter (ii) a first intronic sequence comprising at least a donor splice site, (iii) a second promoter, (iv) a second intronic sequence comprising at least an acceptor splice site; and optionally (v) a nucleotide sequence of interest, wherein preferably said first promoter, said first intronic sequence comprising at least a donor splice site, said second promoter, and said second intronic sequence comprising at least an acceptor splice site are all configured to be operably linked to said optional nucleotide sequence of interest.

Preferably, said first promoter has at least 50% identity to nucleotides 1-969 of SEQ ID NO: 1 or nucleotides 1-614 of SEQ ID NO: 2 over its whole length. Preferably, a nucleotide sequence comprising both said first promoter and said first intronic sequence comprising at least a donor splice site has at least 50% identity to SEQ ID NO: 1 or 2 over its whole length. Preferably, said second promoter has at least 50% sequence identity with SEQ ID NO: 57 or SEQ ID NO: 58 over its whole length.

Preferably, said nucleic acid construct is an isolated construct. Preferably, said nucleic acid construct is a recom-

binant nucleic acid construct. Preferably, said optional nucleotide sequence of interest is a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a heterologous protein or polypeptide.

The present invention further relates to an expression vector comprising a nucleic acid construct or recombinant nucleic acid construct as defined herein.

The present invention further relates to a cell comprising a nucleic acid construct or recombinant nucleic acid construct as defined herein, and/or an expression vector as defined herein.

The present invention also relates to a use of a nucleic acid construct or recombinant nucleic acid construct as defined herein, and/or an expression vector as defined herein and/or a cell as defined herein for the transcription of a nucleotide sequence of interest.

The present invention further relates to a use of a nucleic acid construct or recombinant nucleic acid construct as defined herein, and/or an expression vector as defined herein and/or a cell as defined herein for the expression of a protein or polypeptide of interest.

The present invention further relates to a method for transcription and optionally purifying the produced transcript comprising the step of:

- providing a nucleic acid construct comprising an expression enhancing element, a heterologous promoter and a nucleotide sequence of interest of the invention, wherein said expression enhancing element and said heterologous promoter are operably linked to said nucleotide sequence of interest; and,
- contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- purifying said produced transcript.

The present invention further relates to a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- providing a nucleic acid construct comprising an expression enhancing element, a heterologous promoter and a nucleotide sequence encoding a protein or polypeptide of interest, wherein said expression enhancing element and said heterologous promoter are operably linked to said nucleotide sequence encoding a protein or polypeptide of interest; and,
- contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- purifying said protein or polypeptide of interest.

Preferably, said nucleic acid construct of said method for transcription and/or expression and optionally purifying a transcript and/or protein or polypeptide of interest further comprises an additional expression regulating element operably linked to said nucleotide sequence of interest and/or said nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said additional expression regulating element comprises an intronic sequence. A preferred additional expression regulating element comprises or is an additional expression enhancing element. More preferably, said additional expression regulating element further comprises a translation enhancing element.

The present invention further relates to a nucleic acid molecule that is represented by a nucleotide sequence comprising an expression enhancing element of the invention, i.e. a nucleotide sequence that has at least 50% identity to

SEQ ID NO: 1 or 2 over its whole length. An overview of all SEQ ID NOs is given in Table 1. Preferably, said nucleic acid molecule is an isolated nucleic acid molecule. Preferably, said nucleic acid molecule or isolated nucleic acid molecule is represented by a nucleotide sequence that has at least 50% sequence identity to SEQ ID NO: 1 or 2 over its whole length. Preferably, said nucleic acid molecule or isolated nucleic acid molecule is represented by a nucleotide sequence comprising a sequence derived from the *Cricetulus griseus* gene for polyubiquitin of at most 8000 nucleotides. The present invention further relates to a nucleic acid construct comprising a nucleic acid molecule of the invention. Preferably, said nucleic acid construct is represented by a nucleotide sequence that further comprises a heterologous promoter, wherein preferably said expression enhancing element and said heterologous promoter are configured to be both operably linked to an optional nucleotide sequence of interest. Preferably, said nucleic acid construct further comprises an additional expression regulating element, wherein preferably said expression enhancing element, said heterologous promoter and said additional expression regulating element are configured to be all operably linked to said optional nucleotide sequence of interest. Preferably, said additional expression regulating element further comprises a translation enhancing element. Preferably, said additional expression regulating element comprises an intronic sequence. Preferably, said optional nucleotide sequence of interest is a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a heterologous protein or polypeptide.

Preferably, said nucleic acid construct is a recombinant and/or isolated nucleic acid construct.

The present invention further relates to an expression vector comprising a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or recombinant nucleic acid construct as defined herein, and/or an expression vector as defined herein.

The present invention further relates to a cell comprising a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or recombinant nucleic acid construct as defined herein, and/or an expression vector as defined herein.

The present invention also relates to a use of a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant and/or isolated nucleic acid construct as defined herein, and/or an expression vector as defined herein and/or a cell as defined herein for the transcription of a nucleotide sequence of interest.

The present invention further relates to a use of a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant and/or isolated nucleic acid construct as defined herein, and/or an expression vector as defined herein and/or a cell as defined herein for the expression of a protein or polypeptide of interest.

The present invention further relates to a method for transcription and optionally purifying a produced transcript comprising the step of:

- providing a nucleic acid construct comprising a nucleotide sequence that has at least 50% identity to SEQ ID NO:88 over its whole length and which is operably linked to a nucleotide sequence of interest and,
- contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- purifying said produced transcript.

The present invention further relates to a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- a) providing a nucleic acid construct comprising a nucleotide sequence that has at least 50% identity to SEQ ID NO:88 over its whole length and which is operably linked to a nucleotide sequence of interest and contacting a cell with said nucleic acid construct to obtain a transformed cell, and,
- b) allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- c) purifying said protein or polypeptide of interest.

Preferably, said nucleic acid construct of said method for transcription and/or expression and optionally purifying a transcript and/or protein or polypeptide of interest further comprises an additional expression regulating element operably linked to said nucleotide sequence of interest and/or said nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said additional expression regulating element comprises an intronic sequence. Preferably, said additional expression regulating element further comprises a translation enhancing element.

The present invention further relates to a nucleic acid molecule that is represented by a nucleotide sequence that has at least 50% identity to SEQ ID NO: 88 over its whole length. Preferably, said nucleic acid molecule is an isolated nucleic acid molecule. The present invention further relates to a nucleic acid construct comprising a nucleic acid molecule of the invention. Preferably, said nucleic acid construct is represented by a nucleotide sequence that further comprises an optional nucleotide sequence of interest. Preferably, said nucleic acid construct further comprises an additional expression regulating element, wherein preferably said expression enhancing element is configured to be operably linked to said optional nucleotide sequence of interest. Preferably, said additional expression regulating element further comprises a translation enhancing element. Preferably, said additional expression regulating element comprises an intronic sequence. Preferably, said optional nucleotide sequence of interest is a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a heterologous protein or polypeptide.

Preferably, said nucleic acid construct is a recombinant and/or isolated nucleic acid construct.

The present invention further relates to an expression vector comprising a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant nucleic acid construct as defined herein.

The present invention further relates to a cell comprising a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant and/or isolated nucleic acid construct as defined herein, and/or an expression vector as defined herein.

The present invention also relates to a use of a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant and/or isolated nucleic acid construct as defined herein, and/or an expression vector as defined herein and/or a cell as defined herein for the transcription of a nucleotide sequence of interest.

The present invention further relates to a use of a nucleic acid molecule or an isolated nucleic acid molecule as defined herein, and/or a nucleic acid construct or a recombinant and/or isolated nucleic acid construct as defined

herein, and/or an expression vector as defined herein and/or a cell as defined herein for the expression of a protein or polypeptide of interest.

DESCRIPTION OF THE INVENTION

The inventors identified an expression construct for increasing the expression of a protein or polypeptide of interest. The expression construct of the invention is characterized by two promoters operably linked to a coding sequence of a protein or polypeptide of interest. An expression construct of the invention typically comprises a first promoter followed by a second promoter, a coding sequence of the protein or polypeptide of interest and a polyadenylation sequence, wherein said second promoter is flanked by intronic sequences. Said promoter being flanked by intronic sequences is denominated herein as an intronic promoter. Additional expression regulating sequences may be inserted upstream and downstream of said first and/or second promoter and/or downstream of the polyadenylation sequence. The inventors surprisingly found that an expression construct of the invention comprising a promoter followed by an intronic promoter operably linked to a coding sequence of a protein or polypeptide of interest, results in a significant increase in expression of said protein or polypeptide of interest as compared to an expression construct comprising only one promoter operably linked to said coding sequence. The inventors have found that the expression of initially poorly expressed proteins is increased to appreciable levels when using the combination of a promoter and intronic promoter of the invention instead of a single promoter in an expression construct encoding these proteins, as exemplified in the Examples, more specifically in Example 1. The combination of a promoter and an intronic promoter of the invention in an expression construct for an initially poorly expressed protein facilitates the generation of clonal lines and allows for the generation of clonal lines with increased and relevant expression levels, as exemplified in the Examples, more specifically in Example 3. Furthermore, expression of initially highly expressed proteins is even further increased when using the combination of a promoter and an intronic promoter of the invention instead of a single promoter in an expression construct encoding these proteins as exemplified in the Examples, more specifically in Example 5. Furthermore, an increase in total amount of mRNA and an increase in expression as measured on protein level was found as detailed herein below and exemplified in the Examples enclosed. Furthermore, the percentage of high-producer cell lines in a stably transfected pool is significantly higher as compared to pools with a single promoter operably linked to the coding sequence. As the nucleotide sequence of the invention comprising both a promoter and an intronic promoter operably linked to a nucleotide sequence of interest results in an increase in transcription, the present invention is not limited to the use of this sequence in protein and/or polypeptide expression and/or protein and/or polypeptide production but extends to the use of this combination of a promoter and intronic promoter in methods where higher levels of transcript are desired, for instance in methods for producing noncoding RNA transcripts as further specified herein. Furthermore, a further benefit of the invention is that, apart from an increase in transcription level and/or increase in expression level of the protein or polypeptide of interest, the invention allows for different transcripts to be formed as further detailed herein.

The inventors identified an expression enhancing element for increasing the expression of a protein or polypeptide of interest. The present invention relates to said expression enhancing element. Application of the expression enhancing element of the invention in an expression construct further comprising a heterologous promoter operably linked to a sequence encoding a protein or polypeptide of interest, results in a marked increase in expression of said protein or polypeptide of interest as compared to such expression using a similar expression construct which only differs to the former expression construct in that the expression enhancing element of the invention is absent. The inventors have found that expression of initially poorly expressed proteins is increased to appreciable levels after insertion of the element in an expression construct encoding these proteins as exemplified in the Examples, more specifically in Example 1. Insertion of the expression enhancing element in an expression construct for an initially poorly expressed protein facilitates the generation of clonal lines and allows for the generation of clonal lines with relevant expression levels, as exemplified in the Examples, more specifically in Example 3. Furthermore, expression of initially highly expressed proteins is even further increased after insertion of the element in an expression construct encoding these proteins as exemplified in the Examples, more specifically in Example 5. Furthermore, an increase in total amount of mRNA level and/or an increase in expression as measured on protein level was found as detailed herein below and exemplified in the Examples enclosed. As the expression enhancing element of the invention may result in an increase in transcription, the present invention is not limited to the use of this element in protein and/or polypeptide expression and/or protein and/or polypeptide production but extends to the use of this element in methods where higher levels of transcript are desired, for instance in methods for producing noncoding RNA transcripts as further specified herein.

The inventors further identified a nucleic acid molecule represented by a nucleotide sequence that has at least 50% identity with SEQ ID NO: 88 for increasing the expression of a protein or polypeptide of interest. The use of said nucleotide sequence is attractive as demonstrated in example 11.

First Aspect

In a first aspect, the present invention provides a nucleic acid construct for increasing transcription and/or expression, comprising a first promoter and a second promoter, which are configured to be both operably linked to an optional nucleotide sequence of interest within an expression construct. "Optional" being understood herein as not necessarily being present in an expression construct. For instance, such nucleotide sequence of interest need not be present in a commercialized expression vector, but may be readily introduced by a person skilled in the art before use in a method of the invention.

Preferably, within this first aspect, said nucleotide construct of the invention comprising a first promoter and a second promoter is capable of increasing the transcription of a nucleotide sequence of interest that is under the control of said first promoter and second promoter. Alternatively or in combination with the increased transcription, said nucleotide construct is also preferably capable of increasing expression of a protein or polypeptide of interest encoded by said nucleotide sequence of interest. Preferably, transcription levels are assessed in an expression system using an expression construct comprising said first promoter and second promoter operably linked to a nucleotide sequence of interest using a suitable assay such as RT-qPCR. Preferably,

within this first aspect, the nucleotide construct of the invention comprising the first promoter and second promoter of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% of said nucleotide sequence of interest as compared to transcription using a construct which only differs in that the nucleotide sequence of interest is under the control of a single promoter, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a first promoter and second promoter sequence to be tested operably linked to said nucleotide sequence of interest. Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest which are measured under the same conditions except that said first promoter and second promoter are replaced by a single promoter, preferably a CMV promoter represented by SEQ ID NO: 57, in the expression vector used.

Preferably, within this first aspect, expression levels are established in an expression system using an expression construct comprising said first promoter and second promoter operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an enzyme-linked immunosorbent assay (ELISA) assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the first promoter and second promoter of the invention allow for an increase in expression of protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that the encoding sequence of the protein or polypeptide of interest is under the control of a single promoter, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a first promoter and second promoter sequence to be tested operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector only differs in that said first promoter and second promoter are replaced by a single promoter, preferably a CMV promoter represented by SEQ ID NO: 57, in the expression vector used.

Preferably, within said first aspect, said increase of protein or polypeptide expression is copy number independent as established by an assay suitable to determine copy number dependency by a skilled person such as, but not limit to, a triplex Taqman assay as further detailed in Example 4 of the present invention.

Preferably, within said first aspect, said first promoter is located upstream or at the 5' site of said second promoter. Preferably, said second promoter as defined herein should be devoid of sequence elements that will act as transcription terminators. Transcription terminators well known by the persons skilled in the art are sequences that can result in premature termination of transcription such as, but not limited to, stable hairpin structures, repeat sequences such as long terminal repeats (LTRs) or Alu repeats, polyadenylation motifs and transposable elements.

Within the context of the first aspect of the invention a promoter is a promoter capable of initiating transcription in the host cell of choice. Promoters as used herein include tissue-specific, tissue-preferred, cell-type specific, inducible and constitutive promoters as defined herein in the Definitions section. Promoters that may be comprised within said first or second promoter as defined herein are promoters that may be employed in transcription of nucleotide sequences of interest and/or expression of proteins or polypeptides of interest, preferably in mammalian cells, and include, but are not limited to, the human or murine cytomegalovirus (CMV) promoter, a simian virus (SV40) promoter, a human or mouse ubiquitin C (UBC) promoter, a human or mouse or rat elongation factor alpha (EF1-a) promoter, mouse or hamster beta-actin promoter, or a hamster rpS21 promoter. The Tet-Off and Tet-On responsive elements upstream of a minimal promoter such as a CMV promoter is an example of an inducible mammalian promoter. Examples of suitable yeast and fungal promoters are Leu2 promoter, the galactose (Gall or Ga17) promoter, alcohol dehydrogenase 1 (ADH1) promoter, glucoamylase (Gla) promoter, triose phosphate isomerase (TPI) promoter, translational elongation factor EF-I alpha (TEF2) promoter, glyceraldehyde-3-phosphate dehydrogenase (gpdA) promoter, alcohol oxidase (AOX1) promoter, or glutamate dehydrogenase (gdhA) promoter. An example of a strong ubiquitous promoter for expression in plants is cauliflower mosaic virus (CaMV) 35S promoter.

In an embodiment within said first aspect, said first and said second promoters are similar promoters. Preferably, said first promoter has at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to said second promoter.

In another embodiment within said first aspect, said first promoter and second promoter are distinct or different promoters. Preferably, said first promoter has less than 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% or 5% sequence identity to said second promoter.

In a preferred embodiment within said first aspect, said first promoter sequence comprises or consists of a UBC promoter or a CCT8 promoter and said second promoter comprises or consists of a CMV promoter, or the other way around. Preferably, said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-969 of SEQ ID NO: 1 or with nucleotides 1-614 of SEQ ID NO: 2. Preferably, said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 58, or preferably to SEQ ID NO: 57. Preferred within said first aspect is a nucleotide sequence of the invention wherein said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 57.

identity to nucleotides 1-969 of SEQ ID NO: 1 and said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 57.

Also preferred within said first aspect is a nucleotide sequence of the invention wherein said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with nucleotides 1-614 of SEQ ID NO: 2 and said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 57.

Also preferred within said first aspect is a nucleotide sequence of the invention wherein said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 58, or preferably to SEQ ID NO: 57 and wherein said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-969 of SEQ ID NO 1 or with nucleotides 1-614 of SEQ ID NO 2.

Preferably within said first aspect a nucleotide sequence of the invention wherein said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with to SEQ ID NO: 57 and said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity nucleotides 1-969 of SEQ ID NO: 1. Also preferred is a nucleotide sequence of the invention wherein said first promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 57 and said second promoter comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with nucleotides 1-614 of SEQ ID NO: 2.

It is to be understood that within said first aspect, said first and/or second promoter does not consist only of a promoter enhancer sequence, such as a sequence selected from the group consisting of SEQ ID NO: 52-54. Preferably, said first promoter and second promoter do not consist only of a promoter enhancer sequence, such as a sequence selected from the group consisting of SEQ ID NO: 52-54. Preferably, a nucleotide sequence of the invention does not comprise or consist of SEQ ID NO: 55 or 56.

In a preferred embodiment within said first aspect, said second promoter is flanked by a first intronic sequence at the 5' site or upstream of said second promoter and a second intronic sequence at the 3' site or downstream of said second promoter. Being "flanked" is understood herein as being positioned in between said indicated sequences optionally separated by 1-50, 1-60, 1-70, 1-80, 1-90, 1-100, 1-200, 1-300, 1-400, 1-500, 1-600, 1-700, 1-800, 1-900, 1-1000, 1-5,000 or 1-100,000 nucleotides, these nucleotides being understood to encompass the 5'-UTR. An intronic sequence is understood to be at least part of the nucleotide sequence of an intron. Preferably, said first intronic sequence at the 5' site or upstream of said second promoter comprises at least

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a donor splice site or splice site GT. A donor splice site is understood herein as a splice site that, when combined with an acceptor splice site as defined herein, results in the formation of an intron as defined in the Definition section. Preferably, a nucleotide sequence is an intron if at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the primary RNA loses this sequence by RNA splicing using an assay suitable to detect intron splicing, such as but not limited to reverse-transcriptase polymerase chain reaction (RT-PCR) followed by size or sequence analysis of the RT-PCR. Preferred donor splice sites of the invention are M-W-G-[cut]-G-T-R-A-G-K or M-A-R-[cut]-G-T-R-A-G-K in case the host cell is a mammalian cell, A-G-[cut]-G-T-A-W-K in case the host cell is a plant cell, [cut]-G-T-A-W-G-T-T in case the host cell is a yeast cell and R-G-[cut]-G-T-R-A-G, in case the host cell is an insect cell. “[cut]” is to be understood herein as the specific cut site where splicing will take place. Intron splicing can be assessed functionally using an assay as detailed in the Definition section under “intron”. Most preferably, the donor splice site comprised within the first intronic sequence of the invention is C-T-G-[cut]-G-T-G-A-G-G or A-A-A-[cut]-G-T-G-A-G-G. Preferably, said first intronic sequence consists of said donor splice site or splice site GT. Preferably, said first intronic sequence comprises a single donor splice site as defined herein. Preferably, said first intronic sequence is free of an acceptor splice site as defined herein below.

Preferably, within said first aspect said second intronic sequence at the 3' site or downstream of said promoter comprises at least an acceptor splice site which is understood herein as the splice site AG preferably preceded by a pyrimidine rich sequence or polypyrimidine tract nucleotide sequence, optionally separated from splice site AG by 1-50 nucleotides, and optionally further comprising a branch site comprising the sequence Y-T-N-A-Y, at the 5' site of the polypyrimidine tract nucleotide sequence, wherein the branch site may have the nucleotide sequence C-Y-G-A-C. An acceptor splice site is understood herein as a splice site that, when combined with a donor splice site encompassed within a construct, results in the formation of an intron as defined in the Definition section. Preferably, the acceptor splice site or splice site AG has the sequence [Y-rich]-N-Y-A-G-[cut]. Preferably, the acceptor splice site or splice site AG has the sequence [Y-rich]-N-Y-A-G-[cut]-R in case the host cell is a mammalian cell, [Y-rich]-D-Y-A-G-[cut]-R or [Y-rich]-D-Y-A-G-[cut]-R-W in case the host cell is a plant cell, [Y-rich]-A-Y-A-G-[cut] in case the host cell is a yeast cell and [Y-rich]-N-Y-A-G-[cut] in case the host cell is an insect cell. “[Y-rich]” is to be understood herein as the polypyrimidine tract which is preferably defined as a consecutive sequence of at least 10 nucleotides comprising at least 6, 7, 8, 9 or preferably 10 pyrimidine nucleotides. Preferably, said acceptor splice site or splice site GT has the sequence Y-A-G-[cut]-R. Preferably, said second intronic sequence comprises a single acceptor splice site. In an embodiment, said second intronic sequence is free of a donor splice site as defined herein. In an alternative embodiment, said second intronic sequence comprises both a donor splice site and an acceptor splice site as defined herein. Most preferably, said second intronic sequence is an intron as defined in the Definition section. Preferably, the second promoter and the intronic sequences flanking the second promoter are configured to form an intronic promoter (referred to is FIG. 1). An intronic promoter is known to a person skilled in the art as a promoter located within an intronic sequence. Preferably, said intronic promoter is an

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intron as defined in the Definition section. Preferably, the boundaries of the intronic promoter of the present invention are being formed by the donor splice site of the intronic sequence at the 5' site or upstream of the second promoter of the invention and the acceptor splice site of the intronic sequence at the 3' site or downstream of the second promoter of the invention. The intronic promoter of the invention can have a length that is comparable or similar to naturally occurring introns, preferably comparable or similar to naturally occurring introns in the host cell or organism as defined herein. Preferably, said intronic promoter as defined herein is at most 12,000 nucleotides in length. Preferably, said first intronic sequence at the 5' site or upstream of said second promoter is located at the 3' site or downstream of said first promoter. Preferably, the first promoter and second promoter, the intronic sequences flanking the second promoter, and a nucleotide sequence encoding a protein or polypeptide of interest are configured in such a way that the first promoter is upstream of the second promoter, wherein the second promoter is flanked by said intronic sequences to form an intronic promoter, and wherein said first promoter and second promoter are configured to be both upstream and operably linked to the nucleotide sequence encoding a protein or polypeptide of interest (FIG. 1). The intronic promoter may comprise further expression enhancing elements, but preferably the intronic promoter is free of further splice sites apart from the donor and acceptor splice sites as defined herein within the first and second intronic sequences as defined herein. Preferably, one or more expression enhancing sequences are comprised within said first and/or said second promoter. Without being wished to be bound by any theory, transcription starting from either of the two promoters may result in different transcripts (pre-mRNAs) which, upon splicing result in different mRNAs as illustrated in FIG. 1. In support of this theory, the inventors found that different transcripts are formed using a construct of the invention (referred to is in this respect to FIG. 1, Example 8 and FIG. 10). Furthermore, the increased activity is found to be severely diminished by 4 nucleotides mutation in the intronic promoter which prevents correct intron splicing (referred to this respect is to FIG. 9 and Example 7), also supporting the theory that both promoters are active in the construct. Therefore, a further benefit of the invention is that, apart from an increase in transcription of the nucleotide sequence of interest and/or an increase in expression level of the protein or polypeptide of interest, the invention allows for different transcripts to be formed. “Different transcripts” are understood herein as transcripts that are structurally different or distinct, i.e. having a different or distinct nucleotide sequence. Therefore a further benefit of the invention is to direct or redirect the splicing of a nucleotide sequence of interest. Depending on the location of the intronic splice sites, the transcripts may have a different or distinct UTR sequence and/or a different or distinct coding sequence. It is also possible that only one type of transcript is formed, e.g. in case the 5'-UTR sequences of said first and second intronic sequences are the same. Assessment whether different transcripts are formed can be done using any suitable method known to the person skilled in the art, such as but not limited to Rapid amplification of cDNA ends Polymerase Chain Reaction (RACE-PCR).

Preferably, within said first aspect, said first intronic sequence at the 5' site or upstream of said second promoter, has at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with nucleotides 970-1449 of SEQ ID NO: 1 or at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence

identity with nucleotides 667-1228 of SEQ ID NO: 2, preferably comprising at least a donor splice site or splice site GT.

Preferably, within said first aspect, said intronic sequence downstream or at the 3' site of said second promoter comprises a nucleotide sequence having at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with nucleotides 171-277 of SEQ ID NO: 14, nucleotides 171-274 of SEQ ID NO: 19, nucleotides 133-210 of SEQ ID NO: 20, nucleotides 134-211 of SEQ ID NO: 21, nucleotides 134-226 of SEQ ID NO: 22, nucleotides 134-226 of SEQ ID NO: 23, nucleotides 133-225 of SEQ ID NO: 24, nucleotides 134-226 of SEQ ID NO: 25, nucleotides 146-257 of SEQ ID NO: 26, or nucleotides 147-223 of SEQ ID NO: 27, preferably comprising at least an acceptor splice site AG preceded by a TC-rich nucleotide sequence, optionally separated from splice site AG by 1-50 nucleotides and a branch site comprising the sequence Y-T-N-A-Y or C-Y-G-A-C, at the 5' site of the TC-rich nucleotide sequence.

In a preferred embodiment within said first aspect, said first promoter is flanked at its 3' site by said first intronic sequence. In an embodiment, said first promoter and said first intronic sequence are not aligned in nature but aligned in a construct of the invention by recombination. In another embodiment, said sequence comprising both said first promoter flanked at its 3' site by said first intronic sequence is derived from a naturally occurring sequence. In a preferred embodiment, said nucleotide sequence comprising both a first promoter and a first intronic sequence according to the present invention is a sequence derived from the UBC ubiquitin gene. Preferably, said sequence is derived from a mammalian UBC ubiquitin gene. More preferably, said nucleotide sequence comprising both a first promoter and a first intronic sequence according to the present invention is derived from the *Cricetulus griseus* homologous gene of the human UBC ubiquitin gene, said gene being indicated as the *Cricetulus* sp. gene for polyubiquitin, or CRUPUQ (GenBank D63782). In a preferred embodiment, said nucleotide sequence derived from CRUPUQ is comprising both a first promoter and a first intronic sequence of the invention and is a contiguous sequence of at least 500, 600, 700, 800, 900, 1000 or 1117 in length, preferably at least 1449 nucleotides in length of SEQ ID NO: 1. Preferably, said nucleotide sequence having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1 is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 in length. Most preferably, said sequence being 1449 nucleotides in length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 65% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 70% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 75% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 80% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 85% identity with SEQ ID NO: 1 over its whole length.

nucleotides in length and has at least 85% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 90% identity with SEQ ID NO: 1 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 95% identity with SEQ ID NO: 1 over its whole length. Also preferred is a sequence of at most 8000 nucleotides having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1.

In a further preferred embodiment within said first aspect, said nucleotide sequence comprising both a first promoter and a first intronic sequence according to the present invention is a sequence derived from a CCT8 gene. Preferably, said sequence is derived from a mammalian CCT8 gene. More preferably, said nucleotide sequence comprising both a first promoter and a first intronic sequence according to the present invention is derived from the human or *Homo sapiens* CCT8 gene. In a preferred embodiment, said nucleotide sequence derived from said CCT8 gene comprising both a first promoter and a first intronic sequence of the invention is a contiguous sequence of at least 500, 600, 700, 791 or 1223 in length, preferably at least 1228 nucleotides in length of SEQ ID NO: 2. Preferably, said nucleotide sequence having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 2 is at most 8000 nucleotides in length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 in length. Most preferably, said sequence being 1228 nucleotides in length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 65% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 70% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 75% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 80% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 85% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 90% identity with SEQ ID NO: 2 over its whole length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 95% identity with SEQ ID NO: 2 over its whole length. Also preferred is a sequence of at most 8000 nucleotides having at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 2 over its whole length.

Preferably within said first aspect, said nucleotide sequence comprising a first promoter and a first intronic sequence as defined herein has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to any of SEQ ID NO: 1, 2 and 59-61 over its whole length. Preferably, said nucleotide sequence comprising a first promoter and a first intronic sequence as defined herein comprises or consists of any of the sequences selected from the group consisting of SEQ ID NO: 1, 2 and 59-61. Most preferably, said nucleotide sequence comprising a first promoter and a first intronic sequence as defined herein comprises or consists of any of the sequences selected from the group consisting of SEQ ID NO: 1, 2 and 59.

Preferably, the nucleotide construct of the first aspect further comprises one or more additional expression regulating sequences, wherein preferably said first promoter, said intronic sequences as defined herein, and optionally said additional expression regulating sequence are all configured to be operably linked to an optional nucleotide sequence of interest. An "additional expression regulating sequence" is to be understood herein as a sequence or element in addition to the first and/or second promoter and/or the first and/or second intronic sequence as defined herein above, and may be an additional expression enhancing sequence and/or a distinct expression enhancing sequence. An additional expression regulating sequence as encompassed by the present invention can be, but is not limited to, a transcriptional and/or translational regulation of a gene, including but not limited to, 5'-UTR, 3'-UTR, enhancer, promoter, intron, polyadenylation signal and chromatin control elements such as S/MAR (scaffold/matrix attachment region), ubiquitous chromatin opening element, cytosine phosphodiester guanine island and STAR (stabilizing and anti-repressor element), and any derivatives thereof. Other optional regulating sequences that may be present in the nucleic acid construct of the invention include, but are not limited to, coding nucleotide sequences of homologous and/or heterologous nucleotide sequences, including the Iron Responsive Element (IRE), Translational cis-Regulatory Element (TLRE) or uORFs in 5' UTRs and poly(U) stretches in 3' UTRs. Such one or more additional expression regulating, preferably enhancing elements may be located on any position in the construct, preferably directly aligning or comprised within said first and/or second promoter.

A further preferred regulating sequence within said first aspect comprises or consists of a translation enhancing element. Preferably, a translation enhancing element allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that it is free of said translation enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a translation enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions

except that the expression vector is free of said translation enhancing element to be tested.

Preferably, within said first aspect said translation enhancing element comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity with any of SEQ ID NO: 3-51 over its whole length, or a translation enhancing element that comprises or consists of a nucleotide sequence that comprises:

- 10 i) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides;
- ii) a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, and a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, said first nucleotide sequence not comprising a GAA repeat nucleotide sequence; or,
- iii) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, wherein said GAA repeat nucleotide sequence is located 3' of any one or more of said TC-rich nucleotide sequence, A-rich nucleotide sequences, and/or GT-rich nucleotide sequence.

The GAA repeat nucleotide sequence is defined herein as comprising at least 3 GAA repeats. The GAA repeat nucleotide sequence may comprise an imperfect GAA repeat. The GAA repeat nucleotide sequence may have at least 50% sequence identity, or at least 60% sequence identity, or at least 70% sequence identity, or at least 80% sequence identity, or at least 90% sequence identity or 100% sequence identity with nucleotides 14-50 of SEQ ID NO: 3. The imperfect GAA repeat may comprise the nucleotide sequence (GAA)₃ATAA(GAA)₈.

The TC-rich nucleotide sequence is defined herein as having at least 70%, 80%, 90% or 100% sequence identity with nucleotides 54-68 of SEQ ID NO: 3.

The A-rich nucleotide sequence is defined herein as having at least 70%, 80%, 90% or 100% sequence identity with any one of nucleotides 77-87, nucleotides 93-105, nucleotides 111-121, nucleotides 126-132, or nucleotides 152-169 of SEQ ID NO: 3, respectively.

The GT-rich nucleotide sequence is defined herein as having at least 70%, 80%, 90% or 100% sequence identity with nucleotides 133-148 of SEQ ID NO: 3.

Preferably within said first aspect, said translation enhancing sequence comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 19. Preferably, said translation enhancing sequence is located downstream or at the 3' site of the second promoter sequence of the invention and upstream or at the 5' site of an optional nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said translation enhancing sequence is located downstream or at the 3' site of the second promoter sequence of the invention and upstream or at the 5' site of the second intronic sequence as defined herein.

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Most preferably within said first aspect, said nucleic acid construct of the first aspect of the invention comprising a first promoter, a first intronic sequence, a second promoter and a second intronic sequence, has at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 73, 74, 75, or 76.

Preferably within said first aspect, the nucleic acid construct of the invention further comprises a nucleotide sequence of interest operably linked to and/or under the control of said first and second promoters and optionally said additional expression regulating sequence as defined herein. It is to be understood that said first promoter and second promoter, and optionally said additional expression regulating sequence are all configured to be operably linked to the same, single nucleotide sequence of interest. In a preferred embodiment, said nucleotide sequence of interest is a nucleotide sequence encoding a protein or polypeptide of interest. The protein or polypeptide of interest can be a homologous protein or polypeptide, but in a preferred embodiment of the invention the protein or polypeptide of interest is a heterologous protein or polypeptide. A nucleotide sequence encoding a heterologous protein or polypeptide may be derived in whole or in part from any source known to the art, including a bacterial or viral genome or episome, eukaryotic nuclear or plasmid DNA, cDNA or chemically synthesized DNA. The nucleotide sequence encoding a protein or polypeptide of interest may constitute an uninterrupted coding region or it may include one or more introns bounded by appropriate splice junctions, it can further be composed of segments derived from different sources, naturally occurring or synthetic. The nucleotide sequence encoding the protein or polypeptide of interest according to the method of the invention is preferably a full-length nucleotide sequence, but can also be a functionally active part or other part of said full-length nucleotide sequence. The nucleotide sequence encoding the protein or polypeptide of interest may also comprise signal sequences directing the protein or polypeptide of interest when expressed to a specific location in the cell or tissue. Furthermore, the nucleotide sequence encoding the protein or polypeptide of interest can also comprise sequences which facilitate protein purification and protein detection by for instance Western blotting and ELISA (e.g. c-myc or polyhistidine sequences).

Within the context of the invention, the protein or polypeptide of interest may have industrial or medicinal (pharmaceutical) applications. Examples of proteins or polypeptides with industrial applications include enzymes such as e.g. lipases (e.g. used in the detergent industry), proteases (used inter alia in the detergent industry, in brewing and the like), cell wall degrading enzymes (such as, cellulases, pectinases, beta.-1,3/4- and beta.-1,6-glucanases, rhamnogalacturonases, mannanases, xylanases, pullulanases, galactanases, esterases and the like, used in fruit processing wine making and the like or in feed), phytases, phospholipases, glycosidases (such as amylases, beta-glucosidases, arabofuranosidases, rhamnosidases, apiosidases and the like), dairy enzymes (e.g. chymosin). Mammalian, and preferably human, proteins or polypeptides and/or enzymes with therapeutic, cosmetic or diagnostic applications include, but are not limited to, insulin, serum albumin (HSA), lactoferrin, hemoglobin α and β , tissue plasminogen activator (tPA), erythropoietin (EPO), tumor necrosis factors (TNF), BMP (Bone Morphogenic Protein), growth factors (G-CSF, GM-CSF, M-CSF, PDGF, EGF, and the like), peptide hormones

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(e.g. calcitonin, somatomedin, somatotropin, growth hormones, follicle stimulating hormone (FSH), interleukins (IL-x), interferons (IFN-y), phosphatases, antibodies, and antibody-like proteins such as, but not limited to, multispecific antibodies like DART (Dual-Affinity Re-Targeting) and Tribody protein, and antibody fragments like Fc, Fab, Fab2, Fv and scFv. Also included are bacterial and viral antigens, e.g. for use as vaccines, including e.g. heat-labile toxin B-subunit, cholera toxin B-subunit, envelope surface protein Hepatitis B virus, capsid protein Norwalk virus, glycoprotein B Human cytomegalovirus, glycoprotein S, interferon, and transmissible gastroenteritis corona virusreceptors and the like. Further included are genes coding for mutants or analogues of the said proteins.

Within the context of the invention, in an alternative embodiment, said nucleotide sequence of interest is not a coding sequence for a protein or a polypeptide but may be a functional nucleotide sequence such as, but is not limited to, a sequence encoding a non-coding RNA, wherein a non-coding RNA is understood to be an RNA not coding for a protein or polypeptide. Preferably, said non-coding RNA is a reference sequence or regulatory molecule that may regulate the expression of genes or regulating the activity or localization of proteins or polypeptides. For instance, a non-coding RNA may be an antisense RNA or miRNA molecule. As the first promoter and second promoter of the invention is believed to work at the level of transcription, i.e. the increase in expression by the sequence of the invention comprising said first promoter and second promoter as shown herein is believed to result from an increase in transcription, the construct of the invention can also be used for producing increased levels of transcripts, as well as producing transcripts with different sequences. Transcription levels can be quantified by using regular transcription quantification methods known by the person skilled in the art such as, but not limited to, Northern blotting and RT-qPCR.

Second Aspect

In a second aspect, the present invention provides an expression vector comprising a nucleic acid construct according to the first aspect of the invention. The nucleic acid construct according to the invention is preferably a vector, in particular a plasmid, cosmid or phage or nucleotide sequence, linear or circular, of a single or double stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing any one of the nucleotide sequences of the invention in sense or antisense orientation into a cell. The choice of vector is dependent on the recombinant procedures followed and the host cell used. The vector may be an autonomously replicating vector or may replicate together with the chromosome into which it has been integrated. Preferably, the vector contains a selection marker. Useful markers are dependent on the host cell of choice and are well known to persons skilled in the art and are selected from, but not limited to, the selection markers as defined in third aspect of the invention. A preferred expression vector is the pcDNA3.1 expression vector. Preferred selection markers are the neomycin resistance gene, zeocin resistance gene and blasticidin resistance gene.

Third Aspect

In a third aspect, the present invention provides a cell comprising a nucleic acid construct according to the first aspect of the invention, and/or an expression vector according to the second aspect of the invention as defined herein.

Within the context of the invention, a cell may be a mammalian, including human cell, a plant, animal, insect, fungal, yeast or bacterial cell. A recombinant host cell, such as a mammalian, including human, plant, animal, insect, fungal or bacterial cell, containing one or more copies of a nucleic acid construct according to the invention is an additional subject of the invention. By host cell is meant a cell which contains a nucleic acid construct such as a vector and supports the replication and/or expression of the nucleic acid construct. Examples of suitable bacteria are Gram positive bacteria such as several species of the genera *Bacillus*, *Streptomyces* and *Staphylococcus* or Gram negative bacteria such as several species of the genera *Escherichia* and *Pseudomonas*. Fungal cells include yeast cells. Expression in yeast can be achieved by using yeast strains such as *Pichia pastoris*, *Saccharomyces cerevisiae* and *Hansenula polymorpha*. Other fungal cells of interest include filamentous fungi cells as *Aspergillus niger*, *Trichoderma reesei*, and the like. Furthermore, insect cells such as cells or cell lines from *Drosophila melanogaster*, *Spodoptera frugiperda*, and *Trichoplusia ni*, such as, but not limited to, S2, S9, Sf21, and High Five cells, can be used as host cells. Alternatively, a suitable expression system can be a baculovirus system or expression systems using mammalian cells such as CHO, COS, CPK (porcine kidney), MDCK, BHK, Sp2/0, NS0, and Vero cells. A suitable human cell or human cell line is an astrocyte, adipocyte, chondrocyte, endothelial, epithelial, fibroblast, hair, keratinocyte, melanocyte, osteoblast, skeletal muscle, smooth muscle, stem, synoviocyte cell or cell line. Examples of suitable human cell lines also include HEK 293 (human embryonic kidney), HeLa, Per. C6, CAP (cell lines derived from primary human amniocytes), and Bowes melanoma cells. In an embodiment a human cell is not an embryonic stem cell.

Therefore, another aspect of the invention relates to a host cell that is genetically modified, preferably by a method of the invention, in that a host cell comprises a nucleic acid construct as herein defined above. Host cell is a cell that has been genetically modified. The wording host cell may be replaced by modified cell or transformed cell or recombinant cell or modified host cell or transformed host cell or recombinant host cell. For transformation procedures in plants, suitable bacteria include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

A nucleic acid construct preferably is stably maintained, either as an autonomously replicating element, or, more preferably, the nucleic acid construct is integrated into the host cell's genome, in which case the construct is usually integrated at random positions in the host cell's genome, for instance by non-homologous recombination. Stably transformed host cells are produced by known methods. The term stable transformation refers to exposing cells to methods to transfer and incorporate foreign DNA into their genome. These methods include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethylene-imide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

Alternatively, a protein or polypeptide of interest may be expressed in a host cell, e.g., a mammalian cell, relying on transient expression from vectors.

A nucleic acid construct according to the invention preferably also comprises a marker gene which can provide selection or screening capability in a treated host cell. Selectable markers are generally preferred for host transformation events, but are not available for all host cells. A

nucleic acid construct disclosed herein can also include a nucleotide sequence encoding a marker product. A marker product can be used to determine if the construct or portion thereof has been delivered to the cell and once delivered is being expressed. Examples of marker genes include, but are not limited to the *E. coli lacZ* gene, which encodes β-galactosidase, and a gene encoding the green fluorescent protein.

Within the context of the invention, examples of suitable selectable markers for mammalian cells include, but are not limited to dihydrofolate reductase (DHFR), glutathione synthetase (GS), thymidine kinase, neomycin, neomycin analog G418, hygromycin, blasticidin, zeocin and puromycin.

Other suitable selectable markers include, but are not limited to antibiotic, metabolic, auxotrophic or herbicide resistant genes which, when inserted in a host cell in culture, would confer on those cells the ability to withstand exposure to an antibiotic. Metabolic or auxotrophic marker genes enable transformed cells to synthesize an essential component, usually an amino acid, which allows the cells to grow on media that lack this component. Another type of marker gene is one that can be screened by histochemical or biochemical assay, even though the gene cannot be selected for. A suitable marker gene found useful in such host cell transformation experience is a luciferase gene. Luciferase catalyzes the oxidation of luciferin, resulting in the production of oxyluciferin and light. Thus, the use of a luciferase gene provides a convenient assay for the detection of the expression of introduced DNA in host cells by histochemical analysis of the cells. In an example of a transformation process, a nucleotide sequence sought to be expressed in a host cell could be coupled in tandem with the luciferase gene. The tandem construct could be transformed into host cells, and the resulting host cells could be analyzed for expression of the luciferase enzyme. An advantage of this marker is the non-destructive procedure of application of the substrate and the subsequent detection.

When such selectable markers are successfully transferred into a host cell, the transformed host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media. Two non-limiting examples are CHO DHFR-cells and mouse LTK-cells. These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in non-supplemented media.

The second category is dominant selection which refers to a selection scheme used in any cell type and does not require the use of a mutant cell line. These schemes typically use a drug to arrest growth of a host cell. Those cells which have a novel gene would express a protein conveying drug resistance and would survive the selection. Examples of such dominant selection use the drugs neomycin, (Southern P. and Berg, P., J. Molec. Appl. Genet. 1: 327 (1982)), mycophenolic acid, (Mulligan, R. C. and Berg, P. Science 209: 1422 (1980)) or hygromycin, (Sugden, B. et al., Mol. Cell. Biol. 5: 410-413 (1985)). The three examples employ bacterial genes under eukaryotic control to convey resis-

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tance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid) or hygromycin, respectively. Others include the neomycin analog G418 and puromycin. Other useful markers are dependent on the host cell of choice and are well known to persons skilled in the art.

When a transformed host cell is obtained with a method according to the invention (see below), a host tissue may be regenerated from said transformed cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells.

Resulting transformed host tissues are preferably identified by means of selection using a selection marker gene as present on a nucleic acid construct as defined herein.

Fourth Aspect

In a fourth aspect, the present invention provides a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- a) providing a nucleic acid construct according to the first aspect of the invention comprising a nucleotide sequence encoding a protein or polypeptide of interest; and,
- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- d) purifying said protein or polypeptide of interest.

The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention may be applied on a cell culture, organism culture, or tissue culture. Alternatively, next to the expression in host cells, the protein or polypeptide of interest can be produced in cell-free translation systems using RNAs derived from the nucleic acid constructs of the present invention. The method of the invention may be performed on cultured cells.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to express the protein or polypeptide of interest, and optionally said protein or polypeptide is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to expression of the protein or polypeptide of interest. The person skilled in the art is well aware of techniques to be used for expressing or overexpressing the protein or polypeptide of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to expression of the protein or polypeptide of interest, but in which the protein or polypeptide of interest is automatically (e.g., constitutively) expressed, are also included in the method of the present invention.

Within the context of the invention, purification steps depend on the expressed protein or polypeptide and the host cell used but can comprise isolation of the protein or polypeptide. When applied to a protein/polypeptide, the term "isolation" indicates that the protein or polypeptide is found in a condition other than its native environment. In a preferred form, the isolated protein or polypeptide is substantially free of other proteins, particularly other homologous proteins. It is preferred to provide the protein or polypeptide in a greater than 40% pure form, more preferably greater than 60% pure form. Even more preferably it is preferred to provide the protein or polypeptide in a highly

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purified form, i.e., greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by SDS-PAGE. If desired, the nucleotide sequence encoding a protein or polypeptide of interest may be ligated to a heterologous nucleotide sequence to encode a fusion protein or polypeptide to facilitate protein purification and protein detection on for instance Western blot and in an ELISA. Suitable heterologous sequences include, but are not limited to, the nucleotide sequences coding for proteins such as for instance glutathione-S-transferase, maltose binding protein, metal-binding polyhistidine, green fluorescent protein, luciferase and beta-galactosidase. The protein or polypeptide may also be coupled to non-peptide carriers, tags or labels that facilitate tracing of the protein or polypeptide, both *in vivo* and *in vitro*, and allow for the identification and quantification of binding of the protein or polypeptide to substrates. Such labels, tags or carriers are well-known in the art and include, but are not limited to, biotin, radioactive labels and fluorescent labels.

Preferably, the method of this fourth aspect of the invention allows for an increase in expression of a protein or polypeptide of interest. Preferably, expression levels are established in an expression system using an expression construct according to the first aspect of the invention comprising a first and a second promoter according to the first aspect of the invention, operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct is used in step a) that it is free of said first promoter and said second promoter and is operably linked to a single promoter, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a first promoter and second promoter sequence to be tested operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that said first promoter and second promoter are replaced by a single promoter, preferably a CMV promoter represented by SEQ ID NO: 57, in the expression vector used.

5th Aspect

In a fifth aspect, the present invention provides a method for expressing a protein or polypeptide of interest in an organism, comprising the steps of:

- a) providing a nucleic acid construct according to the first aspect of the invention comprising a nucleotide sequence encoding a protein or polypeptide of interest; and,

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- b) contacting a target cell and/or target tissue of an organism, with said nucleic acid construct to obtain a transformed target cell and/or transformed target tissue, allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- c) allowing said transformed target cell and/or target tissue to develop into a transformed organism; and, optionally,
- d) allowing said transformed organism to express the protein or polypeptide of interest, for example, subjecting said transformed organism to conditions leading to expression of the protein or polypeptide of interest, and optionally recovering said protein or polypeptide.

Within the context of the invention, the target cell may be an embryonal target cell, e.g., embryonic stem cell, for example, derived from a non-human mammalian, such as *bovine*, *porcine*, etcetera species. Preferably, said target cell is not a human embryonic stem cell. In the case of a multicellular fungus, such target cell may be a fungal cell that can be proliferated into said multicellular fungus. When a transformed plant tissue or plant cell (e.g., pieces of leaf, stem segments, roots, but also protoplasts or plant cells cultivated by suspension) is obtained with the method according to the invention, whole plants can be regenerated from said transformed tissue or cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells. The method of the invention may be applied in nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human. Encompassed within the present invention is a method of treatment comprising the method of the present aspect, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. The invention also relates to a construct of the first aspect of the invention for treatment, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. Furthermore, the invention relates to the use of a construct of the first aspect of the invention for the manufacture of a medicament, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide.

Furthermore, a part of the invention is a non-human transformed organism. Said organism is transformed with a nucleotide sequence, recombinant nucleic acid construct, or vector according to the present invention, and is capable of producing the polypeptide of interest. This includes a non-human transgenic organism, such as a transgenic non-human mammalian, transgenic plant (including propagation, harvest and tissue material of said transgenic plant, including, but not limited to, leafs, roots, shoots and flowers), multicellular fungus, and the like.

Preferably, the method of this fifth aspect of the invention allows for an increase in expression of a protein or polypeptide of interest in said organism or at least in one tissue or organelle or organ of said organism. Preferably, expression levels are established in an expression system using an expression construct according to the first aspect of the invention comprising a first and a second promoter according to the first aspect of the invention, operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the

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method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% in said organism or at least in one tissue or organelle or organ of said organism as compared to a method which only differs in that a construct is used in step a) that it is free of said first promoter and said second promoter and is operably linked to a single promoter, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a first promoter and second promoter sequence to be tested operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that said first promoter and second promoter are replaced by a single promoter, preferably a CMV promoter represented by SEQ ID NO: 57, in the expression vector used. Preferably, said increase of protein or polypeptide expression is copy number independent as established by an assay suitable to determine copy number dependency by a skilled person such as, but not limit to, a triplex Taqman assay as further detailed in Example 4 of the present invention.

30 Sixth Aspect

In a sixth aspect, the present invention provides a method for transcription and optionally purifying the produced transcript comprising the step of:

- a) providing a nucleic acid construct according to the first aspect of the invention comprising a nucleotide sequence of interest; and,
- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- d) purifying said produced transcript.

In a preferred embodiment of the method according to the invention a nucleic acid construct as defined above is used. The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention may be applied on a cell culture, organism culture, or tissue culture. The method of the invention may be applied in nucleic acid based vaccination and/or gene therapy preferably in a mammal, preferably in a human. Encompassed within the present invention is a method for treatment comprising or consisting of the method of the present aspect, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. The invention also relates to a construct of the first aspect of the invention for use in treatment, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. Furthermore, the invention relates to the use of a construct of the first aspect of the invention for the manufacture of a medicament, wherein the nucleotide sequence of interest encodes for a therapeutic transcript.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to produce a transcript of the nucleotide sequence of interest, and optionally the produced transcript is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to transcription the nucleotide sequence of interest. The person skilled in the art is well aware of techniques to be used for transcription the nucleotide sequence of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to transcription of the nucleotide sequence of interest, but in which the nucleotide sequence of interest is automatically (e.g., constitutively) transcribed, are also included in the method of the present invention.

Purification steps depend on the transcript produced. The term "isolation" indicates that the transcript is found in a condition other than its native environment. In a preferred form, the isolated transcript is substantially free of other cellular components, particularly other homologous cellular components such as homologous proteins. It is preferred to provide the transcript in a greater than 40% pure form, more preferably greater than 60% pure form. Even more preferably it is preferred to provide the transcript in a highly purified form, i.e., greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by Northern blotting.

Preferably, the method of this aspect of the invention allows for an increase in transcription of a nucleotide sequence of interest. Preferably, transcription levels are established in an expression system using an expression construct according to the first aspect of the invention comprising a first and a second promoter according to the first aspect of the invention operably linked to a nucleotide sequence of interest. Preferably, transcription of said nucleotide sequence of interest is detected by a suitable assay such as RT-qPCR. Preferably, the method of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct is used in step a) that it is free of said first promoter and second promoter and is operably linked to a single promoter, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a first promoter and second promoter sequence to be tested operably linked to said nucleotide sequence of interest. Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest which are measured under the same conditions except that said first promoter and second promoter are replaced by a single promoter, preferably a CMV promoter represented by SEQ ID NO: 57, in the expression vector used.

Seventh Aspect

In a seventh aspect, the present invention provides a method for splicing or redirecting the splicing of a nucleotide sequence of interest, and optionally purifying the produced transcripts comprising the step of:

- a) providing a nucleic acid construct according to the first aspect of the invention comprising a nucleotide sequence of interest; and,
- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,

c) allowing said transformed cell to produce transcripts of the nucleotide sequence of interest resulting in the production of a transcript; and optionally,

d) purifying said produced transcripts.

Preferably within this aspect, said nucleic acid construct used in step a) comprises a nucleotide sequence upstream or at the 5' site of the second intronic sequence of the invention that is different or distinct from the nucleotide sequence upstream or at the 5' site of the first intronic sequence of the invention. Preferably, the nucleotide sequence between first promoter and 5' of said first intronic sequence and the nucleotide sequence between second promoter and 5' of said second intronic sequence differs at least 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% in nucleotide sequence. Preferably, a method of this aspect of the invention wherein such a nucleic acid construct is used results in the production of two different or distinct transcripts, which differ in nucleotide sequence at the 5' site of the transcripts. In case the nucleotide sequence of interest is located downstream or at the 3' site of the second intronic sequence, the resulting transcripts will differ in sequence upstream of said nucleotide sequence of interest as can be detected by any suitable assay known by the person skilled in the art, such as, but not limited to 5'RACE-PCR.

In a preferred embodiment within this aspect, the nucleotide sequence of interest is a sequence encoding a protein or polypeptide of interest. The method of this aspect can be used to produce two proteins or polypeptides with different or distinct N-termini using the construct of the invention. Preferably, a first protein or polypeptide comprising a first N-terminus and a second protein or polypeptide comprising a second N-terminus are produced using the method of this aspect, wherein preferably, a first nucleotide sequence encoding said first N-terminus is located directly upstream or at the 5' site of said first intronic sequence and a second nucleotide sequence encoding said second N-terminus is located directly upstream or at the 5' site of said second intronic sequence. Preferably said first nucleotide sequence encoding said first N-terminus is located downstream or at the 3' site of said first promoter and upstream or at the 5' site of said first intronic sequence. Preferably said second nucleotide sequence encoding said second N-terminus is located downstream or at the 3' site of said second promoter and upstream or at the 5' site of said second intronic sequence. Preferably, said nucleic acid construct further comprises a nucleotide sequence encoding a C-terminus located downstream or at the 3' site of said second intronic sequence. In this embodiment, it is required that said second intronic sequence is an intron as defined in the Definition section. The difference between the N termini may be limited to a signal sequence and result in identical expressed proteins or polypeptides, wherein the localization of the proteins or polypeptides may differ. If performed in an expression system as earlier defined herein, the method of this embodiment preferably results in the production of two proteins or polypeptides of interest, wherein said first protein or polypeptide will comprise said first N-terminus linked to said C-terminus and said second protein or polypeptide will comprise said second N-terminus linked to said C-terminus, as can be detected by any suitable assay known by the person skilled in the art, such as, but not limited to, ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, said assay used to detect the two different or distinct proteins or polypeptides produced is specifically adapted to distinguish between the different

C-termini, as can be detected by any suitable assay known by the person skilled in the art, such as, but not limited to, ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, said assay used to detect the two different or distinct proteins or polypeptides produced is specifically adapted to distinguish between the different

proteins or polypeptides produced, for instance using a detecting antibody specifically binding to either the first or the second N-terminus of proteins or polypeptides produced.

Eight Aspect

In an eighth aspect, the present invention provides a use of a nucleic acid construct according to the first aspect of the invention, and/or a use of an expression vector according to the second aspect of the invention, and/or a use of a cell according to the third aspect of the invention, for the expression of a protein or polypeptide of interest.

Ninth Aspect

In a ninth aspect, the present invention provides for a nucleic acid construct according to the first aspect of the invention, and/or an expression vector according to the second aspect of the invention, and/or a cell according to the third aspect of the invention for use as a medicament. The invention also relates to a method of treatment comprising the administration of a nucleic acid construct according to the first aspect of the invention, and/or an expression vector according to the second aspect of the invention, and/or a cell according to the third aspect of the invention, wherein preferably said administration is to a mammal, more preferably to a human. Preferably, said treatment is nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human. Furthermore, the invention relates to the use of a nucleic acid construct according to the third aspect of the invention, and/or the use of an expression vector according to the second aspect of the invention, and/or the use of a cell according to the third aspect of the invention, for the preparation of a medicament. Preferably said medicament is for nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human.

Tenth Aspect

In a tenth aspect, the present invention provides a nucleic acid molecule that is represented by a nucleotide sequence that comprises or consists of an expression enhancing element for increasing transcription of a nucleotide sequence of interest and/or expression of a protein or polypeptide of interest. Preferably, the expression enhancing element of the invention is capable of increasing the transcription of a nucleotide sequence of interest and/or expression of a protein or polypeptide of interest. Preferably in this aspect, the expression enhancing element of the invention capable of increasing the transcription of a nucleotide sequence of interest and/or expression of a protein or polypeptide of interest is located upstream or at the 5' site of a promoter that is operably linked to said nucleotide sequence of interest.

Preferably within this aspect, transcription levels are established in an expression system using an expression construct comprising said expression enhancing element operably linked to a nucleotide sequence of interest using a suitable assay such a RT-qPCR. Preferably, the expression enhancing element of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to transcription levels using a construct which only differs in that it is free of said expression enhancing element, preferably as exemplified in the Examples which are enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising an expression enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest.

Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest measured under the same conditions except that the expression vector used is free of said expression enhancing element to be tested.

Preferably within this aspect, expression levels are established in an expression system using an expression construct comprising said expression enhancing element operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an enzyme-linked immunosorbent assay (ELISA) assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the expression enhancing element of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that it is free of said expression enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising an expression enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector is free of said expression enhancing element to be tested.

Preferably within this aspect, said nucleic acid molecule is an isolated nucleic acid molecule as defined herein. In a preferred embodiment, said expression enhancing element is a sequence that is derived from the UBC ubiquitin gene. Preferably, said expression enhancing element is derived from a mammalian UBC ubiquitin gene. More preferably, said expression enhancing element is derived from the *Cricetulus griseus* homologous gene of the human UBC ubiquitin gene, said gene being indicated as the *Cricetulus* sp. gene for polyubiquitin, or CRUPUQ (GenBank D63782).

In a preferred embodiment, said expression enhancing element derived from CRUPUQ comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-969 of SEQ ID NO: 1. Preferably, said sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-969 of SEQ ID NO: 1 is a promoter as defined in the Definition section. Preferably, said promoter is capable of initiating transcription of a nucleotide sequence of interest and/or expression of a polypeptide or protein or polypeptide of interest encoded by a nucleotide sequence in a host cell as defined herein below. In a further preferred embodiment, said expression enhancing element derived from CRUPUQ comprises or consists of an intronic sequence. An intronic sequence is understood to be at least

part of the nucleotide sequence of an intron. Preferably, said intronic sequence comprises at least a donor splice site or splice site GT. A donor splice site is understood herein as a splice site that, when combined with an acceptor splice site as defined herein, results in the formation of an intron as defined in the Definition section. Preferably, a nucleotide sequence is an intron if at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the primary RNA loses this sequence by RNA splicing using an assay suitable to detect intron splicing, such as but not limited to reverse-transcriptase polymerase chain reaction (RT-PCR) followed by size or sequence analysis of the RT-PCR. Preferred donor splice sites of the invention are M-W-G-[cut]-G-T-R-A-G-K in case the host cell is a mammalian cell, A-G-[cut]-G-T-A-W-K in case the host cell is a plant cell, [cut]-G-T-A-W-G-T-T in case the host cell is a yeast cell and R-G-[cut]-G-T-R-A-G, in case the host cell is an insect cell. “[cut]” is to be understood herein as the specific cut site where splicing will take place. Intron splicing can be assessed functionally using an assay as detailed in the Definition section under “intron”. Most preferably, the donor splice site comprised within the expression enhancing element of the invention is C-T-G-[cut]-G-T-G-A-G-G. Preferably, said intronic sequence encompassed within said expression enhancing element consists of said donor splice site or splice site GT. Preferably, said intronic sequence encompassed within said expression enhancing element is free of an acceptor splice site as defined herein below. Preferably, said expression enhancing element comprising an intronic sequence has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 970-1449 of SEQ ID NO: 1. In a preferred embodiment, said expression enhancing element comprises or consists of a promoter and an intronic sequence as defined herein. Preferably, the expression enhancing element of the invention has at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element of the invention that has at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1 over its whole length comprises both a promoter and an intronic sequence as defined herein. Preferably, said expression enhancing element for increasing expression is a contiguous sequence of at least 500, 600, 700, 800, 900, 1000, 1100 or 1117 in length, preferably at least 1449 nucleotides in length of SEQ ID NO: 1. Preferably, said expression enhancing element having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1 is at most 8000 nucleotides in length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 in length. Most preferably, said sequence being 1449 nucleotides in length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 65% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 70% identity to SEQ ID NO: 1 over its whole length. Preferably, said

expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 75% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 80% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 85% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 90% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1449 nucleotides in length and has at least 95% identity to SEQ ID NO: 1 over its whole length. Preferably, said expression enhancing element comprises or consists of a sequence that is represented by SEQ ID NO: 1.

In a further preferred embodiment within this aspect, said expression enhancing element of the invention is a sequence derived from the CCT8 gene. Preferably, said element is derived from a mammalian CCT8 gene. More preferably, said expression enhancing element is derived from the human or Homo sapiens CCT8 gene.

In a preferred embodiment within said aspect, said expression enhancing element derived from the Homo sapiens CCT8 gene comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-614 of SEQ ID NO: 2. Preferably, said sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 1-614 of SEQ ID NO: 2 is a promoter as defined in the Definition section. In a further preferred embodiment, said expression enhancing element derived from the Homo sapiens CCT8 gene comprises or consists of an intronic sequence. Preferably, said intronic sequence is an intronic sequence as earlier defined herein comprising at least a donor splice site or splice site GT as earlier defined herein. Preferably said donor splice site has the sequence M-A-R-[cut]-G-T-R-A-G-K, most preferably A-A-A-[cut]-G-T-G-A-G-G. Preferably, said intronic sequence encompassed within said expression enhancing element consists of said donor splice site or splice site GT. Preferably, said expression enhancing element comprising an intronic sequence has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to nucleotides 667-1228 of SEQ ID NO: 2.

In a preferred embodiment within said aspect, said expression enhancing element comprises or consists of a promoter and an intronic sequence as defined herein. Preferably, the expression enhancing element of the invention has at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element of the invention that has at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 2 over its whole length comprises both a promoter and an intronic sequence as defined herein. Preferably, said

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expression enhancing element for increasing expression is a contiguous sequence of at least 500, 600, 700, 791 or 1223 in length, preferably at least 1228 nucleotides in length of SEQ ID NO: 2. Preferably, said expression enhancing element having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 2 is at most 8000 nucleotides in length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 65% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 70% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 75% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 80% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 85% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 90% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element is at most 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1900, 1800, 1700, 1600, 1500 or 1228 nucleotides in length and has at least 95% identity to SEQ ID NO: 2 over its whole length. Preferably, said expression enhancing element comprises or consists of a sequence that is represented by SEQ ID NO: 2.

Further preferred is a nucleotide sequence comprising an expression enhancing element as defined herein that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to any of SEQ ID NO: 59-61 over its whole length. Preferably, said expression enhancing element comprises or consists of any of the sequences selected from the group consisting of SEQ ID NO: 59-61. Most preferably, said expression enhancing element comprises or consists of a sequence that is represented by SEQ ID NO: 59.

Eleventh Aspect

In an eleventh aspect, the present invention provides a nucleic acid construct comprising a nucleic acid molecule according to the tenth aspect of the invention. A nucleic acid construct of the invention comprises an expression enhancing element according to the tenth aspect of the present invention. Preferably, said nucleic acid construct is a recombinant and/or isolated construct as defined herein. Preferably, said nucleic acid construct further comprises a heterologous promoter, wherein preferably said expression enhancing element and said heterologous promoter are configured to be both operably linked to an optional nucleotide sequence of interest as defined herein below. "Heterologous promoter" is to be understood herein as a promoter that is not naturally operably linked to the expression enhancing

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element of the invention, i.e. a contiguous sequence comprising said expression enhancing element and said heterologous promoter does not occur in nature as neighboring sequences but can be synthesized as a recombinant sequence.

Preferably within this aspect, said heterologous promoter is located within a nucleic acid construct of the invention downstream or at the 3' site of the expression enhancing element of the invention. Preferably, said heterologous promoter is located within a construct of the invention upstream or at the 5' site of the nucleotide sequence of the invention encoding a protein or polypeptide of interest. In an embodiment of the invention the heterologous promoter is a promoter capable of initiating transcription in the host cell of choice. Heterologous promoters as used herein include tissue-specific, tissue-preferred, cell-type specific, inducible and constitutive promoters as defined herein. Heterologous promoters and/or regulating sequences that may be employed in expression of polypeptides according to the present invention, preferably in mammalian cells, include, but are not limited to, the human or murine cytomegalovirus (CMV) promoter, a simian virus (SV40) promoter, a human or mouse ubiquitin C promoter, a human or mouse or rat elongation factor alpha (EF1-a) promoter, mouse or hamster beta-actin promoter, or a hamster rpS21 promoter. The Tet-Off and Tet-On elements upstream of a minimal promoter such as a CMV promoter forms an example of an inducible mammalian promoter. Examples of suitable yeast and fungal promoters are Leu2 promoter, the galactose (Gall or Ga17) promoter, alcohol dehydrogenase I (ADH1) promoter, glucoamylase (Gla) promoter, triose phosphate isomerase (TPI) promoter, translational elongation factor EF-I alpha (TEF2) promoter, glyceraldehyde-3-phosphate dehydrogenase (gpdA) promoter, alcohol oxidase (AOX1) promoter, or glutamate dehydrogenase (gdhA) promoter. An example of a strong ubiquitous promoter for expression in plants is cauliflower mosaic virus (CaMV) 35S promoter. Preferably, the nucleic acid construct of the invention comprises a heterologous promoter that is represented by a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% to SEQ ID NO: 58. More preferably, the nucleic acid construct of this aspect of the invention comprises a heterologous promoter that is represented by a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% to SEQ ID NO: 57.

In a further preferred embodiment within this aspect, the nucleic acid construct of the invention further comprises one or more additional expression regulating elements, wherein preferably said expression enhancing element, said heterologous promoter and said one or more additional expression regulating elements are configured to be all operably linked to an optional nucleotide sequence of interest as defined herein below. An "additional expression regulating element" is to be understood herein as an element in addition to the expression enhancing element and/or promoter as defined herein above which may be an additional expression enhancing element or a distinct expression enhancing element or an expression regulating element in its broadest sense. An additional expression regulating element as encompassed by the present invention can be involved in the transcriptional and/or translational regulation of a gene, including but not limited to, 5'-UTR, 3'-UTR, enhancer, promoter, intronic sequence, polyadenylation signal and chromatin control elements such as scaffold/matrix attachment regions, ubiquitous chromatin opening element, cyto-

sine phosphodiester guanine pairs and stabilizing and anti-repressor elements, and any derivatives thereof. Other optional regulating elements that may be present in the nucleic acid construct of the invention include, but are not limited to, coding nucleotide sequences of homologous and/or heterologous nucleotide sequences, including the Iron Responsive Element (IRE), Translational cis-Regulatory Element (TLRE) or uORFs in 5' UTRs and poly(U) stretches in 3' UTRs.

Preferably within this aspect, said additional expression regulating element comprises or consists of an intronic sequence as defined herein. Preferably, the intronic sequence encompassed within the additional expression regulating element comprises at least of an acceptor splice site which is understood herein as to comprise the splice site AG preferably preceded by a polypyrimidine tract nucleotide sequence, optionally separated from splice site AG by 1-50 nucleotides, and optionally further comprising a branch site comprising the sequence Y-T-N-A-Y, at the 5' site of the polypyrimidine tract nucleotide sequence, wherein the branch site may have the nucleotide sequence C-Y-G-A-C. An acceptor splice site is understood herein as a splice site that, when combined with a donor splice site encompassed within a construct, results in the formation of an intron as defined in the Definition section. Preferably, the acceptor splice site or splice site AG has the sequence [Y-rich]-N-Y-A-G-[cut]. Preferably, the acceptor splice site or splice site AG has the sequence [Y-rich]-N-Y-A-G-[cut]-R in case the host cell is a mammalian cell, [Y-rich]-D-Y-A-G-[cut]-R or [Y-rich]-D-Y-A-G-[cut]-R-W in case the host cell is a plant cell, [Y-rich]-A-Y-A-G-[cut] in case the host cell is a yeast cell and [Y-rich]-N-Y-A-G-[cut] in case the host cell is an insect cell. “[Y-rich]” is to be understood herein as a polypyrimidine tract which is preferably defined as a consecutive sequence of at least 10 nucleotides comprising at least 6, 7, 8, 9 or preferably 10 pyrimidine nucleotides. Preferably, said acceptor splice site or splice site GT has the sequence Y-A-G-[cut]-R. Preferably, said intronic sequence encompassed within said additional expression regulating element consists of said acceptor splice site or splice site AG. The intronic sequence preferably comprises or consists of a nucleotide sequence having at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to nucleotides 171-277 of SEQ ID NO: 14, nucleotides 171-274 of SEQ ID NO: 19, nucleotides 133-210 of SEQ ID NO: 20, nucleotides 134-211 of SEQ ID NO: 21, nucleotides 134-226 of SEQ ID NO: 22, nucleotides 134-226 of SEQ ID NO: 23, nucleotides 133-225 of SEQ ID NO: 24, nucleotides 134-226 of SEQ ID NO: 25, nucleotides 146-257 of SEQ ID NO: 26, or nucleotides 147-223 of SEQ ID NO: 27, or nucleotides 970-1449 of SEQ ID NO: 1 or nucleotides 667-1228 of SEQ ID NO: 2. Preferably, said intronic sequence comprised within said additional expression regulating element further comprises a donor splice site as defined herein. Even more preferred, said intronic sequence encompassed within the additional expression regulating element is an intron as defined in the Definition section. Most preferably, the intronic sequence encompassed within the additional expression regulating element comprises or consists of a nucleotide sequence having at least 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to nucleotides 171-277 of SEQ ID NO: 14, nucleotides 171-274 of SEQ ID NO: 19, nucleotides 133-210 of SEQ ID NO: 20, nucleotides 134-211 of SEQ ID NO: 21, nucleotides 134-226 of SEQ ID NO: 22, nucleotides 134-226 of SEQ ID NO: 23, nucleotides 133-225 of SEQ ID NO: 24, nucleotides 134-226 of SEQ ID NO: 25, nucleotides 146-257 of SEQ ID NO: 26, or nucleotides 147-223 of SEQ ID NO: 27, or nucleotides 970-1449 of SEQ ID NO: 1 or nucleotides 667-1228 of SEQ ID NO: 2.

NO: 25, nucleotides 146-257 of SEQ ID NO: 26, or nucleotides 147-223 of SEQ ID NO: 27.

Also preferred within this aspect is an expression regulating element that is a translation enhancing element. Preferably, a translation enhancing element allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that it is free of said translation enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a translation enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector is free of said translation enhancing element to be tested.

Preferably within this aspect, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 3-51 over its whole length. Preferably, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 19 over its whole length. More preferably, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 90% identity to SEQ ID NO: 3-51 over its whole length. Also preferred within this aspect is a translation enhancing element that comprises or consists of a nucleotide sequence that comprises:

- i) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides;
 - ii) a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, and a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, said expression enhancing element not comprising a GAA repeat nucleotide sequence; or,
 - iii) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, wherein said GAA repeat nucleotide sequence is located 3' of any one or more of said TC-rich nucleotide sequence, A-rich nucleotide sequences, and/or GT-rich nucleotide sequence.

The GAA repeat nucleotide sequence, the TC-rich nucleotide sequence, the A-rich nucleotide sequence, the GT-rich

nucleotide sequence have already been defined herein in the first aspect of the invention. These definitions also applied here.

Preferably within said aspect, said additional expression regulating element comprises a translation enhancing element as defined herein and an intronic sequence.

Preferably within said aspect, said additional expression regulating element is located within a nucleic acid construct of the invention downstream or the 3' site of a heterologous promoter. Preferably, said additional expression regulating element is located within a nucleic acid construct of the invention upstream or at the 5' site of a nucleic acid sequence encoding a protein or polypeptide of interest. Moreover, preferably a nucleic acid construct of the invention comprises the following nucleotide sequences indicated here in their relative positions in the 5' to 3' direction: (i) an expression enhancing element, (ii) a heterologous promoter, optionally (iii) an additional expression regulating element, and optionally (iv) a nucleotide sequence of interest, wherein preferably said expression enhancing element, said heterologous promoter and said additional expression regulating element are configured to be all operably linked to said optional nucleotide sequence of interest as defined herein below. It is to be understood that said expression enhancing element, said heterologous promoter, and optionally said additional expression regulating element of the nucleic acid construct of the invention are all configured to be operably linked to the same, single nucleotide sequence of interest.

The inventors found an unexpected synergistic effect when the expression enhancing element of the invention is combined with an additional expression regulating element as defined herein in an expression construct for expressing a protein or polypeptide of interest. In a stably transfected pool with both an expression enhancing element and an additional expression regulating element, the protein yield was significantly higher than the yield expected based on addition of the separate effects of either element. Preferably, said increase of protein or polypeptide expression is copy number independent as established by an assay suitable to determine copy number dependency by a skilled person, such as, but not limit to, a triplex Taqman assay as further detailed in Example 4 of the present invention. Preferably, said nucleic acid construct is a recombinant and/or isolated construct as defined herein. Preferably, said nucleic acid construct further comprises a nucleotide sequence of interest operably linked to and/or under the control of said expression enhancing element, said heterologous promoter and optionally said additional expression regulating element as defined herein. The presence of a nucleotide sequence of interest is optional. "Optional" is to be understood herein as not necessarily being present in an expression construct. For instance, such nucleotide sequence of interest need not be present in a commercialized expression vector, but may be readily introduced by a person skilled in the art before use in a method of the invention. It is to be understood that said expression enhancing element, said heterologous promoter, and optionally said additional expression regulating element are all configured to be operably linked to the same, single nucleotide sequence of interest. Preferably, said nucleic acid construct of the tenth aspect of the invention has at least 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO 73, 74, 75 or 76.

In a preferred embodiment within this aspect, said nucleotide sequence of interest is a nucleotide sequence encoding

a protein or polypeptide of interest. The protein or polypeptide of interest can be a homologous protein or polypeptide, but in a preferred embodiment of the invention the protein or polypeptide of interest is a heterologous protein or polypeptide. A nucleotide sequence encoding a heterologous protein or polypeptide may be derived in whole or in part from any source known to the art, including a bacterial or viral genome or episome, eukaryotic nuclear or plasmid DNA, cDNA or chemically synthesised DNA. The nucleotide sequence encoding a protein or polypeptide of interest may constitute an uninterrupted coding region or it may include one or more introns bounded by appropriate splice junctions. It can further be composed of segments derived from different sources, naturally occurring or synthetic. The nucleotide sequence encoding the protein or polypeptide of interest according to the method of the invention is preferably a full-length nucleotide sequence, but can also be a functionally active part or other part of said full-length nucleotide sequence. The nucleotide sequence encoding the protein or polypeptide of interest may also comprise signal sequences directing the protein or polypeptide of interest when expressed to a specific location in the cell or tissue. Furthermore, the nucleotide sequence encoding the protein or polypeptide of interest can also comprise sequences which facilitate protein purification and protein detection by for instance Western blotting and ELISA (e.g. c-myc or polyhistidine sequences).

The protein or polypeptide of interest in this aspect has already been defined earlier herein in the first aspect of the invention.

In an alternative embodiment, said nucleotide sequence of interest is not a coding sequence for a protein or a polypeptide but may be a functional nucleotide sequence. This alternative embodiment of this aspect has already been defined earlier herein in the first aspect of the invention.

Twelfth Aspect

In a twelfth aspect, the present invention provides an expression vector comprising a nucleic acid construct according to the eleventh aspect of the invention. The expression vector of the invention preferably is a plasmid, cosmid or phage or nucleotide sequence, linear or circular, of a single or double stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing any one of the nucleotide sequences of the invention in sense or antisense orientation into a cell. The choice of vector is dependent on the recombinant procedures followed and the host cell used. The vector may be an autonomously replicating vector or may replicate together with the chromosome into which it has been integrated. Preferably, the vector contains a selection marker. Useful markers are dependent on the host cell of choice and are well known to persons skilled in the art and are selected from, but not limited to, the selection markers as defined in third aspect of the invention. A preferred expression vector is the pcDNA3.1 expression vector. Preferred selection markers are the neomycin resistance gene, zeocin resistance gene and blasticidin resistance gene.

Thirteenth Aspect

In a thirteenth aspect, the present invention provides a cell comprising a nucleic acid molecule according to the tenth aspect of the invention, and/or a nucleic acid construct according to the eleventh aspect of the invention, and/or an expression vector according to the twelfth aspect of the invention as defined herein. The type of cell within the context of this aspect is the same as the one defined in the context of the third aspect. Therefore, another aspect of the

invention relates to a host cell that is genetically modified, preferably by a method of the invention, in that a host cell comprises a nucleic acid construct as defined above in the thirteenth aspect. For transformation procedures in plants, suitable bacteria include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

A nucleic acid construct within the context of this thirteenth aspect is as the one of the third aspect: it is preferably stably maintained, either as an autonomously replicating element, or, more preferably, the nucleic acid construct is integrated into the host cell's genome, in which case the construct is usually integrated at random positions in the host cell's genome, for instance by non-homologous recombination. Stably transformed host cells are produced by known methods. The definition of the term stable transformation and methods encompassed for stable transformation have already been provided under the third aspect.

Alternatively, a protein or polypeptide of interest may be expressed in a host cell, e.g., a mammalian cell, relying on transient expression from vectors.

A nucleic acid construct according to this aspect preferably also comprises a marker gene which can provide selection or screening capability in a treated host cell.

All definitions relating to selectable markers and types of selectable markers including the example of the use of the luciferase gene as selectable marker, the example of a first category of marker based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media, the example of dominant selection have already been provided in the third aspect. They also apply here in the thirteenth aspect of the invention.

When a transformed host cell is obtained with a method according to the invention (see below), a host tissue may be regenerated from said transformed cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells.

Resulting transformed host tissues are preferably identified by means of selection using a selection marker gene as present on a nucleic acid construct as defined herein.

Fourteenth Aspect

In a fourteenth aspect, the present invention provides a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- a. providing a nucleic acid construct according to the eleventh aspect of the invention comprising a nucleotide sequence encoding a protein or polypeptide of interest; and,
- b. contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c. allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- d. purifying said protein or polypeptide of interest.

In a preferred embodiment of the method according to the invention, a nucleic acid construct as defined above in the eleventh aspect of the invention is used. The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention may be applied on a cell culture, organism culture, or tissue culture. Alternatively, next to the expression in host cells the protein or polypeptide of interest can be produced in cell-free translation systems using RNAs derived from the nucleic acid constructs of the present invention. The method of the invention may be performed on cultured cells.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified

DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to express the protein or polypeptide of interest, and optionally said protein or polypeptide is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to expression of the protein or polypeptide of interest. The person skilled in the art is well aware of techniques to be used for expressing or overexpressing the protein or polypeptide of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to expression of the protein or polypeptide of interest, but in which the protein or polypeptide of interest is automatically (e.g., constitutively) expressed, are also included in the method of the present invention.

Purification steps and definitions related to these steps as 20 the definition of an isolated protein or polypeptide are the same as in the method of the fourth aspect and have been earlier defined herein. If desired as defined in the method of 25 the fourth aspect, the nucleotide sequence encoding a protein or polypeptide of interest may be ligated to a heterologous nucleotide sequence to encode a fusion protein or polypeptide to facilitate protein purification and protein detection on for instance Western blot and in an ELISA. Suitable heterologous sequences include, but are not limited to, the nucleotide sequences coding for proteins such as for 30 instance glutathione-S-transferase, maltose binding protein, metal-binding polyhistidine, green fluorescent protein, luciferase and beta-galactosidase. The protein or polypeptide may also be coupled to non-peptide carriers, tags or labels that facilitate tracing of the protein or polypeptide, 35 both *in vivo* and *in vitro*, and allow for the identification and quantification of binding of the protein or polypeptide to substrates. Such labels, tags or carriers are well-known in the art and include, but are not limited to, biotin, radioactive labels and fluorescent labels.

40 Preferably, the method of this fourteenth aspect of the invention allows for an increase in expression of a protein or polypeptide of interest. Preferably, expression levels are established in an expression system using an expression construct according to the eleventh aspect of the invention comprising an expression enhancing element and a heterologous promoter operably linked to a nucleotide sequence 45 encoding a protein or polypeptide of interest according to the eleventh aspect of the invention. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in 50 the art. Preferably, the method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct 55 is used in step a) that it is free of said expression enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) 60 is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising an expression enhancing element to be tested and a CMV 65

promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector is free of said expression enhancing element to be tested. Preferably, said increase of protein or polypeptide expression is copy number independent as established by an assay suitable to determine copy number dependency by a skilled person such as, but not limit to, a triplex Taqman assay as further detailed in Example 4 of the present invention.

Fifteenth Aspect

In a fifteenth aspect, the present invention provides a method for expressing a protein or polypeptide of interest in an organism, comprising the steps of:

- a) providing a nucleic acid construct according to the eleventh aspect comprising a nucleotide sequence encoding a protein or polypeptide of interest of the invention; and,
- b) contacting a target cell and/or target tissue of an organism, with said nucleic acid construct to obtain a transformed target cell and/or transformed target tissue, allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- c) allowing said transformed target cell to develop into a transformed organism; and, optionally,
- d) allowing said transformed organism to express the protein or polypeptide of interest, for example, subjecting said transformed organism to conditions leading to expression of the protein or polypeptide of interest, and optionally recovering said protein or polypeptide.

The target cell may be an embryonal target cell, e.g., embryonic stem cell, for example, derived from a non-human mammalian, such as *bovine*, *porcine*, etcetera species. Preferably, said target cell is not a human embryonic stem cell. In the case of a multicellular fungus, such target cell may be a fungal cell that can be proliferated into said multicellular fungus. When a transformed plant tissue or plant cell (e.g., pieces of leaf, stem segments, roots, but also protoplasts or plant cells cultivated by suspension) is obtained with this method according to the invention, whole plants can be regenerated from said transformed tissue or cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells. This method of the invention may be applied in nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human. Encompassed within the present invention is a method of treatment comprising the method of the present aspect, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. The invention also relates to a construct of the eleventh aspect of the invention for treatment, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. Furthermore, the invention relates to the use of a construct of the eleventh aspect of the invention for the manufacture of a medicament, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide.

Furthermore, an embodiment of the invention is a non-human transformed organism. Said organism is transformed with a nucleotide sequence, recombinant nucleic acid construct, or vector according to the present invention, and is capable of producing the polypeptide of interest. This includes a non-human transgenic organism, such as a trans-

genic non-human mammalian, transgenic plant (including propagation, harvest and tissue material of said transgenic plant, including, but not limited to, leafs, roots, shoots and flowers), multicellular fungus, and the like.

- 5 5 Preferably, the method of this aspect of the invention allows for an increase in expression of a protein or polypeptide of interest in said organism or at least in one tissue or organelle or organ of said organism. Preferably, expression levels are established in an expression system using an 10 expression construct according to the eleventh aspect of the invention comprising an expression enhancing element and a heterologous promoter operably linked to a nucleotide sequence encoding a protein or polypeptide of interest according to the eleventh aspect of the invention. Preferably, 15 said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, this method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% in said organism or at least in one tissue or organelle or organ of said organism, as compared to a method which only differs in that a construct is used in step a) that it is free of said expression enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression 20 of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising an expression 25 enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are 30 compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector is free of said expression enhancing element to be tested.

- 35 40 Preferably, said increase of protein or polypeptide expression is copy number independent as established by an assay suitable to determine copy number dependency by a skilled person such as, but not limit to, a triplex Taqman assay as further detailed in Example 4 of the present invention.

Sixteenth Aspect

In a sixteenth aspect, the present invention provides a method for transcription and optionally purifying the produced transcript comprising the step of:

- 45 50 a) providing a nucleic acid construct according to the eleventh aspect comprising a nucleotide sequence of interest of the invention; and,
- 55 b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- d) purifying said produced transcript.

In a preferred embodiment of this method according to the invention a nucleic acid construct as defined above in the eleventh aspect is used. The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention 60 may be applied on a cell culture, organism culture, or tissue culture. The method of the invention may be applied in nucleic acid based vaccination and/or gene therapy prefer-

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ably in a mammal, preferably in a human. Encompassed within the present invention is a method for treatment comprising or consisting of the method of the present aspect, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. The invention also relates to a construct of the eleventh aspect of the invention for use in treatment, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. Furthermore, the invention relates to the use of a construct of the eleventh aspect of the invention for the manufacture of a medicament, wherein the nucleotide sequence of interest encodes for a therapeutic transcript.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to produce a transcript of the nucleotide sequence of interest, and optionally the produced transcript is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to transcription the nucleotide sequence of interest. The person skilled in the art is well aware of techniques to be used for transcription the nucleotide sequence of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to transcription of the nucleotide sequence of interest, but in which the nucleotide sequence of interest is automatically (e.g., constitutively) transcribed, are also included in the method of the present invention.

Purification steps depend on the transcript produced. The term "isolation" indicates that the transcript is found in a condition other than its native environment. In a preferred form, the isolated transcript is substantially free of other cellular components, particularly other homologous cellular components such as homologous proteins. It is preferred to provide the transcript in a greater than 40% pure form, more preferably greater than 60% pure form. Even more preferably it is preferred to provide the transcript in a highly purified form, i.e., greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by Northern blot.

Preferably, the method of this aspect of the invention allows for an increase in transcription of a nucleotide sequence of interest. Preferably, transcription levels are established in an expression system using an expression construct according to the second aspect of the invention comprising an expression enhancing element and a heterologous promoter operably linked to a nucleotide sequence of interest according to the second aspect of the invention. Preferably, transcription of said nucleotide sequence of interest is detected by a suitable assay such as RT-qPCR. Preferably, the method of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct is used in step a) that it is free of said expression enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising

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an expression enhancing element to be tested and a CMV promoter represented by SEQ ID NO: 57, operably linked to said nucleotide sequence of interest. Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest measured under the same conditions except that the expression vector used is free of said expression enhancing element to be tested.

Seventeenth Aspect

Seventeenth Aspect

10 In an seventeenth aspect, the present invention provides a use of a nucleic acid molecule according to the tenth aspect of the invention, and/or a use of a nucleic acid construct according to the eleventh aspect of the invention, and/or a use of an expression vector according to the twelfth aspect, 15 of the invention, and/or a use of a cell according to the thirteenth aspect of the invention, for the transcription of a nucleotide sequence of interest and/or the expression of a protein or polypeptide of interest.

Eighteenth Aspect

20 In a eighteenth aspect, the present invention provides for
a nucleic acid molecule according to according to the tenth
aspect of the invention, and/or a nucleic acid construct
according to the eleventh aspect of the invention, and/or an
expression vector according to the twelfth aspect of the
invention, and/or a cell according to the thirteenth aspect of
the invention for use as a medicament. The invention also
relates to a method of treatment comprising the administra-
tion of a nucleic acid molecule according to the tenth aspect
of the invention, and/or a nucleic acid construct according to
30 the eleventh aspect of the invention, and/or an expression
vector according to the twelfth aspect of the invention,
and/or a cell according to the thirteenth aspect of the
invention, wherein preferably said administration is to a
mammal, more preferably to a human. Preferably, said
35 treatment is nucleic acid based vaccination and/or gene
therapy preferably in a mammal, most preferably in a
human. Furthermore, the invention relates to the use of a
nucleic acid molecule according to according to the tenth
aspect of the invention, and/or the use of a nucleic acid
40 construct according to the eleventh aspect of the invention,
and/or the use of an expression vector according to the
twelfth aspect of the invention, and/or the use of a cell
according to the thirteenth aspect of the invention, for the
preparation of a medicament. Preferably said medicament is
45 for nucleic acid based vaccination and/or gene therapy
preferably in a mammal, most preferably in a human.

Nineteenth Aspect

In a nineteenth aspect, the present invention provides a nucleic acid molecule that is represented by a nucleotide sequence that has at least 50% identity with SEQ ID NO: 88 for increasing transcription of a nucleotide sequence of interest and/or expression of a protein or polypeptide of interest. Within the context of the nineteenth to twenty seventh aspect, said identity percentage is preferably assessed over the whole length of SEQ ID NO:88. However, it is not excluded that said identity percentage is assessed over part of SEQ ID NO:88 as defined in the section entitled definitions. Preferably, said nucleotide sequence of the invention is capable of increasing the transcription of a nucleotide sequence of interest and/or expression of a protein or polypeptide of interest. Said nucleic acid molecule represented by a nucleotide sequence that has at least 50% identity with SEQ ID NO:88 may be called a transcription regulating sequence.

65 Preferably within this aspect, transcription levels are established in an expression system using an expression construct comprising said nucleotide sequence operably

linked to a nucleotide sequence of interest using a suitable assay such a RT-qPCR. Preferably, the nucleotide sequence of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to transcription levels using a construct wherein the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably as exemplified in example 11 which is enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising the nucleotide sequence of the invention operably linked to said nucleotide sequence of interest. Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest measured under the same conditions except that in the expression vector used the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably as exemplified in example 11 which is enclosed herein.

Preferably within this aspect, expression levels are established in an expression system using an expression construct comprising said nucleotide sequence having at least 50% identity with SEQ ID NO:88 and which is operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an enzyme-linked immunosorbent assay (ELISA) assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the nucleotide sequence having at least 50% identity with SEQ ID NO:88 allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that said nucleotide sequence has been replaced by an alternative sequence, preferably when tested in a system as exemplified in example 11 which is enclosed herein. More specifically, preferably the expression of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said isolated nucleic acid molecule as defined herein. In a preferred embodiment, said nucleotide sequence having at least 50% identity with SEQ ID NO:88 is a sequence that is derived from the UBC ubiquitin gene. Preferably, said nucleotide sequence is derived from a mammalian UBC ubiquitin gene. More preferably, said nucleotide sequence is derived from the *Cricetulus griseus* homologous gene of the human UBC ubiquitin gene, said gene being indicated as the *Cricetulus* sp. gene for polyubiquitin, or CRUPUQ (GenBank D63782).

In a preferred embodiment, said nucleotide sequence comprises or consists of a sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%,

93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with SEQ ID NO: 88 over its whole length. Preferably, said sequence having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with SEQ ID NO: 88 over its whole length comprises a promoter as defined in the Definition section. Preferably, said promoter is capable of initiating transcription of a nucleotide sequence of interest and/or expression of a polypeptide or protein or polypeptide of interest encoded by a nucleotide sequence in a host cell as defined herein below. Preferably, said nucleotide sequence for increasing expression is a contiguous sequence of at least 1450, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 2600 or 2617 in length, preferably at least 2617 nucleotides in length of SEQ ID NO: 88. Preferably, said nucleotide sequence having at least 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 88 is at most 8000 nucleotides in length. Preferably, said nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2617 in length. Most preferably, said sequence being 2617 nucleotides in length. Preferably, nucleotide sequence is at most 8000, 7000, 6000, 5000, 4000, 3000, 2617 nucleotides in length and has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% identity to SEQ ID NO: 88 over its whole length.

Twentieth Aspect

In a twentieth aspect, the present invention provides a nucleic acid construct comprising a nucleic acid molecule according to the nineteenth aspect of the invention. A nucleic acid construct of the invention comprises a nucleotide sequence according to the nineteenth aspect of the present invention. Preferably, said nucleic acid construct is a recombinant and/or isolated construct as defined herein. Preferably, said nucleic acid construct further comprises an optional nucleotide sequence of interest as defined herein below wherein the nucleotide sequence of the invention is operably linked to said optional nucleic acid sequence of interest.

In a further preferred embodiment within this aspect, the nucleic acid construct of the invention further comprises one or more additional expression regulating elements, wherein preferably said nucleotide sequence and said one or more additional expression regulating elements are configured to be all operably linked to an optional nucleotide sequence of interest as defined herein below. An “additional expression regulating element” is to be understood herein as an element in addition to the nucleotide sequence as defined herein above which may be an additional expression regulating element or a distinct expression regulating element or an additional expression enhancing element or a distinct expression enhancing element. An additional expression regulating element as encompassed by the present invention can be involved in the transcriptional and/or translational regulation of a gene, including but not limited to, 5'-UTR, 3'-UTR, enhancer, promoter, intron, polyadenylation signal and chromatin control elements such as scaffold/matrix attachment regions, ubiquitous chromatin opening element, cytosine phosphodiester guanine pairs and stabilizing and anti-repressor elements, and any derivatives thereof. Other optional regulating elements that may be present in the nucleic acid construct of the invention include, but are not limited to, coding nucleotide sequences of homologous and/or heterologous nucleotide sequences, including the

Iron Responsive Element (IRE), Translational cis-Regulatory Element (TLRE) or uORFs in 5' UTRs and poly(U) stretches in 3' UTRs.

Also preferred within this aspect is an expression regulating element that is a translation enhancing element. Preferably, a translation enhancing element allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression of said protein or polypeptide using a construct which only differs in that it is free of said translation enhancing element, preferably when tested in a system as exemplified in the Examples which are enclosed herein. More specifically, preferably the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a translation enhancing element to be tested and a nucleotide sequence having at least 50% identity with SEQ ID NO: 88, operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that the expression vector is free of said translation enhancing element to be tested.

Preferably within this aspect, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 3-51 over its whole length. Preferably, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 19 over its whole length. More preferably, said translation enhancing element comprises or consists of a nucleotide sequence that has at least 90% identity to SEQ ID NO: 3-51 over its whole length. Also preferred within this aspect is a translation enhancing element that comprises or consists of a nucleotide sequence that comprises:

- i) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides;
- ii) a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, and a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, said expression enhancing element not comprising a GAA repeat nucleotide sequence; or,
- iii) a GAA repeat nucleotide sequence, a TC-rich nucleotide sequence comprising at least 8 consecutive C or T nucleotides, at least 3 A-rich nucleotide sequences comprising at least 5 consecutive A nucleotides, a GT-rich nucleotide sequence comprising at least 10 nucleotides, at least 80% of which are G or T nucleotides, wherein said GAA repeat nucleotide sequence is located 3' of any one or more of said TC-rich nucleotide sequence, A-rich nucleotide sequences, and/or GT-rich nucleotide sequence.

The GAA repeat nucleotide sequence, the TC-rich nucleotide sequence, the A-rich nucleotide sequence, the GT-rich nucleotide sequence have already been defined herein in the first aspect of the invention. These definitions also applied here.

Preferably within said aspect, said additional expression regulating element is located within a nucleic acid construct of the invention having at least 50% identity with SEQ ID NO: 88. Preferably, said additional expression regulating element is located within a nucleic acid construct of the invention upstream or at the 5' site of a nucleic acid sequence encoding a protein or polypeptide of interest. Moreover, preferably a nucleic acid construct of the invention comprises the following nucleotide sequences indicated here in their relative positions in the 5' to 3' direction:

- optionally (i) an expression regulating preferably enhancing element,
- (ii) a nucleotide sequence having at least 50% identity with SEQ ID NO:88,
- optionally (iii) an additional expression regulating element, and
- optionally (iv) a nucleotide sequence of interest, wherein preferably said expression enhancing element, said nucleotide sequence having at least 50% identity with SEQ ID NO:88 and said additional expression regulating element are configured to be all operably linked to said optional nucleotide sequence of interest as defined herein below. It is to be understood that said expression enhancing element, said nucleotide sequence having at least 50% identity with SEQ ID NO:88, and optionally said additional expression regulating element of the nucleic acid construct of the invention are all configured to be operably linked to the same, single nucleotide sequence of interest.

The presence of a nucleotide sequence of interest is optional. "Optional" is to be understood herein as not necessarily being present in an expression construct. For instance, such nucleotide sequence of interest need not be present in a commercialized expression vector, but may be readily introduced by a person skilled in the art before use in a method of the invention. It is to be understood that said expression enhancing element, said nucleotide sequence having at least 50% identity with SEQ ID NO:88, and optionally said additional expression regulating element are all configured to be operably linked to the same, single nucleotide sequence of interest.

In a preferred embodiment within this aspect, said nucleotide sequence of interest is a nucleotide sequence encoding a protein or polypeptide of interest. The protein or polypeptide of interest can be a homologous protein or polypeptide, but in a preferred embodiment of the invention the protein or polypeptide of interest is a heterologous protein or polypeptide. A nucleotide sequence encoding a heterologous protein or polypeptide may be derived in whole or in part from any source known to the art, including a bacterial or viral genome or episome, eukaryotic nuclear or plasmid DNA, cDNA or chemically synthesised DNA. The nucleotide sequence encoding a protein or polypeptide of interest may constitute an uninterrupted coding region or it may include one or more introns bounded by appropriate splice junctions. It can further be composed of segments derived from different sources, naturally occurring or synthetic. The nucleotide sequence encoding the protein or polypeptide of interest according to the method of the invention is preferably a full-length nucleotide sequence, but can also be a functionally active part or other part of said full-length nucleotide sequence. The nucleotide sequence encoding the protein or polypeptide of interest may also comprise signal

sequences directing the protein or polypeptide of interest when expressed to a specific location in the cell or tissue. Furthermore, the nucleotide sequence encoding the protein or polypeptide of interest can also comprise sequences which facilitate protein purification and protein detection by for instance Western blotting and ELISA (e.g. c-myc or polyhistidine sequences).

The protein or polypeptide of interest in this aspect has already been defined earlier herein in the first aspect of the invention.

In an alternative embodiment, said nucleotide sequence of interest is not a coding sequence for a protein or a polypeptide but may be a functional nucleotide sequence. This alternative embodiment of this aspect has already been defined earlier herein in the first aspect of the invention.

Twenty First Aspect

In a twenty first aspect, the present invention provides an expression vector comprising a nucleic acid construct according to the twentieth aspect of the invention. The expression vector of the invention preferably is a plasmid, cosmid or phage or nucleotide sequence, linear or circular, of a single or double stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing any one of the nucleotide sequences of the invention in sense or antisense orientation into a cell. The choice of vector is dependent on the recombinant procedures followed and the host cell used. The vector may be an autonomously replicating vector or may replicate together with the chromosome into which it has been integrated. Preferably, the vector contains a selection marker. Useful markers are dependent on the host cell of choice and are well known to persons skilled in the art and are selected from, but not limited to, the selection markers as defined in third aspect of the invention. A preferred expression vector is the pcDNA3.1 expression vector. Preferred selection markers are the neomycin resistance gene, zeocin resistance gene and blasticidin resistance gene.

Twenty Second Aspect

In a twenty second aspect, the present invention provides a cell comprising a nucleic acid molecule according to the nineteenth aspect of the invention, and/or a nucleic acid construct according to the twentieth aspect of the invention, and/or an expression vector according to the twenty first aspect of the invention as defined herein. The type of cell within the context of this aspect is the same as the one defined in the context of the third aspect.

Therefore, another aspect of the invention relates to a host cell that is genetically modified, preferably by a method of the invention, in that a host cell comprises a nucleic acid construct as defined above in the twentieth aspect. For transformation procedures in plants, suitable bacteria include *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*.

A nucleic acid construct within the context of this twentieth aspect is as the one of the third aspect: it is preferably stably maintained, either as an autonomously replicating element, or, more preferably, the nucleic acid construct is integrated into the host cell's genome, in which case the construct is usually integrated at random positions in the host cell's genome, for instance by non-homologous recombination. Stably transformed host cells are produced by known methods. The definition of the term stable transformation and methods encompassed for stable transformation have already been provided under the third aspect.

Alternatively, a protein or polypeptide of interest may be expressed in a host cell, e.g., a mammalian cell, relying on transient expression from vectors.

A nucleic acid construct according to this aspect preferably also comprises a marker gene which can provide selection or screening capability in a treated host cell.

All definitions relating to selectable markers and types of selectable markers including the example of the use of the luciferase gene as selectable marker, the example of a first category of marker based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow independent of a supplemented media, the example of dominant selection have already been provided in the third aspect. They also apply here in the thirteenth aspect of the invention.

When a transformed host cell is obtained with a method according to the invention (see below), a host tissue may be regenerated from said transformed cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells.

Resulting transformed host tissues are preferably identified by means of selection using a selection marker gene as present on a nucleic acid construct as defined herein.

Twenty Third Aspect

In a twenty third aspect, the present invention provides a method for expressing and optionally purifying a protein or polypeptide of interest comprising the step of:

- a. providing a nucleic acid construct according to the twentieth aspect of the invention comprising a nucleotide sequence encoding a protein or polypeptide of interest; and,
- b. contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c. allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- d. purifying said protein or polypeptide of interest.

In a preferred embodiment of the method according to the invention, a nucleic acid construct as defined above in the twentieth aspect of the invention is used. The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention may be applied on a cell culture, organism culture, or tissue culture. Alternatively, next to the expression in host cells the protein or polypeptide of interest can be produced in cell-free translation systems using RNAs derived from the nucleic acid constructs of the present invention. The method of the invention may be performed on cultured cells.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microinjection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to express the protein or polypeptide of interest, and optionally said protein or polypeptide is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to expression of the protein or polypeptide of interest. The person skilled in the art is well aware of techniques to be used for expressing or overexpressing the protein or polypeptide of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to expression of the protein or polypeptide of interest, but in which the protein or polypeptide of interest is automatically

(e.g., constitutively) expressed, are also included in the method of the present invention.

Purification steps and definitions related to these steps as the definition of an isolated protein or polypeptide are the same as in the method of the fourth aspect and have been earlier defined herein. If desired as defined in the method of the fourth aspect, the nucleotide sequence encoding a protein or polypeptide of interest may be ligated to a heterologous nucleotide sequence to encode a fusion protein or polypeptide to facilitate protein purification and protein detection on for instance Western blot and in an ELISA. Suitable heterologous sequences include, but are not limited to, the nucleotide sequences coding for proteins such as for instance glutathione-S-transferase, maltose binding protein, metal-binding polyhistidine, green fluorescent protein, luciferase and beta-galactosidase. The protein or polypeptide may also be coupled to non-peptide carriers, tags or labels that facilitate tracing of the protein or polypeptide, both in vivo and in vitro, and allow for the identification and quantification of binding of the protein or polypeptide to substrates. Such labels, tags or carriers are well-known in the art and include, but are not limited to, biotin, radioactive labels and fluorescent labels.

Preferably, the method of this twenty third aspect of the invention allows for an increase in expression of a protein or polypeptide of interest. Preferably, expression levels are established in an expression system using an expression construct according to the twenty first aspect of the invention comprising a nucleotide sequence having at least 50% identity with SEQ ID NO: 88 operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, the method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct is used in step a) that in said construct the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11, more preferably when tested in a system as exemplified in example 11 which is enclosed herein. More specifically, preferably the expression of nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that in the expression vector the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11.

Twenty Fourth Aspect

In a twenty fourth aspect, the present invention provides a method for expressing a protein or polypeptide of interest in an organism, comprising the steps of:

- providing a nucleic acid construct according to the twentieth aspect comprising a nucleotide sequence encoding a protein or polypeptide of interest of the invention; and,
- contacting a target cell and/or target tissue of an organism, with said nucleic acid construct to obtain a transformed target cell and/or transformed target tissue, allowing said transformed cell to express the protein or polypeptide of interest; and optionally,
- allowing said transformed target cell to develop into a transformed organism; and, optionally,
- allowing said transformed organism to express the protein or polypeptide of interest, for example, subjecting said transformed organism to conditions leading to expression of the protein or polypeptide of interest, and optionally recovering said protein or polypeptide.

The target cell may be an embryonal target cell, e.g., embryonic stem cell, for example, derived from a non-human mammalian, such as *bovine*, *porcine*, etcetera species. Preferably, said target cell is not a human embryonic stem cell. In the case of a multicellular fungus, such target cell may be a fungal cell that can be proliferated into said multicellular fungus. When a transformed plant tissue or plant cell (e.g., pieces of leaf, stem segments, roots, but also protoplasts or plant cells cultivated by suspension) is obtained with this method according to the invention, whole plants can be regenerated from said transformed tissue or cell in a suitable medium, which optionally may contain antibiotics or biocides known in the art for the selection of transformed cells. This method of the invention may be applied in nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human. Encompassed within the present invention is a method of treatment comprising the method of the present aspect, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. The invention also relates to a construct of the twentieth aspect of the invention for treatment, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide. Furthermore, the invention relates to the use of a construct of the twentieth aspect of the invention for the manufacture of a medicament, wherein the protein or polypeptide of interest is a therapeutic and/or immunogenic protein or polypeptide.

Furthermore, an embodiment of the invention is a non-human transformed organism. Said organism is transformed with a nucleotide sequence, recombinant nucleic acid construct, or vector according to the present invention, and is capable of producing the polypeptide of interest. This includes a non-human transgenic organism, such as a transgenic non-human mammalian, transgenic plant (including propagation, harvest and tissue material of said transgenic plant, including, but not limited to, leafs, roots, shoots and flowers), multicellular fungus, and the like.

Preferably, the method of this aspect of the invention allows for an increase in expression of a protein or polypeptide of interest in said organism or at least in one tissue or organelle or organ of said organism. Preferably, expression levels are established in an expression system using an expression construct according to the twenty first aspect of the invention comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, depen-

dent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. Preferably, this method of the invention allows for an increase in protein or polypeptide expression of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% in said organism or at least in one tissue or organelle or organ of said organism, as compared to a method which only differs in that a construct is used in step a) wherein the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11, preferably when tested in a system as exemplified in example 11 which is enclosed herein. More specifically, preferably the expression of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to said nucleotide sequence of interest. Expression is preferably measured by measuring the conversion of any suitable alkaline phosphatase substrate and expression levels are compared to expression levels of said nucleotide sequence of interest which are measured under the same conditions except that in the expression vector said nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11.

Twenty Fifth Aspect

In a twenty fifth aspect, the present invention provides a method for transcription and optionally purifying the produced transcript comprising the step of:

- providing a nucleic acid construct according to the twentieth aspect comprising a nucleotide sequence of interest of the invention; and,
- contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- allowing said transformed cell to produce a transcript of the nucleotide sequence of interest; and optionally,
- purifying said produced transcript.

In a preferred embodiment of this method according to the invention a nucleic acid construct as defined above in the twentieth aspect is used. The method of the invention may be an *in vitro* or *ex vivo* method. The method of the invention may be applied on a cell culture, organism culture, or tissue culture. The method of the invention may be applied in nucleic acid based vaccination and/or gene therapy preferably in a mammal, preferably in a human. Encompassed within the present invention is a method for treatment comprising or consisting of the method of the present aspect, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. The invention also relates to a construct of the twentieth aspect of the invention for use in treatment, wherein the nucleotide sequence of interest encodes for a therapeutic transcript. Furthermore, the invention relates to the use of a construct of the twentieth aspect of the invention for the manufacture of a medicament, wherein the nucleotide sequence of interest encodes for a therapeutic transcript.

The skilled person is capable of transforming cells in accordance with step b). Transformation methods as used in step b) include, but are not limited to transfer of purified DNA via cationic lipid reagents and polyethyleneimide (PEI), calcium-phosphate co-precipitation, microparticle bombardment, electroporation of protoplasts and microin-

jection or use of silicon fibers to facilitate penetration and transfer of DNA into the host cell.

In step c) the transformed cell is allowed to produce a transcript of the nucleotide sequence of interest, and optionally the produced transcript is subsequently recovered. For example, the transformed cell may be subjected to conditions leading to transcription the nucleotide sequence of interest. The person skilled in the art is well aware of techniques to be used for transcription the nucleotide sequence of interest. Methods in which the transformed cell does not need to be subjected to specific conditions leading to transcription of the nucleotide sequence of interest, but in which the nucleotide sequence of interest is automatically (e.g., constitutively) transcribed, are also included in the method of the present invention.

Purification steps depend on the transcript produced. The term "isolation" indicates that the transcript is found in a condition other than its native environment. In a preferred form, the isolated transcript is substantially free of other cellular components, particularly other homologous cellular components such as homologous proteins. It is preferred to provide the transcript in a greater than 40% pure form, more preferably greater than 60% pure form. Even more preferably it is preferred to provide the transcript in a highly purified form, i.e., greater than 80% pure, more preferably greater than 95% pure, and even more preferably greater than 99% pure, as determined by Northern blot.

Preferably, the method of this aspect of the invention allows for an increase in transcription of a nucleotide sequence of interest. Preferably, transcription levels are established in an expression system using an expression construct according to the second aspect of the invention comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to a nucleotide sequence of interest. Preferably, transcription of said nucleotide sequence of interest is detected by a suitable assay such as RT-qPCR. Preferably, the method of the invention allows for an increase in transcription of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to a method which only differs in that a construct is used in step a) wherein said nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11, preferably when tested in a system as exemplified in example 11 which is enclosed herein. More specifically, preferably the transcription of a nucleotide sequence of interest encoding for secreted alkaline phosphatase (SeAP) is measured in a mammalian cell system, most preferably in CHO cells, using a pcDNA3.1 expression vector comprising a nucleotide sequence having at least 50% identity with SEQ ID NO:88 operably linked to said nucleotide sequence of interest. Transcription is preferably measured using RT-qPCR and transcription levels are compared to transcription levels of said nucleotide sequence of interest measured under the same conditions except that in the expression vector used the nucleotide sequence having at least 50% identity with SEQ ID NO:88 has been replaced by an alternative sequence, preferably one of those as described in example 11.

Twenty Sixth Aspect

In an twenty sixth aspect, the present invention provides a use of a nucleic acid molecule according to the nineteenth aspect of the invention, and/or a use of a nucleic acid construct according to the twentieth aspect of the invention, and/or a use of an expression vector according to the twenty first aspect of the invention, and/or a use of a cell according

to the twenty second aspect of the invention, for the transcription of a nucleotide sequence of interest and/or the expression of a protein or polypeptide of interest.

Twenty Seven Aspect

In a twenty seven aspect, the present invention provides for a nucleic acid molecule according to according to the nineteenth aspect of the invention, and/or a nucleic acid construct according to the twentieth aspect of the invention, and/or an expression vector according to the twenty first aspect of the invention, and/or a cell according to the twenty second aspect of the invention for use as a medicament. The invention also relates to a method of treatment comprising the administration of a nucleic acid molecule according to the nineteenth aspect of the invention, and/or a nucleic acid construct according to the twentieth aspect of the invention, and/or an expression vector according to the twenty first aspect of the invention, and/or a cell according to the twenty second aspect of the invention, wherein preferably said administration is to a mammal, more preferably to a human. Preferably, said treatment is nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human. Furthermore, the invention relates to the use of a nucleic acid molecule according to according to the nineteenth aspect of the invention, and/or the use of a nucleic acid construct according to the twentieth aspect of the invention, and/or the use of an expression vector according to the twenty first aspect of the invention, and/or the use of a cell according to the twenty second aspect of the invention, for the preparation of a medicament. Preferably said medicament is for nucleic acid based vaccination and/or gene therapy preferably in a mammal, most preferably in a human.

Definitions

The phrase "nucleic acid" as used herein refers to a naturally occurring or synthetic oligonucleotide or polynucleotide, whether DNA or RNA or DNA-RNA hybrid, single-stranded or double-stranded, sense or antisense, which is capable of hybridization to a complementary nucleic acid by Watson-Crick base-pairing. A nucleic acid of the invention is preferably modified as compared to its naturally occurring counterpart by comprising at least 1, 2, 3, 4, 5, 10, 20, 30 or 50 nucleotide mutations as compared to its naturally occurring counterpart. Preferably, a nucleic acid of the invention does not occur in nature. Nucleic acids of the invention can also include nucleotide analogs (e.g., BrdU), and nonphosphodiester internucleoside linkages (e.g., peptide nucleic acid (PNA) or thiodiester linkages). In particular, nucleic acids can include, without limitation, DNA, RNA, cDNA, gDNA, ssDNA, dsDNA, ssRNA, dsRNA, non coding RNAs, hnRNA, pre-mRNA, matured mRNA or any combination thereof. The terms "nucleic acid sequence" and "nucleotide sequence" as used herein are interchangeable, and have their usual meaning in the art. The term refers to a DNA or RNA molecule in single or double stranded form. An "isolated nucleic acid sequence" refers to a nucleic acid sequence which is no longer in the natural environment from which it was isolated. A nucleic acid molecule is represented by a nucleotide sequence. Furthermore, an element such as, but not limited to an expression enhancing element and a transcription regulating element, is represented by a nucleotide sequence.

A "recombinant construct" (or chimeric construct) refers to any nucleic acid sequence or molecule, which is not normally found in nature in a species, in particular a nucleic acid sequence, molecule or gene in which one or more parts

of the nucleic acid sequence are present that are not associated with each other in nature. For example, a recombinant construct comprises a promoter that is not associated in nature with part or all of the transcribed region or with another regulating region comprised within said recombinant construct. The term "recombinant construct" is understood to include expression constructs in which a promoter or expression regulating sequence is operably linked to one or more sense sequences (e.g. coding sequences) or to an antisense (reverse complement of the sense strand) or inverted repeat sequence (sense and antisense, whereby the RNA transcript forms double stranded RNA upon transcription), or to any other sequence coding for a functional RNA molecule.

A "nucleic acid construct" is defined as a polynucleotide which is isolated from a naturally occurring gene or which has been modified to contain segments of polynucleotides which are combined or juxtaposed in a manner which would not otherwise exist in nature. Optionally, a polynucleotide present in a nucleic acid construct is operably linked to one or more control sequences, which direct the production or transcription of a nucleotide sequence of interest and/or the expression of a peptide or polypeptide of interest in a cell or in a subject

A "vector" or "plasmid" is herein understood to mean a man-made (usually circular) nucleic acid molecule resulting from the use of recombinant DNA technology and which is used to deliver exogenous DNA into a host cell. Vectors usually comprise further genetic elements to facilitate their use in molecular cloning, such as e.g. selectable markers, multiple cloning sites and the like (see below). A nucleic acid construct may also be part of a recombinant viral vector for expression of a protein in a plant or plant cell (e.g. a vector derived from cauliflower mosaic virus, CaMV, or tobacco mosaic virus, TMV) or in a mammalian organism or mammalian cell system (e.g. a vector derived from Moloney murine leukemia virus (MMLV; a Retrovirus) a Lentivirus, an Adeno-associated virus (AAV) or an adenovirus (AdV)).

A "transformed cell" are terms referring to a new individual cell (or organism), arising as a result of the introduction into said cell of at least one nucleic acid molecule, especially comprising a chimeric or recombinant construct encoding a desired protein or a nucleic acid sequence which upon transcription yields an antisense RNA for silencing of a target gene/gene family. The host cell may be a plant cell, a bacterial cell (e.g. an *Agrobacterium* strain), a fungal cell (including a yeast cell), an animal (including insect, mammalian) cell, etc. The transformed cell may contain the nucleic acid construct as an extra-chromosomally (episomal) replicating molecule, as a non-replicating molecule or comprises the recombinant construct integrated in the nuclear or organellar DNA of the host cell. The term "organism" as used herein, encompasses all organisms consisting of more than one cell, i.e. multicellular organisms, and includes multicellular fungi. "Transformation" and "transformed" refers to the transfer of a nucleic acid sequence, generally a nucleic acid sequence comprising a recombinant construct or gene of interest (GOI), into the nuclear genome of a cell to create a "transgenic" cell or organism comprising a transgene. The introduced nucleic acid sequence is generally, but not always, integrated in the host genome. When the introduced nucleic acid sequence is not integrated in the host genome, one may speak of "transfection", "transiently transfected", and "transfected". For the purposes of the present patent specification, the terms "transformation", "transiently transfected", and "transfection" are used interchangeably, and refer to stable

or transient presence of a nucleic acid sequence into a cell or organism. When the cell is a bacterial cell, the term usually refers to an extrachromosomal, self-replicating vector which harbors a selectable antibiotic resistance.

"Sequence identity" or "identity" in the context of amino acid- or nucleic acid-sequence is herein defined as a relationship between two or more amino acid (peptide, polypeptide, or protein) sequences or two or more nucleic acid (nucleotide, polynucleotide) sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between amino acid or nucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Within the present invention, sequence identity with a particular sequence indicated with a particular SEQ ID NO preferably means sequence identity over the entire length of said particular polypeptide or polynucleotide sequence indicated with said particular SEQ ID NO. However, sequence identity with a particular sequence indicated with a particular SEQ ID NO may also mean that sequence identity is assessed over a part of said SEQ ID NO. A part may mean at least 50%, 60%, 70%, 80%, 90% or 95% of the length of said SEQ ID NO. The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The skilled person is capable of identifying such erroneously identified bases and knows how to correct for such errors.

Any nucleotide sequences capable of hybridising to the nucleotide sequences of the invention are defined as being part of the cis-acting elements of the invention. Stringent hybridisation conditions are herein defined as conditions that allow a nucleic acid sequence of at least 25, preferably 50, 75 or 100, and most preferably 150 or more nucleotides, to hybridise at a temperature of about 65° C. or of 65° C. in a solution comprising about 1 M salt or 1 M salt, preferably 6×SSC or any other solution having a comparable ionic strength, and washing at 65° C. in a solution comprising about 0.1 M salt, or 0.1 M salt or less, preferably 0.2×SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having about 90% or more sequence identity or at least 90% sequence identity. Moderate hybridization conditions are herein defined as conditions that allow a nucleic acid sequence of at least 50, preferably 150 or more nucleotides, to hybridise at a temperature of about 45° C. or of 45° C. in a solution comprising about 1 M salt or 1 M salt, preferably 6×SSC or any other solution having a comparable ionic strength, and washing at room temperature in a solution comprising about 1 M salt, or 1 M salt preferably 6×SSC or any other solution having a comparable ionic strength. Preferably, the hybridisation is performed overnight, i.e. at least for 10 hours, and preferably washing is performed for at least one hour with at least two changes of the washing solution. These conditions will usually allow the specific hybridisation of sequences having up to 50% sequence identity. The person skilled in the art will be able to modify these hybridisation conditions in order to specifically identify sequences varying in identity between 50% and 90%.

"Identity" can be readily calculated by known methods, including but not limited to those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New

York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heine, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math., 48:1073 (1988).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include e.g. the GCG program package (Devereux, J., et al., Nucleic Acids Research 12 (1): 387 (1984)), BestFit, BLASTP, BLASTN, and FASTA (Altschul, S. F. et al., J. Mol. Biol. 215:403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215:403-410 (1990). The well-known Smith Waterman algorithm may also be used to determine identity.

Preferred parameters for polypeptide sequence comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: BLOSSUM62 from Hentikoff and Hentikoff, Proc. Natl. Acad. Sci. USA. 89:10915-10919 (1992); Gap Penalty: 12; and Gap Length Penalty: 4. A program useful with these parameters is publicly available as the "Ogap" program from Genetics Computer Group, located in Madison, WI. The aforementioned parameters are the default parameters for amino acid comparisons (along with no penalty for end gaps).

Preferred parameters for nucleic acid comparison include the following: Algorithm: Needleman and Wunsch, J. Mol. Biol. 48:443-453 (1970); Comparison matrix: matches=+10, mismatch=0; Gap Penalty: 50; Gap Length Penalty: 3. Available as the Gap program from Genetics Computer Group, located in Madison, Wis. Given above are the default parameters for nucleic acid comparisons.

Preferred program and parameter for assessing identity for nucleic acid comparison is calculated using EMBOSS Needle Nucleotide Alignment algorithm with the following parameters: DNA full matrix with the following gap penalties: open=10; extend=0.5 as carried out in example 9.

The term "derived from" in the context of being derived from a particular naturally occurring gene or sequence is defined herein as being chemically synthesized according to a naturally occurring gene or sequence and/or isolated and/or purified from a naturally occurring gene or sequence. Techniques for chemical synthesis, isolation and/or purification of nucleic acid molecules are well known in the art. In general, a derived sequence is a partial sequence of the naturally occurring gene or sequence or a fraction of the naturally occurring gene or sequence. Optionally, the derived sequence comprises nucleic acid substitutions or mutations, preferably resulting in a sequence being at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical over its whole length to the naturally occurring gene partial gene or sequence or partial sequence.

"Polypeptide" as used herein refers to any peptide, oligopeptide, polypeptide, gene product, expression product, or protein. A polypeptide is comprised of consecutive amino acids. The term "polypeptide" encompasses naturally occurring or synthetic molecules. A polypeptide is represented by

an amino acid sequence. A polynucleotide is represented by a nucleotide sequence. A polypeptide is represented by an amino acid sequence.

The term "homologous" when used to indicate the relation between a given nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organisms of the same species, preferably of the same variety or strain. If homologous to a host cell, a nucleic acid sequence of interest, preferably encoding a polypeptide will typically be operably linked to another promoter sequence or, if applicable, another secretory signal sequence and/or terminator sequence than in its natural environment.

When used to indicate the relatedness of two nucleic acid sequences the term "homologous" means that one single-stranded nucleic acid sequence may hybridise to a complementary single-stranded nucleic acid sequence. The degree of hybridisation may depend on a number of factors including the extent of identity between the sequences and the hybridisation conditions such as temperature and salt concentration as discussed later. Preferably, the region of identity is greater than 5 bp, more preferably the region of identity is greater than 10 bp.

The term "heterologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean a nucleic acid or polypeptide molecule from a foreign cell which does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or which is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which they are introduced, but have been obtained from another cell or synthetically or recombinantly produced.

When used to indicate the relatedness of two nucleic acid sequences, the term the term "heterologous sequence" or "heterologous nucleic acid" is one that is not naturally found operably linked as neighboring sequence of the other sequence. As used herein, the term "heterologous" may mean "recombinant". "Recombinant" refers to a genetic entity distinct from that generally found in nature. As applied to a nucleotide sequence or nucleic acid molecule, this means that said nucleotide sequence or nucleic acid molecule is the product of various combinations of cloning, restriction and/or ligation steps, and other procedures that result in the production of a construct that is distinct from a sequence or molecule found in nature.

"Operably linked" is defined herein as a configuration in which a control sequence or regulating sequence is appropriately placed at a position relative to the nucleotide sequence of interest, preferably coding for the polypeptide of interest such that the control or regulating sequence directs or affects the transcription and/or production or expression of the nucleotide sequence of interest, preferably encoding a peptide or polypeptide of the invention in a cell and/or in a subject. For instance, a promoter is operably linked to a coding sequence if the promoter is able to initiate or regulate the transcription or expression of a coding sequence, in which case the coding sequence should be understood as being "under the control of" the promoter. When one or more nucleotide sequences and/or elements comprised within a construct are defined herein to be "configured to be operably linked to an optional nucleotide sequence of interest", said nucleotide sequences and/or elements are understood to be configured within said con-

struct in such a way that these nucleotide sequences and/or elements are all operably linked to said nucleotide sequence of interest once said nucleotide sequence of interest is present in said construct.

"Promoter" refers to a nucleic acid sequence located upstream or 5' to a translational start codon of an open reading frame (or protein-coding region) of a gene and that is involved in recognition and binding of RNA polymerase II and other proteins (trans-acting transcription factors) to initiate transcription. The term promoter refers to a nucleic acid fragment that functions to control the transcription of one or more genes, located upstream with respect to the direction of transcription of the transcription initiation site of the gene, and is structurally identified by the presence of a binding site for DNA-dependent RNA polymerase, transcription initiation sites and any other DNA sequences, including, but not limited to transcription factor binding sites, repressor and activator protein binding sites, and any other sequences of nucleotides known to one skilled in the art to act directly or indirectly to regulate the amount of transcription from the promoter. The promoter does not include the transcription start site (TSS) but rather ends at nucleotide -1 of the transcription site, and does not include nucleotide sequences that become untranslated regions in the transcribed mRNA such as the 5'-UTR. Promoters of the invention may be tissue-specific, tissue-preferred, cell-type specific, inducible and constitutive promoters. Tissue-specific promoters are promoters which initiate transcription only in certain tissues and refer to a sequence of DNA that provides recognition signals for RNA polymerase and/or other factors required for transcription to begin, and/or for controlling expression of the coding sequence precisely within certain tissues or within certain cells of that tissue. Expression in a tissue-specific manner may be only in individual tissues or in combinations of tissues. Tissue-preferred promoters are promoters that preferentially initiate transcription in certain tissues. Cell-type-specific promoters are promoters that primarily drive expression in certain cell types. Inducible promoters are promoters that are capable of activating transcription of one or more DNA sequences or genes in response to an inducer. The DNA sequences or genes will not be transcribed when the inducer is absent. Activation of an inducible promoter is established by application of the inducer. Constitutive promoters are promoters that are active under many environmental conditions and in many different tissue types. Preferably, capability to initiate transcription is established in an expression system using an expression construct comprising said promoter operably linked to a nucleotide sequence of interest using a suitable assay such as a RT-qPCR or Northern blotting. A promoter is said to be capable to start transcription if a transcript can be detected or if an increase in a transcript level is found of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to transcription using a construct which only differs in that it is free of said promoter. In a further preferred embodiment, capability to initiate expression is established in an expression system using an expression construct comprising said promoter operably linked to a nucleotide sequence encoding a protein or polypeptide of interest. Preferably, said protein or polypeptide of interest is a secreted protein or polypeptide and expression of said protein or polypeptide of interest is detected by a suitable assay such as an ELISA assay, Western blotting or, dependent on the identity of the protein or polypeptide of interest, any suitable protein identification and/or quantification assay known to the person skilled in the art. A promoter is

said to be capable to initiate expression if the protein or polypeptide of interest can be detected or if an increase in a expression level is found of at least 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 500%, 1000%, 1500% or 2000% as compared to expression using a construct which only differs in that it is free of said promoter. As a first and second promoter of the invention, an induced or constitutive promoter or a combination thereof may be used in the present invention.

An "intron" is a nucleotide sequence within a primary RNA transcript that is removed by RNA splicing or intron splicing while the final mature RNA product is being generated. Assessment whether intron splicing occurs can be done using any suitable method known to the person skilled in the art, such as but not limited to reverse-transcriptase polymerase chain reaction (RT-PCR) followed by size or sequence analysis of the RT-PCR product. Preferably, a nucleotide sequence is an intron if at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the primary RNA loses this sequence by RNA splicing using an assay suitable to detect intron splicing as indicated above. Preferably, an intron comprises a splice site GT at the 5' end of the nucleotide sequence, and a splice site AG at the 3' end of the nucleotide sequence, which splice site AG is preceded by a pyrimidine rich nucleotide sequence or poly-pyrimidine tract, optionally separated from splice site AG by 1-50 nucleotides. An intron may further comprise a branch site comprising the sequence Y-T-N-A-Y, at the 5' side of the poly-pyrimidine tract. The branch site may have the nucleotide sequence C-Y-G-A-C. An "intronic sequence" is understood to be at least part of the nucleotide sequence of an intron.

"Expression" will be understood to include any step involved in the production of the peptide or polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification and secretion.

Optionally, a promoter represented by a nucleotide sequence present in a nucleic acid construct is operably linked to another nucleotide sequence encoding a peptide or polypeptide as identified herein.

An expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of a nucleotide sequence encoding a polypeptide of the invention in a cell and/or in a subject.

As used herein, the "5'-UTR" is the sequence starting with nucleotide 1 of the mRNA and ending with nucleotide -1 of the start codon. It is possible that a regulating part of the promoter is comprised within the nucleotide sequence becoming a 5'-UTR; however, in such case, the 5'-UTR is still not part of the promoter as herein defined.

The term "control sequences" is defined herein to include all components, which are necessary or advantageous for the expression of a polynucleotide or a polypeptide. Each control sequence may be native or foreign to the nucleic acid sequence harboring or encoding the polynucleotide or the polypeptide. Such control sequences include, but are not limited to, a leader, optimal translation initiation sequences (as described in Kozak, 1991, J. Biol. Chem. 266:19867-19870), a polyadenylation sequence, a pro-peptide sequence, a pre-pro-peptide sequence, a promoter, a signal sequence, and a transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals.

The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitat-

ing ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence, which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcriptional control sequences, which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence, which shows transcriptional activity in the cell including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the cell.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence of interest, preferably encoding a polypeptide of interest. Any terminator, which is functional in the cell, may be used in the present invention.

The control sequence may also be a suitable leader sequence, a non-translated region of a mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence of interest, preferably encoding a polypeptide of interest. Any leader sequence, which is functional in the cell, may be used in the present invention.

The control sequence may also be a polyadenylation sequence, a sequence which is operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add adenine residues to transcribed mRNA. Any polyadenylation sequence, which is functional in the cell, may be used in the present invention.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a product or a composition or a nucleic acid molecule or a peptide or polypeptide of a nucleic acid construct or vector or cell as defined herein may comprise additional component(s) than the ones specifically identified; said additional component(s) not altering the unique characteristic of the invention. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

TABLE 1

Sequence identification	
SEQ ID NO:	Description
1	Expression enhancing element 1
2	Expression enhancing element 2
3	UN1
4-13	sequences derived from UN1
14	UN2
15	UN1dGAA
16	UN2dGAA
17	R3
18	fUN1
19	UN2-2

TABLE 1-continued

Sequence identification	
SEQ ID NO:	Description
20	UN2-3
21	UN2-4
22	UN2-5
23	UN2-6
24	UN2-7
25	UN2-8
26	UN2-9
27	UN2-10
28	UN1dGAA-2
29	UN1dGAA-3
30	UN1dGAA-4
31	UN1dGAA-5
32	UN1dGAA-6
33	UN2dGAA-2
34	UN2dGAA-3
35	UN2dGAA-4
36	UN1shuffle
37	UN1shuffle-2
38	UN1shuffle-3
39	UN1shuffle-4
40	UN1shuffle-5
41	UN1shuffle-6
42	UN2shuffle-1
43	UN2shuffle-2
44	CAA1
45	CAA2
46	CAA3
47	CAA4
48	CAA5
49	CAA6
50	TATA1
51	TATA2
52	CMV promoter enhancer sequence
53	UBC enhancer region
54	CMV promoter enhancer sequence construct
55	construct
56	CMV promoter sequence
57	Minimal CMV promoter sequence
58	EEE1-Xt
59	EEE1-80
60	EEE1-60
61	EEE1-50
62	EEE1-SL
63	HC RACE primer
64	Light chain vector sequence
65	Heavy chain vector sequence
66	HuMab1 protein light chain
67	HuMab1 protein heavy
68	HuMab2 protein light chain
69	HuMab2 protein and heavy chain
70	pcDNA3.1(+)
71	SeAP protein
72	EEE1 + CMV + TEE
73	EEE1-Xt + CMV + TEE
74	EEE1-80 + CMV + TEE
75	EEE1-60 + CMV + TEE
76	pPNic384
77	pPNic602 insert
78	EF1a promoter
79	EEE1-A1
80	EEE1-A2
81	EEE1-A3
82	EEE1-B1
83	EEE1-B2
84	EEE1-B3
85	EEE1-B4
86	EEE1-B5
87	Transcription regulating sequence
88	

FIGURES

FIG. 1. Schematic map of intronic promoter construct and different transcripts. The construct comprises 2 promoters.

Transcription by Promoter 1 results in a primary transcript including the intron that contains the complete Promoter 2 sequence and is bordered by 5' and 3'-splice sites. After intron splicing, said primary transcript results in a mRNA without said intron (Transcript 1) encoding a "Gene". Transcription from Promoter 2 also results in a mRNA (Transcript 2) encoding the same "Gene".

FIGS. 2a-2b. Schematic map of EEE1 (FIG. 2a) and EEE2 (FIG. 2b) elements showing some features of the UBC and CCT8 genes relevant to their promoter activity in a genomic context. Features include the predicted transcription start site (TSS), 5'-UTRs, exon and intron information.

FIG. 3. Schematic map of an expression vector for an Ig light chain (IgLC) with the EEE1 sequence integrated upstream of the CMV promoter.

FIGS. 4a-4b. Comparison of HuMab1 production by CHO-S pools stably transfected with Reference or EEE1 constructs. Expression vector without (FIG. 4a) and with (FIG. 4b) additional expression regulating element. The bars represent the average exhaust titers of 4 pools derived from 2 independent transfections. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIG. 5. Comparison of HuMab1 production by CHO-S pools stably transfected with Reference or EEE2 constructs. The bars represent the average exhaust titers of 4 pools derived from 2 independent transfections. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIG. 6. Analysis of HuMab1 production by top-10 CHO-S clonal cell lines stably transfected with EEE1-TEE constructs harboring EEE1. Cells were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIGS. 7a-7b. Comparison of HuMab2 production by CHO-S pools stably transfected with EEE1 in reference vector (FIG. 7a, left panel) and in vector with additional regulating element (FIG. 7a, right panel). The bars represent the average exhaust titers of 4 pools derived from 2 independent transfections. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium. (FIG. 7b) Analysis of HuMab2 production by top-12 Reference and EEE1-TEE CHO-S clonal cell lines. Cells were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIG. 8. Comparison of SeAP activity in the exhaust media of CHO-S pools stably transfected with Reference or EEE1 constructs. Expression vector without (left panel) and with (right panel) additional expression regulating element. The bars represent the average activities of 4 pools derived from 2 independent transfections, measured using the SEAP Reporter Gene Assay Kit, Abcam. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIG. 9. Comparison of SeAP activity in the exhaust media of CHO-S pools stably transfected with constructs containing different versions of the EEE1 element. The bars represent the average activities of 4 pools derived from 2 independent transfections, measured using the SEAP Reporter Gene Assay Kit, Abcam. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium.

FIGS. 10a-10b. 5'-RACE amplification of 5'-ends of heavy chain transcripts from CHO-S clones stably transfected with EEE1-TEE constructs expressing HuMab1 (FIG. 10a). Two bands are detected on agarose gel, corresponding to the transcripts generated by the CMV promoter (Transcript 1) and the UBC promoter (Transcript 2). The size difference between Transcript 1 and Transcript 2 is explained in a schematic map of intronic promoter construct and different transcripts (FIG. 10b). The construct comprises

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2 promoters, UBC and CMV. The CMV promoter is linked to a TEE sequence which also comprises a short intron. Transcription by the CMV promoter results, after intron splicing, in mRNAs with TEE as 5'-UTR (Transcript 1). The UBC promoter is linked to a partial UBC 5'-UTR region which comprises a 5' splice donor site and precedes the CMV promoter. Transcription by the UBC promoter results in mRNAs with the UBC 5'-UTR sequence (Transcript 2). The large intron which is spliced from the primary transcript runs from the 5'-splice donor sequence in the UBC sequence to the 3'-splice acceptor site in the TEE and contains the complete CMV sequence.

FIG. 11. Effect of EEE in *Pichia pastoris* expressing recombinant human interleukin 8. Each bar represents the average expression of 10 independent clones.

The invention will be explained in more detail in the following Examples section, with reference to the appended figures. The examples serve for illustration purposes only, and do not intend to limit the present invention in any way.

EXAMPLES

The expression enhancing element represented by SEQ ID NO: 1 is based on the Chinese hamster (*Cricetulus griseus*) ubiquitin-C (UBC) gene. It comprises the predicted promoter sequence and part of the 5'-untranslated region (FIG. 2A). The expression enhancing element represented by SEQ ID NO: 2 is based on the human CCT8 gene (chaperonin containing TCP1, subunit 8). It comprises the predicted promoter sequence and part of the 5'-untranslated region as well as a short sequence encoding 27 amino acids and part of the first intron (FIG. 2B).

Example 1

Expression plasmids were constructed based on the pcDNA3.1 expression vector (SEQ ID NO: 71). The vector was modified by removing the f1-ori. Coding sequences for an IgG1 (HuMab1) heavy (represented by a sequence that is at least 96% identical SEQ ID NO: 68) and light chain (represented by a sequence that is at least 99% identical SEQ ID NO: 67) genes were inserted in this vector (the light chain coding sequence was inserted in the vector represented by SEQ ID NO: 65 and the heavy chain coding sequence was inserted in the vector represented by SEQ ID NO: 66), resulting in the Reference constructs. To generate the EEE1 (Expression Enhancing Element 1) vectors, the EEE1-sequence (SEQ ID NO: 1) was inserted upstream of the CMV promoter (SEQ ID NO: 57) (FIG. 3). EEE2 (SEQ ID NO: 2) was introduced in a similar way, resulting in the EEE2 vectors. A vector with an additional expression regulating element was generated by replacing the pcDNA3.1 5'-UTR for SEQ ID NO: 19 (transcription enhancing element; TEE).

CHO-S cells (Life Technologies) were maintained per manufacturer's instructions. Duplicate transfections were performed using 3E7 cells, 50 µg of linearized DNA and FreeStyle MAX Reagent (Life Technologies). Post-transfection pools were split in two and selected in CD FortiCHO medium supplemented with 8 mM glutamine and 800 µg/ml G418. Selected pools were seeded in 30 ml of the same medium at a density of 3E5 cells/ml in 125 ml shake-flasks. The HuMab1 exhaust titers were determined by ELISA (FIG. 4). Exhaust titers of the Reference pools were too low for accurate determination. This indicates that the antibody is poorly expressed. The EEE1 pools produced approximately 1 µg/ml (FIG. 4A). A similar effect was observed in the vector with an additional expression regulating element.

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In this vector the introduction of EEE1 increased the production from approximately 0.2 µg/ml to 9-12 µg/ml in stable pools from three independent transfection experiments (FIG. 4B). These data show that a poorly expressed antibody can be expressed at significantly higher levels by introduction of the EEE1 element.

Example 2

The effect of introducing the EEE2 element was studied in a vector harboring an additional expression regulating element (See Example 1, FIG. 4B). CHO-S cells were transfected either with the reference or with the EEE2 constructs as described previously. Antibody exhaust titers of the stably transfected EEE2 pools were over 20 times higher than the Reference pools (FIG. 5).

Example 3

The EEE1-TEE and Reference pools generated previously were seeded in six 96-well plates at a density of 0.5 cell/well in CD FortiCHO selection medium. The Reference cells showed impaired growth as compared to the EEE1-TEE clones and thus no HuMab1 was produced. Clones of EEE1-TEE showed normal growth and HuMab1 production (See below). 100 Clonal EEE1-TEE lines were assessed for HuMab1 production in microtiter plates. The 10 clones with highest specific productivity were expanded to 125 ml shake-flasks. The clones were seeded in 30 ml of CD FortiCHO selection medium at a density of 3E5 cells/ml and HuMab1 exhaust titers were determined by ELISA (FIG. 6). Clones produced up to 0.25 µg/ml HuMab1. These data indicate that the EEE1 can facilitate the generation of clonal lines and allows the generation of clonal lines with relevant expression levels.

Example 4

The copy number of antibody expressing EEE comprising clones was determined. The PrimerExpress program (Life Technologies) was used to design Taqman primers and probes specific for the heavy- and light chains of HuMab1 and β2 microglobulin. The primers were combined in a triplex Taqman assay to measure gene copies in gDNA samples of EEE1-TEE HuMab1 clones and pools. The gene copy numbers were compared with HuMab1 titers (Table 2). Clonal cell lines producing similar HuMab1 titers had different numbers of light and heavy chain gene copies (Clone 1 and 2). Also, clones producing very different HuMab1 titers had similar gene copy numbers (Clone 3 and 4). In pools relatively high numbers of light and heavy chain genes were paired with relatively low expression levels. These data (Table 2) indicate that there is no correlation between EEE comprising gene copy number and HuMab1 expression levels.

TABLE 2

	Titers of IgG1 and gene copy numbers		
	IgG titer	LC	HC
Clone1	123.7	36.8	25.1
Clone2	118.2	1.7	1.7
Clone3	143.6	3.1	1.2
Clone4	7.2	4.6	0.7
Pool	9.0	17.5	21.1

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Example 5

The HuMab1 heavy and light chain genes of the previous examples were replaced by heavy and light chain genes (SEQ ID NO's: 69 and 70) encoding a biosimilar antibody (HuMab2 derived from DrugBank Accession Number DB00072). The constructs were used to generate CHO-S pools as described previously. Using ELISA, the exhaust titers were determined. The data (6.3 µg/ml without enhancing element) indicate that this antibody is produced to a higher level than the antibody from the previous examples. Without any additional expression regulating element introduction of the EEE resulted in a 3.7 fold increase (FIG. 7A, left panel), in the modified vector the increase is 7 fold (FIG. 7A, right panel). Since stand-alone the additional expression regulating element results in a 40% increase, the data also indicate a synergistic effect between the EEE and the additional expression regulating element. Clonal lines were isolated from the Reference and EEE1-TEE pools as described previously. The best EEE1-TEE clones produced 3-fold higher HuMab2 titers as compared to the best Reference clones (FIG. 7B). These data indicate that the EEE1 element can be successfully applied in enhancing recombinant protein expression from stable cell lines.

Example 6

The HuMab1 light chain gene of the constructs from Example 1 was replaced by the gene encoding secreted alkaline phosphatase (SeAP; SEQ ID NO: 72). The constructs were used to generate CHO-S pools as described previously. The SeAP activity was measured in the exhaust medium using the SEAP Reporter Gene Assay Kit, Abcam. The EEE1 pools showed 2-fold higher activity as compared to the Reference pools (FIG. 8). In the EEE1-TEE pools the increase was almost 4-fold as compared to the Reference pool. These data show that EEE1 enhances the expression of a single subunit non-antibody protein in a transfected cell line.

Example 7

The SeAP constructs used in Example 6 all comprised the CMV promoter. Two TEE vector variants were made that contained the human EF-1 α promoter instead of CMV (SEQ ID NO: 79). The constructs were used to generate CHO-S pools as described previously. The SeAP activity was measured in the exhaust medium using the SEAP Reporter Gene Assay Kit, Abcam. The EEE1-TEE pool with EF-1 α as

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intronic promoter produced 2.8-fold higher SeAP activity as compared to the Reference EF-1 α promoter pool without EEE1. These data show that EEE1 enhances the expression of a protein in an intronic promoter construct when the intronic promoter is not the CMV promoter, such as the EF-1 α promoter.

Example 8

The EEE1 element of the EEE1 SeAP-expression vector was replaced by the following variants: 1. EEE1-80 represented by SEQ ID NO: 60 has a 290 bp truncation from the 5'-end; 2. EEE1-60 represented by SEQ ID NO: 61 with a 580 bp truncation from the 5'-end; 3. EEE1-50 represented by SEQ ID NO: 62 with a 725 bp truncation from the 5'-end; 4. EEE1-Xt represented by SEQ ID NO: 59 with a 800 bp extension from the genomic *C. griseus* UBC sequence at the 5'-end; 5. EEE1-SL (SEQ ID NO: 63) has all major predicted splice donor and acceptor sites mutated. SeAP activity in the supernatant of cells transfected with the EEE1 element was set at 100%, which decreased to 39% activity without the EEE1 element (FIG. 9). The 5' truncations of the EEE1-80 and EEE1-60 constructs gradually decreased activity but still showed enhanced activity as compared to the No-EEE construct. The EEE1-50 element decreased SeAP activity to 40%, which is similar to the No-EEE construct. The EEE1-Xt construct showed almost 40% increased activity as compared to the EEE1 construct. The data suggest that sequences with more than 50% identity to EEE1 can function as expression enhancing elements. The EEE1-Xt construct produced almost 40% increased activity as compared to the EEE1 construct, which shows that additional enhancer sequences reside in the region upstream of the genomic sequence from which EEE1 was taken. The activity of the EEE1 element is severely impaired by 4 nt mutations of the EEE1-SL construct which prevent correct intron splicing, resulting in a significant reduction in SeAP expression as compared to the EEE1 construct.

Example 9

The EEE1 element of the EEE1 SeAP-expression vector was replaced by 9 variants of the EEE1 element, which can be grouped based on 2 different types of mutations. The first type of EEE1 variants (EEE1-A) all had changes within the EEE1 or EEE1-Xt element with more than 30 percent of nucleotides mutated, each in another of the 3 regions which each consisted of at least 244 bp. The second type of EEE1 variants (EEE1-B) also had the same size as the EEE1 element (1,449 bp) with at least 96 percent sequence identity, with mutations that targeted different functional sequences of the EEE1 sequence. The different mutations are listed in Table 3.

TABLE 3

Modifications of EEE1				
Type A: More than 30% mutated in 3 regions of EEE1				
Variant	SEQ ID NO:	Identity to EEE1 ¹⁾	Modified EEE1 region	Size modified region (bp)
EEE1-A1	80	71.6% ²⁾	5' promoter region	1,526
EEE1-A2	81	95.0%	3' promoter region	244
EEE1-A3	82	81.8%	intron region	480

TABLE 3-continued

Modifications of EEE1			
Type B: Mutations that target specific domains of EEE1			
Variant	SEQ ID NO:	Identity to EEE1 ¹⁾	Modification of EEE1 sequence
EEE1-B1	83	97.9%	1: 7 nt changed in nt 144-152 2: 4 nt changed in nt 612-615 3: 4 nt changed in nt 667-670 4: 6 nt changed in nt 816-823 5: 5 nt changed in nt 1,106-1,112 6: 5 nt changed in nt 1,432-1,438
EEE1-B2	84	96.5%	50 single bp mutations = 50% of CG's mutated in nt 227-1,409; predicted transcription factor binding sites maintained.
EEE1-B3	85	99.7%	5 single bp mutations = 50% of CG's mutated in nt 549-603
EEE1-B4	86	96.5%	50 single bp mutations = 50% of CG's mutated in nt 227-1,409; 8 predicted sites for transcription factors SP1, HSF, and NFkB affected.
EEE1-B5	87	96.0%	51 bp mutations in 12 regions with predicted transcription factor binding activity spanning nt 105-1,449 were mutated.
EEE1-B6 ³⁾	63	99.7%	4 single bp mutations eliminating predicted and known splice-donor or acceptor sites, including known donor site (nt 970), nt 545 and 552 in promoter region, nt 1,267 in intron region.

¹⁾Identity calculated using EMBOSS Needle Nucleotide Alignment algorithm with the following parameters:

DNAfull matrix with the following gap penalties: open = 10; extend = 0.5

²⁾99% identity EEE1-A1 calculated relative to EEE1-Xt³⁾This is referred to as EEE1-SL in Example 8

SeAP activity in the supernatant of cells transfected with the different variants was measured. Activity of cells with EEE1 element was used as reference (100%). Without EEE1 element the activity was 24% in this experiment. SeAP activity of cells transfected with the EEE1-A2 and EEE1-A3 constructs was decreased to 75% and 48% relative to EEE1, respectively (Table 4). This is higher than the 24% activity observed with the No-EEE construct in this experiment. The EEE1-A1 construct decreased SeAP activity to 30% relative to the EEE1-Xt construct on which it is based, which is still higher than the No-EEE construct which produces only 18% of SeAP activity relative to the EEE1-Xt construct. The data show that EEE1 variants with as little as 72% overall identity and locally 50% identity to the genomic UBC sequence can function as expression enhancing elements.

SeAP activity of cells transfected with the EEE1-B1 to B6 constructs was decreased by up to 42% relative to the EEE1 construct (Table 4). The data show that mutations in regions with a predicted functionality in the intronic promoter activity of the EEE1 element can significantly limit the expression enhancement capability of the EEE1 element. For instance, mutating 4 nt involved in intron-splicing resulted in 38% decreased SeAP titers (EEE1-B6). Mutation of different sets of CpG's also resulted in decreased SeAP titers (B1, B2, B4).

TABLE 4

SeAP activity of EEE1 variants	
Construct	Activity relative to EEE1 (%) ¹⁾
No-EEE1	24
EEE1	100
EEE1-A2	75
EEE1-A3	48
EEE1-B1	58

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TABLE 4-continued

SeAP activity of EEE1 variants	
Construct	Activity relative to EEE1-Xt (%) ¹⁾
EEE1-B2	60
EEE1-B3	73
EEE1-B4	58
EEE1-B5	84
EEE1-B6	62
Activity relative to EEE1-Xt (%) ¹⁾	
No-EEE1	18
EEE1-Xt	100
EEE1-A1	30

¹⁾Values represent the average activities of 4 pools derived from 2 independent transfections, measured using the SEAP Reporter Gene Assay Kit, Abcam. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium

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Example 10

A EEE1-TEE CHO-S clone from Example 3 was grown and cells were harvested in log-phase. Total RNA was isolated from the cells using AllPrep DNA/RNA Mini Kit (Qiagen). cDNA was synthesized using the Epicentre Exact Start Eukaryotic mRNA 5' and 3' RACE Kit. First strand cDNA was amplified using the 5' RACE primer from the kit combined with a heavy chain specific primer (SEQ ID NO: 64) and ZymoTaq DNA polymerase. The PCR product was analyzed on 1.2% agarose gels, showing two discrete bands (FIG. 10A) which were separately isolated and inserted in a PCR4-TOPO vector (Life Technologies). Sequencing analysis revealed that the upper band seen on the agarose gel corresponds to the transcript initiated from the CMV promoter. The lower band corresponds to the transcript initiated from the UBC promoter. Both products have the predicted intronic sequence spliced out correctly. The differences in

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size correspond to the different lengths of the 5'-UTRs, as depicted in FIG. 10B. The data show that both promoters contribute to transcription.

Example 11

CHO-S pools stable transfected with constructs with three different single promoters were compared by the SeAP activity in the supernatant. Pools were grown in 125 ml shake-flasks in 30 ml CD FortiCHO selection medium and SeAP activity was measured using the SEAP Reporter Gene Assay Kit (Abcam) in 4 pools per construct derived from 2 independent transfections. The constructs either contained the CMV promoter (Example 6), the EF-1 α promoter (Example 7), or the UBC promoter (Example 11). The UBC promoter produced 2.7-fold higher SeAP activity as compared to the CMV promoter construct. The UBC promoter produced 6.0-fold higher SeAP activity as compared to the EF-1 α promoter. The data shows that the expression with the UBC promoter alone is higher as compared to the CMV promoter or the EF-1 α promoter alone.

Example 12 Methanol Induced Secretion of IL8 in *Pichia pastoris* GS115 Integrative Transformants

Plasmids for stable transformation of *Pichia pastoris* with human interleukin 8 (hIL-8) expression constructs were generated in plasmid pPIC9K (Life Technologies). Insertion of the hIL-8 gene in pPIC9K resulted in plasmid pPNic384 (SEQ ID NO: 77), which contains the hIL-8 gene under control of the AOX1 promoter. The EEE1 sequence was

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inserted upstream of the AOX1 promoter as a AatII-AleI fragment (SEQ ID NO: 78) in pPNic384, resulting in plasmid pPNic602.

The expression vectors were linearized by digestion with Sall and transformed into *P. pastoris* strain GS115 using electroporation as recommended (Invitrogen, 2008). Transformants were plated on RDB agar plates (Regeneration Dextrose Medium, a medium lacking histidine). After incubation at 30° C. for 48 h, large colonies were observed. A control transformation without DNA was performed resulting in no colonies. Randomly 10 clones per construct were picked from the transformation plate and grown to saturation in 800 μ l BMG (Buffered minimal medium with 1% glycerol), in 2 ml deep well plates. The plate was kept in a shaking incubator (Infors-HT Microtron) set at 30° C., 1000 rpm for 18 hours. The optical density of the culture was between 5-10 absorbance units at 600 nm. The cells were harvested and the medium replaced by 800 μ l of BMM (Buffered minimal medium with 0.5% methanol) in 2 ml deep well plates. The cells were grown in the shaking incubator and every 24 hours 0.5% methanol (final concentration) was added to the culture to maintain induction. After 72 hours of methanol induction the culture supernatant were collected and assayed for secreted hIL8 yields using the AlphaLISA hIL8 kit (Perkin Elmer). The data show (FIG. 11) that there is a significant difference between the IL8 yields of the reference and the EEE1 transformants, suggesting that the EEE1 sequence upstream of the promoter improves the hIL8 yields compared to expression plasmid without the EEE1 sequence.

SEQUENCE LISTING

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<160> NUMBER OF SEQ ID NOS: 88

<210> SEQ ID NO 1
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1

<400> SEQUENCE: 1

tttcaggcaa ccagagctac atagttagat cctgtctcaa caaaaataaa ataatctaag      60
gcttcaaagg gttcaatctc ttaggttagct aaatatgaac aaaatttggg aatatgtgacc      120
tttccttag tgacagtcag atagaacctt ctcgagtgc aaggcaccaa gtgcaaacag      180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtcgaat gccaactgtt      240
ttcaagaact gtgtggattt ttctgcctgt aacgaattca gattcatttt tc当地actcg      300
gggagagttt tccccctta taatttttt tttaaattta tttaactttt ttctgttccc      360
cttggttga gaattgcaga gtcatccacc ctgtcacagt gccaggaggc tcagggatgg      420
gcccaggggc ctggggggc tgaaggggct ggggaagcga gggctccaaa gggaccccag      480
tgtggcagga gccaaagccc taggtcccta gaacgcagag gccaccggc cccccccagac      540
gggtaagcg ggtgggtgtc tggggcgcga agccgactg cgcatgcgcc gaggtccgt      600
ccggccgcgc tgatccaagc cgggttctcg cggcgcactg gtcgtgattt acaagtccaca      660
cacgctgatc cttccgcggc gcccacagg gtcacagctt ttccccccc cacaaggccc      720
cctactctct gggcaccaca cacgaacatt ctttgcgcgt gacccgttgc gctctagtc      780
ggcgccctcg gtgcagagac tggAACGGCC ttgggaagta gtccttaacc gcattccgc      840

```

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ggagggatcg tcgggagggc gtggcttctg aggattataat aaggcgactc cgggggggtc	900
ttagctagt ccgtcgaga cccgagttca gtgcgcgtt ctctgtgagg actgtgcgg	960
ccgcccgtgg tgaggagaag ccgcgcgtt tggcgtagct gagagacggg gagggggcgc	1020
ggacacgagg ggcagccgc ggcctggaeg ttctgtttcc gtggccgcg aggaaggcga	1080
ctgtccttag goggaggacc cagcgcaag atggcgccca agtggaaagcc tgagggata	1140
ggcgagggc cctgaggcgc tcgcacgggt tggggggaa gcagggccgc gagcagctg	1200
cagccggaa cgtcgccca accccttatt ttttttagc ggttgcgggc cgtaggtgcc	1260
tccgaagtga gagccgtggg cgtttactg tcgggagagg tcggteggat ttcatccgt	1320
tgctaaagac ggaagtgcga ctgagacggg aaaaaaaaaa agtcgggttg tggcggttga	1380
acctggacta aggccacat gacgtcgccg ttctatggg ctcataatgg gtggtagga	1440
cattccct	1449

<210> SEQ ID NO 2
<211> LENGTH: 1228
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE2

<400> SEQUENCE: 2	
gtaaagcaga tcacacagaa tatggcacac ttgagcactt gatgtgtact acattactct	60
tagtgcacat ttaattatc gtgcgcattc ccagcgcttc ctatgggcc caacacagag	120
cggacgccta gagacaattt tggggatgg ggcagatgtc ctgcctcggg aaaaaaaaaaag	180
cacacctgcc ctgacgttgg tggctgggtc tggaaagatac gtggaaattha agctaaggat	240
gtgtggcttc cagatcaaaa accgcaaaaa tctaacgcggc tgactactga ctacggtcag	300
agagcacaga ctggagcaac ctctcacggc ctgggctgtc tgccgtgcg tgagccagaa	360
acccgagggg ctccctggc cccgcctatc gatgcacccg atcggggatc gtcagcttgg	420
ttctggcac agaggttgc ttttcgcga tgcttcagac ctggcggcag ggaaagggtg	480
ggctaattgg agagccagga agagcgttag gccccccac gctgcgttcc cagaaggctg	540
tgcgtgtcc tgcgttcctc cgggtcttc cgagcggctcg cgtgaactgc ttccagcagg	600
ctggccatgg cgcttcacgt tcccaaggct cccggcttgc cccagatgtc caaggaggg	660
gcgaaagtaa gggctgaagg aaaggaatga ggtggagcg tcagcatagg gctggccgg	720
cggccggcga gtagggggc ctactaacgg gctgagcgtg ctgcctggc tcagcggccg	780
ggggaaagaga agattccaga aaggaggtg attttggaaag ggctggccca cccggacctg	840
cgggcacttc ttttttcccg cgacccggag aaggccgagg gatcggccgc acgatcgaca	900
ttgtacacct tgaaggtgga cggatgtgaa gcccgcgtg cgtttgcct ccacccgtaa	960
atggggctaa ggcccgtaac ccttaagga ggttgtgagg gtgaaattga ataacgtaga	1020
tgaattgtc ttgagaactg cgacgtcgat tattcacatag ctcgcgagtt gttagatggg	1080
gaagaacgag aactagccga tccagagaag agatggggaa aaggccgg gtcttggtg	1140
cttgcgttcc agtggaaaac atacggctt cagtttagt gacagaagcc atgcgttga	1200
gcacaaatgag ttccggtccc aacttatg	1228

<210> SEQ ID NO 3
<211> LENGTH: 173
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence

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<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 3

```
caagctctag caggaagaag aaataagaag aagaagaaga agaagaagaa gcgttcctc      60
tttttttgtt gagataaaaa aataaaactc caaaaaaaaa gaaaatcatc aaaaaaaca   120
atttcaaaaa gagtttttgtt gtttggggat taaagaataa aaaaaacaac gtc          173
```

<210> SEQ ID NO 4
<211> LENGTH: 175
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 4

```
caagctctag caggaagaag aaataagaag aagaagaaga agaagaagaa gcgttcctc      60
tttttttgtt gagataaaaa aataaaactc caaaaaaaaa gaaaatcatc aaaaaaaca   120
atttcaaaaa gagtttttgtt gtttggggat taaagaataa aaaaaacaac gtccc        175
```

<210> SEQ ID NO 5
<211> LENGTH: 189
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 5

```
agatcaactag aagcttcaag ctctagcagg aagaagaaat aagaagaaga agaagaagaa    60
gaagaagcgt ctcctttct tcttgtgaga gtaaaaaata aaactccaa aaaaaagaaaa     120
atcatcaaaa aaacaaattt caaaaagagt ttttgtt ggggattaaa gaataaaaaa     180
aacaacgcc                                         189
```

<210> SEQ ID NO 6
<211> LENGTH: 189
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 6

```
agatcaactag aagcttcaag ctctagcagg aagaagaaag aagaagaaga agaagaagaa    60
gaagaagcgt ctcctttct tcttgtgaga gtaaaaaaga aaactccaa aaaaaagaaaa     120
atcatcaaaa aaacaaattt caaaaagagt ttttgtt ggggattaaa gaagaaaaaa     180
aacaacgcc                                         189
```

<210> SEQ ID NO 7
<211> LENGTH: 191
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 7

```
agatcaactag aagcttcaag ctctagcagg aagaagaaat aagaagaaga agaagaagaa    60
gaagaagcgt ctcctttct tcttgtgaga gtaaaaaata aaactccaa aaaaaagaaaa     120
atcatcaaaa aaacaaattt caaaaagagt ttttgtt ggggattaaa gaataaaaaa     180
```

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aacaacaggc c

191

<210> SEQ ID NO 8
<211> LENGTH: 284
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 8

cttttcgca acgggttgc cgccagaaca caggtgtcgtaggaatttag ctggta	60
atacgactca ctataggagg accaagctg gctaggtaa ctggtagcc aagctctagc	120
aggaagaaga aataagaaga agaagaagaa gaagaagaag cgtctccctct tcttcttg	180
agagtaaaaa ataaaactcc caaaaaaaaaa aaaaatcatca aaaaaaaaaa ttcaaaaag	240
agtttttgtg tttggggatt aaagaataaa aaaaacaacg tccc	284

<210> SEQ ID NO 9
<211> LENGTH: 230
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 9

aaccactgc ttactggctt atcgaaatta atacgactca ctataggagg accaagctc	60
tagcaggaag aagaaataag aagaagaaga agaagaagaa gaagcgtctc ctttctct	120
tgtgagagta aaaaataaaaa ctccccaaaa aaagaaaatc atcaaaaaaaaaa caaatttcaa	180
aaagaggttt tgtgtttggg gattaaagaa taaaaaaaaac aacctccacc	230

<210> SEQ ID NO 10
<211> LENGTH: 177
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 10

caagctctag cagcaacaac aaataacaac aacaacaaca acaacaacaa gcgtctctc	60
tttttttgtt gagataaaaa aataaaactc caaaaaaaaa gaaaatcatc aaaaaaaaaa	120
atttcaaaaa gagtttttgtt gtttggggat taaagaataa aaaaaaaaaac ctccacc	177

<210> SEQ ID NO 11
<211> LENGTH: 191
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 11

agatcaactag aagcttcaag ctctagcagg aagaagaaat aagaagaaga agaagaataa	60
gaagaagcgt ctcgtttct tcttgtgaga gtaaaaaata aaactccaa aaaaaaaaaa	120
atcatcaaaa aaagaaattt caaaaagagt ttttgtttt ggggattaaa gaataaaaaa	180
aacaacaggc c	191

<210> SEQ ID NO 12
<211> LENGTH: 189
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence

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<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 12

```
agatcaactag aagcttcaag ctcttagcagg aagaagaaat aataagaaga agaagaataa      60
gaaagaagcgt ctcctttct tcttgaga gtaaaaaata aaactccaa aaaaaataaa      120
atcatcaaaa aaataaattt caaaaagagt ttttgtttt ggggattaaa gaataaaaaa      180
aacaacgc      189
```

<210> SEQ ID NO 13
<211> LENGTH: 173
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TEE

<400> SEQUENCE: 13

```
caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgtctcctc      60
tttttttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca      120
atttcaaaa gagttttgt gtttgggat taaaagaakaa aaaaaacaac gtc      173
```

<210> SEQ ID NO 14
<211> LENGTH: 274
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2

<400> SEQUENCE: 14

```
caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgtctcctc      60
tttttttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca      120
atttcaaaa gagttttgt gtttgggat taaaagaakaa aaaaaacaac aggtgagtaa      180
gcgcagttgt cgtctcttgc ggtgccgttg ctgggtctca caccttttag gtctgttctc      240
gtttccgtt ctgactctct cttttcggt gcag      274
```

<210> SEQ ID NO 15
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA

<400> SEQUENCE: 15

```
ggcgtctcct ctttttttg tgagagtaaa aaataaaact cccaaaaaaaa akaaaatcat      60
aaaaaaaaaca aatttcaaaa agagtttttg tgtttgggat taaaagaakaa aaaaaacaac      120
gtc      123
```

<210> SEQ ID NO 16
<211> LENGTH: 257
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2dGAA

<400> SEQUENCE: 16

```
aaaagtatcaa caaaaagct tcgtctcctc tttttttgt gagagtaaaa aakaaaactc      60
aaaaaaaaaaa kaaaatcatc aaaaaaacaac atttcaaaa gagttttgt gtttgaagt      120
```

-continued

caggactcta gctttctact gtagtatcct ctaaaggact gctgttctgt gcacccctt	180
cctttgttta tcatacgcgca cgacaagagt actaactaat taacttaggg ggattaaaga	240
akaaaaaaaaa caacaaa	257

<210> SEQ ID NO 17
<211> LENGTH: 173
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: R3

<400> SEQUENCE: 17

caagctctag cacgttcctt cttttcttg tgagagtaaa aaakaaaact cccaaaaaaaa	60
akaaaaatcat caaaaaaaaaa aatttcaaaa agagttttt tgtttgggaa ttaaagaaka	120
aaaaaaaaacaa ggaagaagaa akaagaagaa gaagaagaag aagaagaagc ctc	173

<210> SEQ ID NO 18
<211> LENGTH: 384
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: FUN1

<400> SEQUENCE: 18

caagctctag caggaagaag aaataagaag aagaagaaga agaagaagaa gcgttcctc	60
tttttttgtt gagagtaaaa aataaaactc caaaaaaaaa gaaaatcatc aaaaaaacaa	120
atttcaaaaa gagtttttgtt gtttgggat taaagaataaa aaaaaacaac gtctggacaa	180
accacaacta gaatgcagtg aaaaaatgc tttatgttga aaatttgc tgctattgct	240
ttatgtttaa ccattataag ctgcaataaa caagtttaca acaacaatg cattcattt	300
atgtttcagg ttcaggggaa ggtgtggag gtttttaaa gcaagtaaaa cctctacaaa	360
tgtggtaaaa tcgataagga tcgg	384

<210> SEQ ID NO 19
<211> LENGTH: 277
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-2

<400> SEQUENCE: 19

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgttcctc	60
tttttttgtt gagagtaaaa aakaaaactc caaaaaaaaa gaaaatcatc aaaaaaacaa	120
atttcaaaaa gagtttttgtt gtttgggat taaagaagaa aaaaaacaac aggtgagtaa	180
gcgcagttgt cgtctttgc ggtgccgtt ctggttctca caccttttag gtctgttctc	240
gtcttcgtt ctgactctct cttttcggtt gcaggcc	277

<210> SEQ ID NO 20
<211> LENGTH: 252
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-3

<400> SEQUENCE: 20

aagctctagc aggaagaaga akaagaaga agaagaagaa gaagaagaag cgttcctc	60
tcttttttgtt agagtaaaaa aakaaaactcc caaaaaaaaa aaaaatcatca aaaaaacaaa	120

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tttcaaaaag agtaggttaag attatctt cccaaaattt attactttt tattgaacaa	180
ttattaacca atcatggctt aacgaaaaac aggttttg tttggggatt aaagaakaaa	240
aaaaacaaaa ca	252

<210> SEQ ID NO 21
<211> LENGTH: 254
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-4

<400> SEQUENCE: 21

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgttcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
atttcaaaaa gagtaggtaa gattatctt tcccaaaattt gattactttt attattgaac	180
aattactaac atttcatggc ttaacgaaaa acaggtttg tggttgggaa ttaaaaka	240
aaaaaaaaacaa aaca	254

<210> SEQ ID NO 22
<211> LENGTH: 266
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-5

<400> SEQUENCE: 22

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgttcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
atttcaaaaa gagtgaggtt agattatcg tattttaaattt atttattttt tctttccat	180
tttttggct aacatttcc atggtttat gatatcatgc aggtacgttt tggtttgg	240
gattaaagaa kaaaaaaaaac aaaaca	266

<210> SEQ ID NO 23
<211> LENGTH: 266
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-6

<400> SEQUENCE: 23

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgttcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
atttcaaaaa gagtgaggtt agattatcg tattttaaattt atttattttt tctttccat	180
tttttggct aacatttcc taggttttat tatatctagc aggtacgttt tggtttgg	240
gattaaagaa kaaaaaaaaac aaaaca	266

<210> SEQ ID NO 24
<211> LENGTH: 265
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-7

<400> SEQUENCE: 24

aagctctagc aggaagaaga aakaagaaga agaagaagaa gaagaagaag cgttcctc	60
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tcttcttgta agagtaaaaa akaaaactcc caaaaaaaaaak aaaatcatca aaaaaacaaa	120
tttcggaaag agtgaggtaa gattatcgat atttaaatta ttatattctt ctttccatt	180
ttttggcta acatttcct aggtttatt atatctagca ggtacgttt gtgttgggg	240
ataaaagaak aaaaaaaaca aaaca	265

<210> SEQ ID NO 25
<211> LENGTH: 266
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-8

<400> SEQUENCE: 25

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgtctcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
attcaaaaa gagtgaggta agattatcga tatttaattt atttatttct tctttccat	180
ttttggctt aacatttcc tagttttat tatatctagc aggtacgttt tgtgttggg	240
gattaaagaa kaaaaaaaaac aaaacc	266

<210> SEQ ID NO 26
<211> LENGTH: 287
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-9

<400> SEQUENCE: 26

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgtctcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
attcaaaaa gagtttttgtt gtttgtaagt caggactcta gctttctact gtatgtatcct	180
ctaaaggact gctgttctgt gcacccctt ccttgcata tcatacgca cgacaagagt	240
actaactaat taacttaggg ggattaaaga akaaaaaaaaa caacaaa	287

<210> SEQ ID NO 27
<211> LENGTH: 251
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2-10

<400> SEQUENCE: 27

caagctctag caggaagaag aaakaagaag aagaagaaga agaagaagaa gcgtctcctc	60
ttcttcttgt gagagtaaaa aakaaaactc caaaaaaaaa kaaaatcatc aaaaaaaca	120
attcaaaaa gagtttttgtt gtttggttaa gtaattgcct tactcgaaa ataatcaatc	180
atcataactaa cgcaagaggc gctgatattg cggttataca gggattaaag akaaaaaaaaa	240
acaacgtcac c	251

<210> SEQ ID NO 28
<211> LENGTH: 143
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA-2

<400> SEQUENCE: 28

aaagtatcaa caaaaaagct tcgtctcctc ttcttcttgt gagagtaaaa aakaaaactc	60
---	----

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ccaaaaaaaaaaa kaaaaatcatc aaaaaaaacaa atttcaaaaa gagttttgt gtttgggat	120
taaagaakaa aaaaaacaac aaa	143

<210> SEQ ID NO 29
<211> LENGTH: 123
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA-3

<400> SEQUENCE: 29

tcgtctc cttttttgt gagagtaaaa aakaaaactc caaaaaaaaaaa kaaaaatcatc	60
aaaaaaaaacaa atttcaaaaa gagttttgt gtttgggat taaagaakaa aaaaaacaac	120
gtc	123

<210> SEQ ID NO 30
<211> LENGTH: 134
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA-4

<400> SEQUENCE: 30

caagctctag cacgtctc ctttttttg tgagagtaaa aaakaaaact cccaaaaaaaa	60
akaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttggga ttaaagaaka	120
aaaaaaaaacaa cgcc	134

<210> SEQ ID NO 31
<211> LENGTH: 140
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA-5

<400> SEQUENCE: 31

ggcaagctc agcacgtctc ctcttttct tgtgagagta aaaaakaaaa ctccaaaaaa	60
aaakaaaatc atcaaaaaaa caaattcaa aaagagttt tgtgtttggg gattaaagaa	120
aaaaaaaaac aacctccacc	140

<210> SEQ ID NO 32
<211> LENGTH: 130
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1dGAA-6

<400> SEQUENCE: 32

caagcgtctc ctcttttct tgtgagagta aaaaakaaaa ctccaaaaaa aaakaaaatc	60
atcaaaaaaa caaattcaa aaagagttt tgtgtttggg gattaaagaa kaaaaaaaaac	120
aacctccacc	130

<210> SEQ ID NO 33
<211> LENGTH: 178
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2dGAA-2

<400> SEQUENCE: 33

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```
cgtctccctc tcttttgcg agataaaaaa akaaaactcc caaaaaaaaaak aaaatcatca 60  
aaaaaaaaaaa ttcaaaaag agtttttg tttggggatt aaagaakaaa aaaaacaacc 120  
tcgtcgctgt tgcccgattcg cgtacgata cgcccttgtgc tgacacttct gttagcacc 178
```

```
<210> SEQ ID NO 34
<211> LENGTH: 238
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2dGAA-3
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<400> SEQUENCE: 34

```
caagctctag cacgtctcct cttttcttg tgagagataaa aaakaaaact cccaaaaaaaaa 60  
akaaaaatcat caaaaaaaaaa aatttcaaaa agagttttg tgtttggga ttaaagaaka 120  
aaaaaaaaacaa cagggtgagta agcgcaaggtt tcgtctcttgc gggtggcggtt gctggttctc 180  
acacctttta qqtctgtttct cqtcttcgt tctgactctc tcttttcgt tqcqaggcc 238
```

<210> SEQ ID NO 35

```
<211> LENGTH: 229
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2dGAA-4
```

<400> SEQUENCE: 35

atcaagtc t agcacgtc tc ctcttctt ct tgtagagta aaaaakaaaa cttccaaaaaa	60
aaakaaaaatc atcaaaaaaa caaatttcaa aaagagttagt gtaagattat cgatatttaa	120
attattttat tcttctttc cattttttg gctaacattt tccttaggttt tattatatct	180
agcaggtagc ttttgtgtt ggggattaaa gaakaaaaaa aacaaaaca	229

<210> SEQ ID NO 36

```
<211> LENGTH: 188
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1shuffle
```

<400> SEQUENCE: 36

caagctctag cacgtctcct ctttttcttg tgagagataaa aaakaaaact cccaaaaaaaa	60
akaaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttggga ttaaagaaka	120
aaaaaaaaacaa ggaagaagaa akaagaagaa gaagaagaag aagaagaagg gcggccgccc	180
ctttcacc	188

<210> SEQ ID NO 37

```
<211> LENGTH: 173
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UNIshuffle-2
```

<400> SEQUENCE: 37

caagctctag cacgtctcct ctttttcttg tgagagtaaa aaakaaaact cccaaaaaaa	60
akaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttggga ttaagaaka	120
aaaaaaaaaca ggaagaagaa akaagaagaa gaagaagaag aagaagaagc gcc	173

<210> SEQ ID NO 38
<211> LENGTH: 154

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<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1shuffle-3

<400> SEQUENCE: 38

```
gaagctctag cacgtctcct cttttcttg tgagagtaaa aaakaaaact cccaaaaaaa      60
akaaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttgggta ttaagaaka      120
aaaaaaaaacaa ggaagaagaa gaagaagaag cgcc                                154
```

<210> SEQ ID NO 39
<211> LENGTH: 179
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1shuffle-4

<400> SEQUENCE: 39

```
ggcaagctct agcacgtctc ctttttctt tgtgagagta aaaaakaaaa ctccaaaaaa      60
aaaaakaaaatc atcaaaaaaa caaatttcaa aaagagtttt tggtttggg gattaaagaa      120
aaaaaaaaacaa aaggaagaag aaakaagaag aagaagaaga agaagaagaa gcctccacc      179
```

<210> SEQ ID NO 40
<211> LENGTH: 160
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1shuffle-5

<400> SEQUENCE: 40

```
ggcaagctct agcacgtctc ctttttctt tgtgagagta aaaaataaaaaa ctccaaaaaa      60
aaagaaaaatc atcaaaaaaa caaatttcaa aaagagtttt tggtttggg gattaaagaa      120
aaaaaaaaacaa aaggaagaag aagaagaaga agcctccacc                                160
```

<210> SEQ ID NO 41
<211> LENGTH: 177
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN1shuffle-6

<400> SEQUENCE: 41

```
caagctctag cacgtctcct cttttcttg tgagagtaaa aaakaaaact cccaaaaaaa      60
akaaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttgggta ttaagaaka      120
aaaaaaaaacaa ggaagaagaa akaagaagaa gaagaagaag aagaagaagc ctccacc      177
```

<210> SEQ ID NO 42
<211> LENGTH: 277
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2shuffle-1

<400> SEQUENCE: 42

```
caagctctag cacgtctcct cttttcttg tgagagtaaa aaakaaaact cccaaaaaaa      60
akaaaaatcat caaaaaaaca aatttcaaaa agagttttg tgtttgggta ttaagaaka      120
aaaaaaaaacaa ggaagaagaa akaagaagaa gaagaagaag aagaagaagc aggtgagtaa      180
gcgcagttgt cgtctttgc ggtgccgtt ctggttctca caccttttag gtctgttctc      240
```

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gtcttcgtt ctgactctc cttttcggt gcaggcc	277
---------------------------------------	-----

<210> SEQ ID NO 43
<211> LENGTH: 267
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: UN2shuffle-2

<400> SEQUENCE: 43

atcaagctct agcacgtctc ctcttcttct tgtgagagta aaaaakaaaa ctcccaaaaa	60
aaakaaaaatc atcaaaaaaaaa caaatttcaa aaagagttag gtaagattat cgatattaa	120
attatttatt tcttcttttc cattttttg gctaacattt tccttaggtt tattatatct	180
agcaggtacg ttttgtttt ggggattaaa gaakaaaaaa aacaaggaag aagaakaag	240
aagaagaaga agaagaagaa gaaaaca	267

<210> SEQ ID NO 44
<211> LENGTH: 264
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA1

<400> SEQUENCE: 44

caagctctac caccaagaac aaacaacaac aacatatata aaacaacaac caccatctcc	60
tcttcttctt gtcaactcca aaatcaaact cccaaaaaaaaa agcaaatcat caaaagttag	120
gtaagattat cgatatttaa attatttatt tcttcttttc cattttttg gctaacattt	180
tccttaggtt tattatatct agcaggtacg aaatttcaaa caacaacaac aaacaacaaa	240
caacattaac atcatatcaa aacc	264

<210> SEQ ID NO 45
<211> LENGTH: 188
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA2

<400> SEQUENCE: 45

caacacctctac caccaacaac aaacaacaac aacaacaaca acaacaacaa ccctctccac	60
atctccctct cagagtaaaa aacaaaactc cccaaaaaaaaa gaaaatcatc aaaaaaacaa	120
atttcaaaaa gacttcttct cattccttat taaaagaacaa aaaaaacaag gcggccgccc	180
ccttcacc	188

<210> SEQ ID NO 46
<211> LENGTH: 207
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA3

<400> SEQUENCE: 46

gtatTTTAC aacaattacc aacaacaaca aacaacaaac aacattacaa ttactatTTA	60
caattacaag cgtctctct tcttcttgtg agagtaaaaa ataaaactcc caaaaaaaaaag	120
aaaaatcatca aaaaaacaaa ttcaaaaag agttttgtg tttggggatt aaagaataaa	180
aaaaacaagg cggccgcccc cttcacc	207

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```

<210> SEQ ID NO 47
<211> LENGTH: 188
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA4

<400> SEQUENCE: 47
caagctctac caccaagaac aaacaacaac aacatatata aaacaacaac caccatctcc      60
tcttcttctt gtcaactcca aaatcaaact cccaaaaaaaa agcaaatcat caaaaccaca      120
aatttcaaac aacaacaaca aacaacaaac aacattaaca tcatatcaag gcggccgccc      180
ccttcacc                                         188

<210> SEQ ID NO 48
<211> LENGTH: 175
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA5

<400> SEQUENCE: 48
caacacctac caccaacaac aaacaacaac aacaacaaca acaacaacaa ccctctccac      60
atotccctct cagagtaaaa aacaaaattg aaaaaaaaaa gattttataa taaaaacaaa      120
tttcaaaaag aattcaactc attcaatatt acaacaagaa caaaggaggt cacat      175

<210> SEQ ID NO 49
<211> LENGTH: 177
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CAA6

<400> SEQUENCE: 49
caagctctac caccaagaac aaacaacaac aacatatata aaacaacaac caccatctcc      60
tcttcttctt gtcaactcca aaatcaaact cccaaaaaaaa agcaaatcat caaaaccaca      120
aatttcaaac aacaacaaca aacaacaaac aacattaaca tcatatcaac ctccacc      177

<210> SEQ ID NO 50
<211> LENGTH: 175
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TATA1

<400> SEQUENCE: 50
caagctctag caggaagaag aaataagaag aagaagaaga agaagaagaa gcgtctcctc      60
ttttcttcta cagagtaaaa aataactttt ataataaaaga aaatcatcaa aaaaacaaat      120
ttcaaaaaga gttttgtgt ttggggatta aagaataaaa aaaaggaggt cacat      175

<210> SEQ ID NO 51
<211> LENGTH: 177
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: TATA2

<400> SEQUENCE: 51
caagctctag caggaagaag aaataagaag aagtatataa aagaagaaga agcgtctcct      60
tttcttcttg tgaagtaaaa aataaaaactc cccaaaaaaaaa gaaaatcatc aaaaaacaa      120

```

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atttcaaaaa gagttttgt gtttggggat taaagaataa aaaaaacaac ctccacc 177

<210> SEQ ID NO 52
<211> LENGTH: 315
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: fragment

<400> SEQUENCE: 52

```
ggagttccgc gttacataac ttacggtaaa tggcccgect ggctgaccgc ccaacgaccc      60
ccgcccattg acgtcaataa tgacgtatgt tcccatagta acgccaatag ggacttcca      120
ttgacgtcaa tgggtggagt attacggta aactgcccac ttggcagttac atcaagtgtta      180
tcatatgccaa agtacgcccc ctattgacgt caatgacggt aaatggcccg cctggcatta      240
tgcccagttac atgaccttat gggacttcc tacttggcag tacatctacg tattagtcat      300
cgctattacc atggc      315
```

<210> SEQ ID NO 53
<211> LENGTH: 303
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: fragment

<400> SEQUENCE: 53

```
ggcctccgcg cgggttttg ggcctcccg cggggcgcccc ctcctcacg gcgagcgctg      60
ccacgtcaga cgaaggcgccg agcgagcgcc ctgatcccttc cgcccgacg ctcaggacag      120
cgccccgctg ctcataagac tcggccttag aaccccgatg tcaagcagaag gacatTTTg      180
gacgggactt ggggtgactct agggcaactgg ttttcttcc agagagcgga acaggcgagg      240
aaaagtagtc ctttctcgcc gattctgcgg agggatctcc gtggggcggt gaacgcccgt      300
gat      303
```

<210> SEQ ID NO 54
<211> LENGTH: 305
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: fragment

<400> SEQUENCE: 54

```
cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaaacgacc cccgccccatt      60
gacgtcaata atgacgtatg ttcccatagt aacgccaata gggacttcc attgacgtca      120
atgggtggag tatttacggta aactgcccc cttggcagta catcaagtgt atcatatgcc      180
aagtacgccc cttattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta      240
catgaccta tgggacttcc ctacttggca gtacatctac gtattagtca tcgttattac      300
catga      305
```

<210> SEQ ID NO 55
<211> LENGTH: 1428
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: fragment

<400> SEQUENCE: 55

```
cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaaacgacc cccgccccatt      60
```

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gacgtcaata atgacgtatg ttcccatagt aacgccaata gggacttcc attgacgtca	120
atgggtggag tatttacggt aaactgccc cttggcagta catcaagtgt atcatatgcc	180
aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt atgcccagta	240
catgaccta tgggacttcc ctacttggca gtacatctac gtattagtca tcgctattac	300
catgaattgg tttgatctga ttataaccta ggtcgaggaa ggtttcttca actcaaattc	360
atccgcctga taatttctt atatttcct aaagaaggaa gagaagcgca tagaggagaa	420
ggaaaataat ttttaggag ctttcttac ggctatgagg aatttgggc tcagttgaaa	480
agcctaaact gcctctcggt aggttggcg cggcgaacta ctttcagcg cgcacggaga	540
cggcgtctac gtgaggggtg ataagtgacg caacactcg tgcataaatt tgccctccgc	600
agccccggagc atttaggggc gggtggcttt gttgggttag cttgtttgtg tccctgtggg	660
tggacgtggc tggtgattgg caggatcctg gtatccgcta acaggtactg gcccccagcc	720
gtaacgacct tgggggggtg tgagggggg gaatgggtga ggtcaagggt gaggcttctt	780
ggggttgggt gggccgctga ggggaggggcg tgggggaggg gaggggcgagg tgacgcggcg	840
ctggggcctt ccggggacgt gggcccttgc gacctgaggg gggcgaggc gggtggcgcg	900
cgggggttga cggaaaactaa cggacgccta accgatggc gattctgtcg agtttactc	960
ggggggggagg cggaaaagag gtatgggtg tgggttctgg aagccttac tttggaatct	1020
cagtgtgaga aaggtgcccc ttcttgcgtt tcaatggat ttttatttgc cgagtctgt	1080
gggtttgggt ttgtttcag tttgcctaac accgtgctta ggtttgggc agattggagt	1140
tccggtcgggg gagttgaat atccggaaaca gttagtgggg aaagctgtgg acgattggta	1200
agagagcgct ctggattttc cgctgttgc gttgaaacct tgaatgacga atttcgtatt	1260
aagtgactta gccttgcata attgagggga ggttgcggg atattaacgt atttaaggca	1320
tttgaagga atagttgcta attttgaaga atattaggtg taaaagcaag aaatacaatg	1380
atccctgaggt gacacgctta ttgtttactt tttaaactagg tcagcatg	1428

<210> SEQ ID NO 56

<211> LENGTH: 1055

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: fragment

<400> SEQUENCE: 56

ggagttccgc gttacataac ttacggtaaa tggcccgccc ggctgaccgc ccaacgaccc	60
ccgcccattt acgtcaataa tgacgtatgt tcccatagta acgccaatag ggacttcca	120
ttgacgtcaa tgggtggagt atttacggta aactgcccac ttggcagttac atcaagtgt	180
tcatatgcca agtacgcccc ctattgacgt caatgacggt aaatggcccg cctggcatta	240
tgcccagttac atgaccttat gggacttcc tacttggcag tacatctacg tattagtcat	300
cgcttattacc atggtcgagg tgagcccad gttctgcttc actctccca tctcccccc	360
ctccccccccc ccaattttgt atttattttat ttttaattt ttttgtgcag cgatggggc	420
gggggggggg gggggcgccg gccaggcgccg gcggggcgccg gcgaggggcg gggcgccg	480
aggccggagag gtgcggcgcc agccaatcg agcggcgccg tccgaaagtt tcctttatg	540
gcgaggcgccg ggcggcgccg gcccataaa aagcgaagcg cgccggcgccg gggagtcgct	600
gcccgcgtcc ttgcggccgt gccccgcctt gccgcgcctt cgccgcgcgc gccccggctc	660

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tgactgaccg	cgttactaaa	acaggtaagt	ccggcctccg	cgcgggtt	tggcgcctcc	720
cgcggggcgc	ccccctctca	cgggagcgc	tgcacgtca	gacgaagggc	gcagegagcg	780
tcctgatcct	tccgccccga	cgctcaggac	ageggcccgc	tgctcataag	actcggcctt	840
agaaccccag	tatcagcaga	aggacatttt	aggacgggac	ttgggtgact	ctagggcact	900
ggttttttt	ccagagagcg	gaacaggcga	ggaaaagtag	tccctctcg	gcgattctgc	960
ggagggatct	ccgtggggcg	gtgaacgcgc	atgatgcctc	tactaaccat	gttcatgttt	1020
tctttttttt	tctacaggc	ctgggtga	aacag			1055

<210> SEQ ID NO 57
<211> LENGTH: 753
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: CMV promoter

<400> SEQUENCE: 57

tcaatattgg	ccattagcca	tattattcat	tggttatata	gcataaatca	atattggcta	60
ttggccattg	catacgttgt	atctatatca	taatatgtac	atttatatgt	gctcatgtcc	120
aatatgaccg	ccatgttggc	attgattatt	gactagttat	taatagtaat	caattacggg	180
gtcattagtt	catagccat	atatggagtt	ccgcgttaca	taacttacgg	taaatggccc	240
gcctggctga	ccgccccaa	cccccggccc	attgacgtca	ataatgacgt	atgttccat	300
agtaacgcca	atagggactt	tccattgacg	tcaatgggt	gagtatttac	ggtaaaactgc	360
ccacttggca	gtacatcaag	tgtatcatat	gccaagtccg	ccccctat	acgtcaatga	420
cggtaaatgg	ccgccttggc	attatgccc	gtacatgacc	ttacgggact	ttcctacttg	480
gcagtagatc	tacgttattag	tcatcgctat	taccatgtg	atgcgggttt	ggcagtagacac	540
caatggcggt	ggatagcggt	ttgactcacg	gggatttcca	agtctccacc	ccattgacgt	600
caatggaggt	ttgttttggc	acccaaatca	acgggacttt	ccaaaatgtc	gtaataaccc	660
cgcggcggtt	acgcaaatgg	gcggtagg	tgtacggtgg	gagggtctata	taagcagagc	720
tcgttttagt	aaccgtcaga	tcactagaag	ctt			753

<210> SEQ ID NO 58
<211> LENGTH: 69
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: minimal CMV promoter

<400> SEQUENCE: 58

taggcgtgta	cgggtggagg	tctatataag	cagagctcg	ttagtgaacc	gtcagatcac	60
tagaagctt						69

<210> SEQ ID NO 59
<211> LENGTH: 2248
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-Xt

<400> SEQUENCE: 59

tggtgaccct	gtctaaaaaa	accctaaaaa	agtgtggga	ttagtggcat	gcaccaccat	60
tcccacaaaa	ggtttatttt	taataatatg	tgtgtgagtg	tgtatcacta	tgagtatatg	120
tcaatatgt	tcaatgtccc	cagggacatt	taaagagccc	ctgaagctgg	agtcataggc	180

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cattatgaac tgcctgacat ggctaattggg aattgaactc agatttctg gaagttatac	240
ctgtcttac tgctgagcca tgtctctgaa gaccccaggg atttttttt tttttgaga	300
caggtatttt ctgtatagcc ctggctgtcc taaaagact ctctatatgt agaccaggct	360
tgcctggagc ttggatatgc acctgtttct gcctcaggaa tggtgggatt gaagggtgtc	420
accaccacat ccgctaacaat gcacaattct taatgggaaa atatcttatt taatgaatga	480
aaggtttggg ggtatggatgt agcttaatgg aaaatgactg aagattcaa ttaaaaatct	540
ggggcttagc tgccgggtgg gtgggtcctg ccttagtcc cagtaactggg gagggcagg	600
aaggaggatc tctgtgagtt cgaggccagc tggctataaa cgtgagttcc aggacagcca	660
gagatacaca gacaaaccct gtctcaccaa aacaaaacaa caacaacaac aacaaatctg	720
ggacgttaggc ttgggtgggt ggcacacatt ttgattccag cacttggaaag gaaggccct	780
gcatggtcta catagcttgtt ttcaggcaac cagagctaca tagttagatc ctgtctcaac	840
aaaaataaaa taatctaagg cttcaaaaggg ttcaatctct taggttagcta aatatgaaca	900
aaatttggga aatgtgaccc ttccottagt gacagtcaga tagaacottc tcgagtgc当地	960
ggacaccaag tgccaaacagg ctcaagaaca gcctggaaag gtctagtgtc atggggcttc	1020
aggtcgaatg ccaactgttt tcaagaactg tggatgtttt tctgcgtgtc acgaattcag	1080
attcattttt caaaactcggtt ggagatttt ccccccttat aatttttttt ttaaattttt	1140
taaaactttgt ttgcgtcccc ttgtttttag aattgcagag tcatccaccc tgcacagtg	1200
ccagggagct caggatggg cccaggggcc tggggggctg gaaggggctg gggaaagcgg	1260
ggctccaaag ggacccctgt gtggcaggag ccaagccct aggtccctag aacgcagg	1320
ccaccggggc ccccccagacg gggtaagcgg gtgggtgtct gggcgcgaa gcccactgc	1380
gcatgcgcg aggtccgcgc cggccgcget gatccaaagcc gggttctcgc gcccacccgg	1440
tctgtattgtca aagtcacac acgtctgttcc ctcgcgggg cccacagggt tcacaccc	1500
tccctccccc acaaagcccc ctactctctg ggcacccacac acgaacatcc ttgagcgtg	1560
accttggatgtt ctctagtcag gcgcctccgg tgcagagact ggaacggct tggaaagtag	1620
tccctaaccg catttcccgcg gagggatcgt cggggggcg tggcttctga ggattatata	1680
aggcgactcc gggggggct tagctgttc cgtcgagac cccagttcag tcgcgcgtt	1740
tctgtgagga ctgcgtccgc cggcgctgtt gaggagaagc cggcgccgtt ggctgtactg	1800
agagacgggg agggggcgccg gacacgggg gcagcccgcc gctggacgt tctgtttccg	1860
tggcccgccgaa ggaaggcgac tgcctgtggg cggaggaccg agccgcaga tggccggccaa	1920
gtggaaaggct gagggatag gcgagccgc ctgaggccgtt ccacgggtt gggggggaaag	1980
caggcccccgccg aggccgcgttcc gtcggccaa ccccttatttttttgcgg	2040
gttgcggccgc gtaggtgcctt ccgaagtggag agccgtgggg gtttgcgtt cggggaggtt	2100
cggtcggatttttcatccgtt gctaaagacg gaagtgcac tgcacgggaa agggggggaa	2160
gtcggttgggtt ggcgggttggaa cctggactaa ggccgcacatg acgtcgccgtt ttctatggc	2220
tcataatggg tggtgaggac attccct	2248

<210> SEQ ID NO 60
 <211> LENGTH: 1159
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: EEE1-80

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<400> SEQUENCE: 60

tcaaaaactcg	gggagagttt	tccccctta	taattttttt	tttaaattta	ttaaacttg	60
tttcgttccc	cttgtttga	gaattgcaga	gtcatccacc	ctgtcacagt	gccagggagc	120
tcagggatgg	gcccaggggc	ctggcggggc	tgaaggggct	gggaaagcga	gggctccaaa	180
gggaccccg	tgtggcagga	gccaagccc	taggtcccta	gaacgcagag	gccaccggga	240
ccccccagac	ggggtaagcg	ggtggtgtc	tggggcgega	agccgcactg	cgcacgcgcc	300
gaggtccgct	cggccgcgc	tgatccaago	cgggttctcg	cggccacactg	gtcgtagtt	360
acaagtaca	cacgctgatc	cctccgcggg	gccgcacagg	gtcacagcct	ttccccctcc	420
cacaaagccc	cctactctct	gggcaccaca	cacgaacatt	ccttgagcgt	gaccttgg	480
gctctagtca	ggcgcctccg	gtgcagagac	tggAACGGC	ttggaaagta	gtccccataacc	540
gcatttccgc	ggagggatcg	tcgggagggo	gtggcttctg	aggattata	aaggcgactc	600
cgggcggg	ttagctagtt	ccgtcggaga	cccgagttca	gtcgccgctt	ctctgtgagg	660
actgctgcgc	ccggccgtgg	tgaggagaag	ccgcgcgcgt	tggcgtagct	gagagacggg	720
gagggggcgc	ggacacgagg	ggcagccgc	ggcctggacg	ttctgtttcc	gtggcccg	780
aggaaggcga	ctgtcctgag	gcccgggacc	cagcggcaag	atggcggcca	agtggaaagcc	840
tgaggggata	ggcgagcggc	cctgaggcgc	tcgacgggg	tgggggggaa	gcaggccgc	900
gaggcagctg	cagccggaa	cgtcggccca	accccttatt	tttttgacg	gtttgcggc	960
cgttaggtgc	tccgaagtga	gagccgtggg	cgtttgactg	tggggagagg	tcggcggat	1020
ttcatccgt	tgctaaagac	ggaagtgcga	ctgagacggg	aagggggggg	agtcggttgg	1080
tggcggttga	acctggacta	aggcgacat	gacgtcgcgg	tttctatgg	ctcataatgg	1140
tggtgtgagga	cattttccct					1159

<210> SEQ ID NO 61

<211> LENGTH: 869

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: EEE1-60

<400> SEQUENCE: 61

cgcacatgcgc	gagggtccgt	ccggccgcgc	tgatccaago	cgggttctcg	cggccacactg	60
gtcgtagtt	acaagtaca	cacgctgatc	cctccgcggg	gccgcacagg	gtcacagcct	120
ttccccctcc	cacaaagccc	cctactctct	gggcaccaca	cacgaacatt	ccttgagcgt	180
gaccttgg	gctctagtca	ggcgcctccg	gtgcagagac	tggAACGGC	ttggaaagta	240
gtccccataacc	gcatttccgc	ggagggatcg	tcgggagggo	gtggcttctg	aggattata	300
aaggcgactc	ccggcggg	ttagctagtt	ccgtcggaga	cccgagttca	gtcgccgctt	360
ctctgtgagg	actgctgcgc	ccgcgcgtgg	tgaggagaag	ccggcgacgt	tggcgtagct	420
gagagacggg	gagggggcgc	ggacacgagg	ggcagccgc	ggcctggacg	ttctgtttcc	480
gtggcccg	aggaaggcga	ctgtcctgag	gcccgggacc	cagcggcaag	atggcggcca	540
agtggaaagcc	tgggggata	ggcgagcggc	cctgaggcgc	tcgacgggg	tgggggggaa	600
gcaggccgc	gaggcagctg	cagccggaa	cgtcggccca	accccttatt	tttttgacg	660
gtttgcggc	cgttaggtgc	tccgaagtga	gagccgtggg	cgtttgactg	tggggagagg	720
tcggcggat	tttcatccgt	tgctaaagac	ggaagtgcga	ctgagacggg	aagggggggg	780
agtcggttgg	tggcggttga	acctggacta	aggcgacat	gacgtcgcgg	tttctatgg	840

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ctcataatgg gtgggtgagga catttcctt 869

<210> SEQ ID NO 63
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-SL

<400> SEQUENCE: 63

ttcaggcaa ccagagctac atagtggat cctgtctcaa caaaaataaa ataatctaag 60
gcttcaaagg gttcaatctc ttaggttagct aaatatgaac aaaatttggg aatgtgacc 120
tttccttag tgacagtcag atagaacctt ctgcgtgca aggacaccaa gtgcaaacag 180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtcgaat gccaaacttgtt 240
ttcaagaact gtgtggatt ttctgcctgt aacgaattca gattcatttt tcaaaactcg 300
gggagagttt tccccctta taatttttt tttaaatttt ttaaactttt tttcgcccc 360
cttggggatggaa gtcatccacc ctgtcacagt gccaggggc tcagggatgg 420
gcccaaaaaa ctggcgcccc tgaaggggct ggggaagcga gggctccaaa gggacccccag 480
tgtggcagga gccaaagccc taggtcccta gaacgcagag gccacccgggaa ccccccagac 540
ggggaaagcg gttgggtgtc tggggcgcgca agccgcactg cgcatcgcc gaggtccgct 600
ccggcccgcc tcatccaagc cgggttctcg cgccgcacccgt gtcgtgattt acaagtcaca 660
cacgctgatc cttccgcggg gcccacagg gtcacagccctt tccccctccc cacaagccc 720
cctactctctt gggcaccaca cacgaacatt ctttgagcgt gacccgttgc gctctgtca 780
ggcgccctccg gtgcagagac tggAACGGCC ttgggaagta gtcccttaacc gcattccgc 840
ggggggatcg tcgggggggc gtgggttctg aggattat aaggccactc cgggggggtc 900

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ttagctagtt ccgtcggaga cccgagttca gtgcgcgtt ctctgtgagg actgctgccg	960
cgcgcgcgtgc tgaggagaag cgcgcgcgt tggcgtacgt gagagacggg gagggggcgc	1020
ggacacgagg ggcageccgc ggcctggaeq ttctgttcc gtggcccgcg aggaaggcga	1080
ctgtccttag gcgaggacc cagggcaag atggcggcca aatggaaagcc tgagggata	1140
ggcgagccgc cctgaggcgc tcgacgggt tggggggaa gcaggccgc gaggcactg	1200
cagccggaa cgtgcggcca accecttatt tttttgacg ggttgcgggc cgttaggtgcc	1260
tccgaattga gagccgtgg cgtttactg tcgggagagg tcggteggat tttcatccgt	1320
tgctaaagac ggaagtgcga ctgagacggg aagggggggg agtcgggtgg tggcggttga	1380
acctggacta aggccacat gacgtcgcgg ttctatggg ctcataatgg gtggtaggaa	1440
cattccct	1449

<210> SEQ ID NO 64
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: HC RACE PRIMER

<400> SEQUENCE: 64

gctgggtgcccgcc aggtccttag cgcaatagta cac	33
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<210> SEQ ID NO 65
<211> LENGTH: 4128
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Light chain vector sequence without the coding sequence

<400> SEQUENCE: 65

tgcaggccgc cgctttcagg caaccagago tacatagtga gatcctgtct caacaaaaat	60
aaaataatct aaggcttcaa agggttcaat ctcttagtta gctaaatatg aacaaaattt	120
ggaaatgtc acctttccct tagtgcacgt cagatagaac cttctcgagt gcaaggacac	180
caagtgc当地 caggctcaag aacagcctgg aaaggtctag tgctatggg cttcaggctg	240
aatgccaact gtttcaaga actgtgtgaa ttttctgca tgtaacgaat tcagattcat	300
tttcaaaac tcggggagag tttccccct ttataatttt tttttaaat ttattaaact	360
ttgtttcggtt cccctgttt tgagaattgc agagtcatcc accctgtcac agtgcaggg	420
agctcaggaa tggggccagg ggcctggcgg ggctgaaggg gctgggaag cgagggctcc	480
aaaggaccc cagtgtggca ggagccaaag ccctaggctcc ctagaacgc当地 gaggccaccc	540
ggacccccc当地 gacggggtaa ggggggggt gtctggggcg cgaagccgc当地 ctgcgc当地 catgc	600
ggcgaggctcc gtcggccgc当地 cgctgatcca agccgggtcc tcgcgc当地 gagggctgc当地	660
ttgacaagtc acacacgctg atccctccgc gggccgc当地 agggtcacag ctttccccct	720
ccccacaaag cccctactc tctggccacc acacacgaaat ttcccttgc当地 cgtgacccctg	780
ttggctctag tcaggccct cccgtgcaga gactggaaacg gcttggaaat gtgtcccta	840
accgcattc cgcggaggaa tcgtcggagg ggcgtggctt ctgaggatataaggcga	900
ctccggccgg gtccttagcta gttccgtcgg agacccgagtc agtgcgc当地 cttctctgt	960
aggactgctg cccgc当地 cccgc当地 tggtgaggag aagccgc当地 gcttggcgta gctgagagac	1020
ggggaggggg cgcggacacg agggccagcc cgcggccctgg acgttctgtt tccgtggccc	1080

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gcgaggaagg cgactgtcct gagggggagg acccagccgc aagatggcg ccaagtggaa	1140
gcctgagggg atagggggc ggcctgagg cgctcgacgg ggttgggggg gaaggaggcc	1200
cgcgaggcag ctgcagccgg gaacgtgcgg ccaaccctt attttttttg acgggttcg	1260
ggccgttaggt gcctccgaag tgagggcgt gggcgtttg ctgtcgggag aggtcggtcg	1320
gattttcatc cgttgtaaa gacggaaatg cgactgagac gggaaaggggg gggagtcgg	1380
tggtggeggg tgaacctgga ctaaggcgca catgacgtcg cgggttctat gggctataa	1440
tgggtgggtga ggacatttcc ctgtttaaac ttAAACAAGT ttgtacaAAA aagcaggcta	1500
gatcttcaat attggccatt agccatatta ttcatgttggt atatagcata aatcaatatt	1560
ggctattggc cattgcatac gttgtatcta tatcataata tgtacatttatttggctca	1620
tgtccaatat gaccgcatttggcatttga ttattgtacta gtttataata gtaatcaattt	1680
acgggggtcat tagttcatag cccatatacg gagttccgcg ttacataact tacggtaat	1740
ggcccgcctg gctgaccgc caacgacccc cggccatttga cgtcaataat gacgtatgtt	1800
ccccatagtaa cgccaaatagg gactttccat tgacgtcaat ggggtggagta tttacggtaa	1860
actgcccact tggcagtaca tcaagtgtat catatgccaat gtccgcgggg tattgacgtc	1920
aatgacggta aatggccgc ctggcattat gcccagtaca tgaccttacg ggactttcc	1980
acttggcagt acatctacgt attagtcatac gctattacca tagtgatgcg gttttggcag	2040
tacaccaatg ggcgtggata gcggtttgac tcacggggat ttccaaatgtt ccacccatt	2100
gacgtcaatg ggagttgtt ttggcaccaa aatcaacggg actttccaaa atgtcgtaat	2160
aaccccgccc cgttgcgc aatggggcggtt aggctgtac ggtggggaggt ctatataagc	2220
agagctcggt tagtgaaccg tcagatcact agaagcttaa tacgactcac tatagggaga	2280
cccaagctgg cttagcttta aacggggccct cttagtaacg cccggcgtgt gctggattc	2340
ggcttaactc tagaccatgg ggcgcgcgg ttcagcctcg actgtgcctt ctgttgcca	2400
gccatctgtt gtttgcctt ccccccgtgc ttccctgacc ctggaaagggtt ccactccac	2460
tgtcctttcc taataaaatg agggaaattgc atcgcattgt ctgagtaggt gtcatttat	2520
tctgggggggtt ggggtggggc aggacagca gggggaggat tgggaagaca atagcaggca	2580
tgtggggat ggggtgggtt ctatggcttc tgaggcggaa agaaccagct ggatccatcc	2640
gttagatatac tggtaatgtt gtgtcagttt ggggtgtggaa agtccccagg ctccccagca	2700
ggcagaagta tgcaaagcat gcatctcaat tagtcagcaa ccaggtgtgg aaagtcccc	2760
ggctccccag caggcagaag tatgcacaaatc atgcacatcata attagtcagc aaccatagtc	2820
ccgccccctaa ctccgcctat cccgcctcta actccgcctt gttccgcctt ttctccggcc	2880
catgcctgac taatttttt tatttatgca gaggccgagg cccgccttcgc ctctgagcta	2940
ttccagaagt agtgaggagg cttttttggg ggcctaggct tttgcaaaaa gctcccgaaa	3000
gtttgtatatac ctatttcgg atctgtatcaa gagacaggat gaggatgtt tcacatgatt	3060
gaacaagatg gattgcacgc aggttctccg gccgcgtggg tggagaggctt attccgtat	3120
gactgggcac aacagacaat cggctgtct gatgcgcggc tgggtggctt gtcagcgcag	3180
gggcgcgggg ttcttttgtt caagaccgac ctgtccgggtt ccctgaatga actgcaggac	3240
gaggcagcgc ggctatcggt gctggccacg acgggcgttc cttgcgcagc tgggttcgac	3300
gttgcactg aagcggaaag ggactggctg ctattggggc aagtgcgggg gcaggatctc	3360
ctgtcatctc accttgcctt tgccgagaaa gtatccatca tggctgtatgc aatgcggcgg	3420
ctgcatacgc ttgatccggc tacctgcccc ttcgaccacc aagcgaacaca tcgcacatcg	3480

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cgagcacgta ctcggatgga agccgggtctt gtcgatcagg atgatctgga cgaagagcat 3540
 cagggggctcg cgccagccga actgttcgccc aggctcaagg cgccatgcc cgacggcgag 3600
 gatctcgatcg tgacacatgg cgatgcctgc ttgccgataata tcatggtgga aaatggccgc 3660
 ttttctggat tcatcgactg tggccggctg ggtgtggcg accgctatca ggacatagcg 3720
 ttggctaccc gtgatattgc tgaagagctt ggccggcgaat gggctgaccg cttccctcg 3780
 ctttacggta tcgccccctcc cgattcgcag cgatcgccct tctatgcct tcttgacgag 3840
 ttcttcctagg taccacgaga tttcgattcc accggccgcct tctatgaaag gttgggcttc 3900
 ggaatcgttt tccgggacgc cggtctggat atcctccagc gccgggatct catgtggag 3960
 ttcttcgccc accccaactt gtttattgca gcttataatg gttacaataa aagcaatagc 4020
 atcacaaaattt tcaacaataa agcattttttt tcaactgcatt cttagttgtgg tttgtccaaa 4080
 ctcatcaatg tatcttatca tgtctcaggt tgatgagcat attttacc 4128

<210> SEQ ID NO 66
 <211> LENGTH: 2647
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Heavy chain vector sequence without the coding sequence

<400> SEQUENCE: 66

tgcaggcggc cgctttcagg caaccagago tacatagtga gatcctgtct caacaaaaat 60
 aaaataatct aaggctcaa agggttcaat ctcttagtta gctaaatatg aacaaaattt 120
 gggaaatgtg acctttcct tagtgacagt cagatagaac cttctcgagt gcaaggacac 180
 caagtgc当地 caggctcaag aacagcctgg aaaggtctga tgctatgggg cttcaggctcg 240
 aatgccaact gtttcaaga actgtgtggaa ttttctgcc tgtaacgaat tcagattcat 300
 ttttcaaaac tcggggagag ttttccccct ttataatttt ttttttaaat ttattaaact 360
 ttgtttcggtt ccccttggtt tgagaattgc agagtcattcc accctgtcac agtgcacagg 420
 agctcaggga tggggccagg ggcctggcg ggctgaagggg gctggggaaag cgaggcgctcc 480
 aaagggaccc cagtgaggca ggagccaaag ccctaggctcc ctagaacgc当地 gagggccaccc 540
 ggacccccc当地 gacggggtaa gccccgggggt gtctggggcc cgaagccgc当地 ctgcgc当地 600
 gccgaggctcc gctccggccg cgctgatcca agccgggttc tgc当地ccgac ctggctgtga 660
 ttgacaagtc acacacgctg atccccccgc gggccgc当地 agggtcacag ctttccccct 720
 ccccacaaag cccctactc tctgggcacc acacacgaaac attccttgc当地 cgtgacccctg 780
 ttggctctag tcaggcgcct cccgtgc当地 gactggaaacg gcttggggaa gtatcccta 840
 acccgatttc cggggaggga tcgtggggag ggctgggtt ctgaggattataaggc当地 900
 ctccgggggg gtc当地tgc当地 gtc当地tgc当地 agaccggagtc tcaactgc当地 cttctctgt 960
 aggactgtc cccggccgc tggtgaggag aagccggccg gcttggcgta gctgagagac 1020
 ggggggggggg cccggacacgc agggccagcc cccggccctgg acgttctgtt tccgtggccc 1080
 gcgaggaaagg cccactgtccct gaggcggagg acccagccgc aagatggccg ccaagggtggaa 1140
 gcctgagggg ataggc当地 ggc当地tgc当地 cccctgagg cccctggccg ggttgggggg gaaggcaggcc 1200
 cccggaggccg ctgc当地ccgg gaacgtgc当地 cccacccctt attttttttgc acggggccg 1260
 ggccgttaggt gtc当地ccgaaag tgagagccgt gggcgttgc ctgtggggag aggtcggtcg 1320
 gatccatc ctttgc当地aa gacggaaatg cccactgc当地 gggaaagggggg gggaggccg 1380

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tggtggcggt tgaacctgga ctaaggcgca catgacgtcg cggtttctat gggctataa 1440
 tgggtggta ggacatttcc ctgtttaaac ttaaacaagt ttgtacaaa aagcaggcta 1500
 gatcttcaat attggccatt agccatatta ttcatgggtt atatagcata aatcaatatt 1560
 ggctattggc cattgcatac gttgtatcta tatcataata tgtacattt tattggctca 1620
 tgtccaatat gaccgccatg ttggcattga ttattgacta gttattaata gtaatcaatt 1680
 acggggtcat tagttcatag cccatataatg gagttcccg 1740
 ggcccgctg gctgaccgccc caacgaccgc cgccattga cgtcaataat gacgtatgtt 1800
 cccatagtaa cgccaatatgg gacttccat tgacgtcaat gggtgaggt tttacggtaa 1860
 actgcccact tggcagtaca tcaagtgtat catatgccaa gtccgcccc tattgacg 1920
 aatgacggta aatggccgc ctggcattat gcccagtaca tgaccttacg ggactttcct 1980
 acttggcagt acatctacgt attagtcacg gctattacca tagtgtatgcg gtttggcag 2040
 tacaccaatg ggcgtggata ggcgtttgac tcacggggat ttccaagttt ccacccatt 2100
 gacgtcaatg ggagtttggtt ttggcaccaa aatcaacggg actttccaaa atgtcgtaat 2160
 aaccccgcccc cggtgacgca aatggggcggt aggccgtgtac ggtgggaggt ctatataagc 2220
 agagctcggt tagtgaaccg tcagatcaact agaagcttaa tacgactcac tataaggaga 2280
 cccaaagctgg cttagcgttta aacggggccct ctagtaacgg ccggcagttt gctggaaattc 2340
 ggcttaactc tagaccatgg ggcggccgg ttcagcctcg actgtgcctt cttagttgcca 2400
 gccatctgtt gtttgcctt ccccggttcc ttcccttgcctt ctggagggtt ccactccac 2460
 tgcctttcc taataaaatg agggaaattgc atgcattgtt ctgagtaggt gtcatttttat 2520
 tctgggggggtt gggggggggc aggacagcaa gggggaggat tgggaagaca atagcaggca 2580
 tgctggggat gcggtgggtt ctatggcttc tgaggcggaa agaaccagctt ggtccatcc 2640
 gtttagat 2647

<210> SEQ ID NO 67

<211> LENGTH: 235

<212> TYPE: PRT

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: HuMab1 protein light chain

<400> SEQUENCE: 67

Met	Gly	Trp	Ser	Cys	Ile	Ile	Leu	Phe	Leu	Val	Ala	Thr	Ala	Thr	Gly
1					5				10			15			

Val	His	Ser	Ala	Gln	Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Val
						20		25		30					

Ser	Ala	Ser	Val	Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln
						35		40		45					

Gly	Ile	Ser	Ser	Trp	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala
					50		55		60						

Pro	Lys	Leu	Leu	Ile	Tyr	Ala	Ala	Ser	Ser	Leu	Gln	Ser	Gly	Val	Pro
65					70			75		80					

Ser	Arg	Phe	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile		
					85		90		95						

Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln	Ala
					100		105		110						

Asn	Asn	Phe	Pro	Leu	Thr	Phe	Gly	Gly	Thr	Lys	Val	Glu	Ile	Lys	
					115		120		125						

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Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
130 135 140

Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe
145 150 155 160

Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln
165 170 175

Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser
180 185 190

Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu
195 200 205

Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser
210 215 220

Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230 235

<210> SEQ ID NO 68

<211> LENGTH: 474

<212> TYPE: PRT

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: HuMab1 protein heavy chain

<400> SEQUENCE: 68

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1 5 10 15

Val His Ser Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu Val Gln
20 25 30

Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
35 40 45

Ser Asn Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50 55 60

Glu Trp Val Ser Ala Ile Ser Ala Ser Gly His Ser Thr Tyr Leu Ala
65 70 75 80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn
85 90 95

Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Ala Lys Asp Arg Glu Val Thr Met Ile Val Val Leu Asn
115 120 125

Gly Gly Phe Asp Tyr Trp Gly Gln Gly Thr Arg Val Thr Val Ser Ser
130 135 140

Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
145 150 155 160

Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
165 170 175

Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
180 185 190

Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
195 200 205

Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
210 215 220

Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
225 230 235 240

Arg Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
245 250 255

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Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
260 265 270

Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
275 280 285

Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
290 295 300

Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
305 310 315 320

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
325 330 335

His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
340 345 350

Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
355 360 365

Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
370 375 380

Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
385 390 395 400

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
405 410 415

Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
420 425 430

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
435 440 445

Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
450 455 460

Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
465 470

<210> SEQ ID NO 69
<211> LENGTH: 233
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: HuMab2 protein light chain

<400> SEQUENCE: 69

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1 5 10 15

Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
20 25 30

Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val
35 40 45

Asn Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
50 55 60

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg
65 70 75 80

Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser
85 90 95

Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr
100 105 110

Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr
115 120 125

Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu
130 135 140

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Lys Ser Gly Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro
145 150 155 160

Arg Glu Ala Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly
165 170 175

Asn Ser Gln Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
180 185 190

Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His
195 200 205

Lys Val Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val
210 215 220

Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230

<210> SEQ_ID NO 70

<211> LENGTH: 470

<212> TYPE: PRT

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: HuMab2 protein heavy chain

<400> SEQUENCE: 70

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr Gly
1 5 10 15

Val His Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
20 25 30

Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile
35 40 45

Lys Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
50 55 60

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala
65 70 75 80

Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn
85 90 95

Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val
100 105 110

Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr
115 120 125

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
130 135 140

Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly
145 150 155 160

Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
165 170 175

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
180 185 190

Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
195 200 205

Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
210 215 220

Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Pro
225 230 235 240

Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
245 250 255

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp
260 265 270

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Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
 275 280 285
 Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly
 290 295 300
 Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
 305 310 315 320
 Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
 325 330 335
 Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro
 340 345 350
 Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu
 355 360 365
 Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn
 370 375 380
 Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
 385 390 395 400
 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
 405 410 415
 Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
 420 425 430
 Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
 435 440 445
 Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
 450 455 460
 Ser Leu Ser Pro Gly Lys
 465 470

<210> SEQ_ID NO 71
 <211> LENGTH: 5428
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: pcDNA3.1(+) cloning vector
 <400> SEQUENCE: 71

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ccgcataagg	taagccagtt	ctgtccctgt	cttgggtgtt	ggagggtcg	ct gagtagtgcg	120
cgagcaaaat	ttaagctaca	acaaggcaag	gcttgaccga	caattgcatg	aagaatctgc	180
ttagggttag	gcgttttgcg	ctgtttcgcg	atgtacgggc	cagatatacg	cgttgacatt	240
gattattgac	tagttattaa	tagtaatcaa	ttacggggtc	attagttcat	agcccatata	300
tggagttccg	cgttacataa	cttacggtaa	atggcccgcc	tggctgacccg	cccaacgacc	360
cccgccccatt	gacgtcaata	atgacgtatg	ttcccatagt	aacgccaata	gggacttcc	420
attgacgtca	atgggtggag	tatcacgtt	aaactgccc	cttggcagta	catcaagtgt	480
atcatatgcc	aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gcctggcatt	540
atgcccagta	catgacctta	tgggacttcc	ctacttggca	gtacatctac	gtattagtca	600
tcgcttattac	catggtgatg	cgggtttggc	agtacatcaa	tgggcgtgga	tagcggttg	660
actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc	720
aaaatcaacg	ggactttcca	aaatgtcgta	acaactccgc	cccattgacg	caaatggcg	780
gtaggcgtgt	acggtgggag	gtctatataa	gcagagctct	ctggctaact	agagaaccca	840
ctgcttactg	gcttatcgaa	attaatacga	ctcaactata	ggagacccaa	gctggctagc	900

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gtttaaactt aagcttgta ccgagctcg	atccactagt ccagtgttgt ggaattctgc	960
agatatatccag cacagtggcg	gccgctcgag tctagaggcg ccgttaaac ccgctgatca	1020
gcctcgactg tgccttctag ttgcgcagca	tctgttgtt gcccctcccc cgtgcctcc	1080
ttgaccctgg aagggtccac	tcccactgtc ctttcctaataa aaaaatgagga aattgcac	1140
cattgtctga gtaggtgtca	ttcttattctg ggggggtgggg tggggcagga cagcaagggg	1200
gaggattggg aagacaata	gagcatgtc gggatgegg tgggccttat ggcttctgag	1260
gcggaaagaa ccagctgggg	ctctaggggg tatccccacg cgcctgttag cgccgcatta	1320
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aaaaaaacttgc	attagggtga tggttca	1560
cgcccttgc	cgttggagtc cacgttctt aatagtggac tcttgcacca aactggaa	1620
acactcaacc	ctatctcggt ctattcttt gatttataag ggattttgcc gatttcgccc	1680
tattggtaa	aaaatgagct gatthaacaa aaatttaacg cgaattaatt ctgtggaa	1740
tgtgtcagtt	agggtgtggaa aagtccccag gctccccacg aggcagaagt atgcaaa	1800
tgcatactcaaa	ttagtcagca accagggtgtg gaaagtcccc aggctcccc gcaggcagaa	1860
gtatgc	aaag catgcatactc aattagtcag caaccatagt cccgcctta actccgc	1920
tccgc	ccctt aactccgc agtcccggcc attctccgc ccatggctga ctaattttt	1980
tatattatgc	agaggccgag gccgcctcg cctctgagct attccagaag tagtgaggag	2040
gctttttgg	aggcctaggc ttttgcaaaa agctcccgag agcttgcata tccatttcg	2100
gatctgatca	agagacagga tgaggatcg ttcgcata gtaacaagat ggattgcac	2160
cagggtctcc	ggccgcgtt ggagagggc tattcgctca tgactggca caacagacaa	2220
tcggctgc	tgcgcgcgtt gttccggc tgtcagcgca gggcgcccg gttttttg	2280
tcaagaccga	cctgtccggt gccctgaatg aactgcagga cgaggcagcg cggctatcg	2340
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ctgcgc	gagatccatc atggctgatc caatgcggc gtcgcatacg cttgatccgg	2520
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aactgttgc	caggctcaag gcgcgcgtc ccgcgcgcg ggtctcgatc gtgacccatg	2700
gcgtgc	cttgcgcata atcatggcgg aaaatggccg cttttcgat ttcatcgact	2760
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ctgaagacgt	tgccggcgaa tgggtgcacc gcttcctcgat gtttacggat atcgccgc	2880
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gggttcgaa	atgaccgacc aagcgacgc caacctgcata tcacgagatt tcgattccac	3000
cgcgccttc	tatgaaaggt tgggttcgg aatcgatcc cgggcgcg gctggatgt	3060
cctccagcg	ggggatctca tgctggatctt cttgcgcac cccaaacttgt ttattgcagc	3120
ttataatggt	tacaaataaa gcaatagcat cacaatttc acaaataaag catttttc	3180
actgcattct	atgtgtggtt tgtccaaact catcaatgtc tcttatcatg tctgtatacc	3240
gtcgacccct	agctagagct tggcgtaatc atggcatacg ctgtttccgt tggaaattg	3300

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ttatccgctc acaattccac acaacatacg agccggaagc ataaagtgt aagcctgggg	3360
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ggaaacctg tcgtgccagc tgcatataatg aatcgccaa cgccgggg gaggcggtt	3480
gcgtattggg cgctttccg cttccgtct cactgactcg ctgcgctcg tcgttccgt	3540
gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag aatcaggga	3600
taacgcagga aagaacatgt gagcaaaagg ccagcaaaag gccaggaaacc gtaaaaaggc	3660
cgcgttgcgt gcgttttcc ataggctccg cccccctgac gaggcatcaca aaaatcgacg	3720
ctcaagtca ggtggcgaa acccgacagg actataaaaga taccaggcgt ttccccctgg	3780
aagctccctc gtgcgtctc ctgttccgac cctgcccgtt accggatacc tgtccgcctt	3840
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gtaggtcggt cgctccaagc tgggtgtgt gcacgaaccc cccgttcagc ccgaccgctg	3960
cgccttatcc ggttaactatc gtcttgagtc caaccggta agacacgact tatcgccact	4020
ggcagcagcc actggtaaca ggattagcag agegaggat gttaggcgtg ctacagagtt	4080
cttgaagtgg tggcctaact acggotacac tagaagaaca gtatggta tctgcgtct	4140
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agaagatcct ttgatctttt ctacggggtc tgacgctcag tggAACGAAA actcacgtt	4320
agggattttg gtcatgagat tatcaaaaag gatcttcacc tagatectt taaattaaaa	4380
atgaagttt aaatcaatct aaagtatata ttagttaact tggctgaca gttaccaatg	4440
cttaatcaatgtt gaggcaccta tctcagcgt ctgtcttattt cgatccatcca tagttgcctg	4500
actccccgtc gtgttagataa ctacgatacg ggagggctt ccattggcc ccagtgc	4560
aatgataccg cgagacccac gtcacccgc tccagattt tcagcaataa accagccagc	4620
cggaaaggcc gaggcagaa gtggccctgc aactttatcc gcctccatcc agtctattaa	4680
tttgtgcgg gaaagcttagag taagtagtgc gccagttat agtttgcgc acgttgtgc	4740
cattgctaca ggcatcggtt tgacgctcgtc gtgtttggt atggcttcat tcaatccgg	4800
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ttagtactca accaagtcat tctgagaata gtgtatgcgg cgaccgagtt gctttggcc	5040
ggcgtcaata cgggataata ccgcgcacaca tagcagaact ttAAAAGTGC tcatcatgg	5100
aaaacgtct tcggggcgaa aactctcaag gatcttacgg ctgttgcgtt ccagtgcgt	5160
gttaaccact cgtgcaccca actgtatctc agcatctttt actttacca ggcgttctgg	5220
gtgagcaaaa acaggaaggc aaaatgcgc aaaaaaggga ataaggccga cacggaaatg	5280
ttgaataactc atactttcc ttttcaata ttattgttgcg atttacagg gttattgtct	5340
catgagcgga tacatatttgc aatgtatgtt gaaaaataaa caaatagggg ttccgcgcac	5400
atttccccgtt aaagtgcac ctgcgtc	5428

<210> SEQ ID NO 72

<211> LENGTH: 519

<212> TYPE: PRT

<213> ORGANISM: Artificial sequence

<220> FEATURE:

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<223> OTHER INFORMATION: SeAP protein

<400> SEQUENCE: 72

Met	Leu	Leu	Leu	Leu	Leu	Gly	Leu	Arg	Leu	Gln	Leu	Ser	Leu		
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Gly	Ile	Ile	Pro	Val	Glu	Glu	Asn	Pro	Asp	Phe	Trp	Asn	Arg	Glu	
	20					25			30						
Ala	Ala	Glu	Ala	Leu	Gly	Ala	Ala	Lys	Lys	Leu	Gln	Pro	Ala	Gln	Thr
	35					40			45						
Ala	Ala	Lys	Asn	Leu	Ile	Ile	Phe	Leu	Gly	Asp	Gly	Met	Gly	Val	Ser
	50					55			60						
Thr	Val	Thr	Ala	Ala	Arg	Ile	Leu	Lys	Gly	Gln	Lys	Lys	Asp	Lys	Leu
	65					70		75					80		
Gly	Pro	Glu	Ile	Pro	Leu	Ala	Met	Asp	Arg	Phe	Pro	Tyr	Val	Ala	Leu
	85					90			95						
Ser	Lys	Thr	Tyr	Asn	Val	Asp	Lys	His	Val	Pro	Asp	Ser	Gly	Ala	Thr
	100					105			110						
Ala	Thr	Ala	Tyr	Leu	Cys	Gly	Val	Lys	Gly	Asn	Phe	Gln	Thr	Ile	Gly
	115					120			125						
Leu	Ser	Ala	Ala	Ala	Arg	Phe	Asn	Gln	Cys	Asn	Thr	Thr	Arg	Gly	Asn
	130					135			140						
Glu	Val	Ile	Ser	Val	Met	Asn	Arg	Ala	Lys	Lys	Ala	Gly	Lys	Ser	Val
	145					150			155				160		
Gly	Val	Val	Thr	Thr	Thr	Arg	Val	Gln	His	Ala	Ser	Pro	Ala	Gly	Thr
	165					170			175						
Tyr	Ala	His	Thr	Val	Asn	Arg	Asn	Trp	Tyr	Ser	Asp	Ala	Asp	Val	Pro
	180					185			190						
Ala	Ser	Ala	Arg	Gln	Glu	Gly	Cys	Gln	Asp	Ile	Ala	Thr	Gln	Leu	Ile
	195					200			205						
Ser	Asn	Met	Asp	Ile	Asp	Val	Ile	Leu	Gly	Gly	Arg	Lys	Tyr	Met	
	210					215			220						
Phe	Arg	Met	Gly	Thr	Pro	Asp	Pro	Glu	Tyr	Pro	Asp	Asp	Tyr	Ser	Gln
	225					230			235				240		
Gly	Gly	Thr	Arg	Leu	Asp	Gly	Lys	Asn	Leu	Val	Gln	Glu	Trp	Leu	Ala
	245					250			255						
Lys	Arg	Gln	Gly	Ala	Arg	Tyr	Val	Trp	Asn	Arg	Thr	Glu	Leu	Met	Gln
	260					265			270						
Ala	Ser	Leu	Asp	Pro	Ser	Val	Thr	His	Leu	Met	Gly	Leu	Phe	Glu	Pro
	275					280			285						
Gly	Asp	Met	Lys	Tyr	Glu	Ile	His	Arg	Asp	Ser	Thr	Leu	Asp	Pro	Ser
	290					295			300						
Leu	Met	Glu	Met	Thr	Glu	Ala	Ala	Leu	Arg	Leu	Leu	Ser	Arg	Asn	Pro
	305					310			315				320		
Arg	Gly	Phe	Phe	Leu	Phe	Val	Glu	Gly	Arg	Ile	Asp	His	Gly	His	
	325					330			335						
His	Glu	Ser	Arg	Ala	Tyr	Arg	Ala	Leu	Thr	Glu	Thr	Ile	Met	Phe	Asp
	340					345			350						
Asp	Ala	Ile	Glu	Arg	Ala	Gly	Gln	Leu	Thr	Ser	Glu	Glu	Asp	Thr	Leu
	355					360			365						
Ser	Leu	Val	Thr	Ala	Asp	His	Ser	His	Val	Phe	Ser	Phe	Gly	Gly	Tyr
	370					375			380						
Pro	Leu	Arg	Gly	Ser	Ser	Ile	Phe	Gly	Leu	Ala	Pro	Gly	Lys	Ala	Arg
	385					390			395				400		

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Asp Arg Lys Ala Tyr Thr Val Leu Leu Tyr Gly Asn Gly Pro Gly Tyr
405 410 415

Val Leu Lys Asp Gly Ala Arg Pro Asp Val Thr Glu Ser Glu Ser Gly
420 425 430

Ser Pro Glu Tyr Arg Gln Gln Ser Ala Val Pro Leu Asp Glu Glu Thr
435 440 445

His Ala Gly Glu Asp Val Ala Val Phe Ala Arg Gly Pro Gln Ala His
450 455 460

Leu Val His Gly Val Gln Glu Gln Thr Phe Ile Ala His Val Met Ala
465 470 475 480

Phe Ala Ala Cys Leu Glu Pro Tyr Thr Ala Cys Asp Leu Ala Pro Pro
485 490 495

Ala Gly Thr Thr Asp Ala Ala His Pro Gly Tyr Ser Arg Val Gly Ala
500 505 510

Ala Gly Arg Phe Glu Gln Thr
515

<210> SEQ ID NO 73
<211> LENGTH: 2522
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1+CMV+TEE

<400> SEQUENCE: 73

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tttccttag tgacagtcag atagaacctt ctgcgtgca aggacaccaa gtgcaaacag	180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtcgaat gccaaactgtt	240
ttcaagaact gtgtggattt ttctgcctgt aacgaattca gattcatttt tc当地actcg	300
gggagagttt tccccctta taatttttt tt当地aaattta tt当地actttt tttcgcccc	360
cttggtttga gaattgcaga gtcatccacc ctgtcacagt gccaggagc tcaggatgg	420
gcccaggggc ctggcggggc tgaaggggct gggaaagcga gggctccaaa gggacccag	480
tgtggcagga gccaaagccc taggtcccta gaacgcagag gccaccggg ccccccagac	540
ggggtaagcg ggtgggtgtc tggggcgcga agccgcactg cgcatgcgcc gaggtccgct	600
ccggccgcgc tgatccaagc cgggttctcg cgccgacactg gtcgtgattt acaagtccaca	660
cacgctgtatc cttccgcggg gcccacagg gtcacagctt ttccctccc cacaaagccc	720
cctactctct gggcaccaca cacgaacatt ctttgagcgt gacattgtt gctctagtc	780
ggggcctccg gtgcagagac tggAACGGCC ttgggaagta gtc当地taacc gc当地tccgc	840
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ccggccgtgg tgaggagaag ccgcgcgcgt tggcgtagct gagagacggg gagggggcgc	1020
ggacacgagg ggcagccgc ggcctggacg ttctgttcc tt当地ccgcg aggaaggcga	1080
ctgtccgtgg gcccggggacc cagcggcaag atggcggccca agtggaaagcc tgaggggata	1140
ggcgagcggc cctgaggcgc tc当地gggggt tggggggaa gcaggcccgc gagggcagctg	1200
cagccggaa cgtgcggcca acccatttatt tt当地tgcgtt ggttgcgggc cgttaggtgcc	1260
tccgaagtga gagccgtggg cgtttgcactg tc当地ggagagg tc当地gtggat tt当地atccgt	1320
tgctaaagac ggaagtgcga ctgagacggg aagggggggg agtccgggttgg tggcgggtga	1380

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acctggacta	aggcgcacat	gacgtcgccg	tttctatggg	ctcataatgg	gtggtgagga	1440
cattttccctg	ttaaaactta	aacaagtttg	tacaaaaaaag	caggctagat	cttcaatatt	1500
ggccatttagc	catattatttc	attggttata	tagcataaaat	caatattggc	tattggccat	1560
tgcatacgtt	gtatctatat	cataatatgt	acatttatat	tggctcatgt	ccaatatgac	1620
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caataggggc	tttccattga	cgtcaatggg	tggagtattt	acggtaaaact	gcccaacttgg	1860
cagttacatca	agtgtatcat	atgccaagtc	cgccttat	tgacgtcaat	gacggtaaat	1920
ggcccgccctg	gcattatgcc	cagttacatga	ccttacggga	ctttccctact	tggcagtaca	1980
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gtggatagcg	gtttgactca	cggggatttc	caagtctcca	ccccatttgc	gtcaatggga	2100
gtttgttttgc	acacaaaat	caacgggact	ttccaaaatg	tgcataataac	cccgccccgt	2160
tgcacgc	gggcggtagg	cgtgtacgg	ggggaggctta	tataaggcaga	gctcgtttag	2220
tgaaccgtca	gatcaactaga	agcttcaago	tctacggaga	agaagaaaga	agaagaaagaa	2280
gaagaagaag	aagaagcg	tccttcttctt	cttgcggat	taaaaaagaa	aactccaaa	2340
aaaaagaaaa	tcatcaaaa	aacaaatttgc	aaaaagagtt	tttgtgttttgc	gggattaaag	2400
aagaaaaaaa	acaacagg	agtaagcg	gttgcgtct	cttgcgggtc	cgttgcttgt	2460
tctcacac	tttaggtctg	ttctcg	ccgttctgac	tctcttttgc	tcgttgcagg	2520
cc						2522

<210> SEQ ID NO 74
<211> LENGTH: 3321
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-Xt+CMV+TEE

<400> SEQUENCE: 74

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tgcctggagc	ttggatatgc	acctgcttct	gcctcaggaa	tggtgggatt	gaagggtgtc	420
accaccacat	cgccta	acatgc	taatgggtt	atatcttatt	taatgaatga	480
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ggacgttaggc	ttgggtgtgg	ggcacacatt	ttgattccag	cacttggaa	gaaggcct	780
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ccagggagct cagggatggg cccagggcc tggcggggct gaaggggctg gggaaagc当地	1260
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gtggaaagccct gaggggatag gcgagcggcc ctgaggc当地 cgacgggg当地 ggggggg当地 ag	1980
caggccgc当地 aggcaagctgc agccgggaaac gtgc当地 ggccaa cccctt当地 tttt当地 gaggg当地	2040
gttgc当地 gggcc local gtaggtgc当地 cc当地 gaaggatgag agccgtggcc gttt当地 gactgt cgccgagaggt当地	2100
cggtc当地 ggatt ttc当地 atccgc当地 gctaaagacg gaagtc当地 gac tgagacgg当地 aggggggg当地 ag	2160
gtc当地 gggtt当地 ggccggtgaa cctggactaa ggcc当地 cacatg acgtc当地 gggtt当地 ttctatggc当地	2220
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cccattgc当地 tcaatgggag tttt当地 ttggatgc当地 cccatgc当地 ttatgc当地 tttatgc当地 gggtt当地	2940
cgttaataacc cccccc当地 ctgg gacgc当地 aaatgc当地 ggccggttaggc gtgtacgggt ggaggcttat	3000
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gaagaaagaa aagaagaagaa aagaagaaga agaagcgtctt cctt当地 ttgc当地 gaggt当地	3120
aaaaaaagaaa actcccaaaa aaaagaaaat catcaaaaaa acaaatttca aaaagagttt当地	3180
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<210> SEQ ID NO 75
<211> LENGTH: 2232
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-80+CMV+TEE

<400> SEQUENCE: 75

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tcaggatgg	gcccaggggc	ctgggggggc	tgaaggggct	ggggaaagcga	gggctccaaa	180
gggaccccag	tgtggcagga	gccaaagccc	taggtcccta	gaacgcagag	gccaccggga	240
ccccccagac	gggtaagcg	ggtgtgggtc	tggggcgcga	agccgcactg	cgcacatgc	300
gaggtccgct	cggccgcgc	tgatccaago	cgggttctcg	cgcgcacactg	gtcggtattg	360
acaagtca	cacgctgate	cctccgcggg	ccgcgcacagg	gtcacagcct	ttccctcccc	420
cacaaagccc	cctactctct	gggcaccaca	cacgaacatt	cottgagcgt	gaccttggtg	480
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tgaggggata	ggcgagcggc	cctgaggcgc	tcgacggggt	tgggggggaa	gcaggccgc	900
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cgttaggtgc	tccgaagtga	gagccgtggg	cgtttgactg	tggggagagg	tggcgccgt	1020
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cttcaatatt	ggccatttagc	catattattc	attggttata	tagcataaat	caatattggc	1260
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gacggtaaat	ggccgcctg	gcattatgc	cagtacatga	cottacggga	cttccctact	1680
tggcagtaca	tctacgtatt	agtcatcgct	attaccatag	tgtgcgggt	ttggcagtac	1740
accaatgggc	gtggatagcg	gtttgactca	cggggatttc	caagtctcca	ccccattgac	1800
gtcaatggga	gtttgttttgc	acacaaaaat	caacgggact	ttccaaatgt	tcgtaataaac	1860
ccgcgcgcgt	tgacgcaaat	gggcggtagg	cgtgtacggt	gggaggtcta	tataaggcaga	1920

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aactcccaa aaaaagaaaa tcatcaaaaa aacaatttc aaaaagagg ttgtgttt	2100
gggattaaag aagaaaaaaaa acaacaggtg agtaagcgca gttgtcgct cttgegggtc	2160
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<210> SEQ ID NO 76

<211> LENGTH: 1942

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: EEE1-60+CMV+TEE

<400> SEQUENCE: 76

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tccccctccc cacaagcccc cctactctctt gggcaccaca cacgaacatt ccttgagcgt	180
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ctcataatgg gtggtgagga cattccctg tttaaactta aacaagttt tacaaaaaaag	900
caggctagat cttcaatatt ggccatttgc catatttttc attggttata tagcataaat	960
caatattggc tattggccat tgcatacgat gtatctatat cataatatgt acatttat	1020
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ccccatttgc gtcattggaa gtttgcgttgc gacccaaat caacggact ttccaaatg	1560
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tataaggcaga gtcgttttag tgaaccgtca gatcaactaga agcttcaagc tctacgcagg	1680
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140

taaaaaaagaa aactccaaa aaaaagaaaa tcatcaaaaa aacaaattc aaaaagagtt 1800
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<210> SEQ ID NO 77
 <211> LENGTH: 9513
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: pPNic384

<400> SEQUENCE: 77

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cgggttaggaa tggagegggc aaatgcttac ctctggacc ttcaagaggt atgttagggtt	6720
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cagtctatta attgttgcgg ggaagctaga gtaagtagttt cgccagttaa tagttgcgc	8640
aacgttggtg ccattgtcg aggcatcggt gttgcacgct cgtcggttgg tatggctca	8700
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gcgggttagct ctttcgggtcc tccgcgtt gtcagaagta agttggcgc acgtgttatca	8820
ctcatgggtt tggcagcact gcataattct cttactgtca tgccatccgt aagatgtttt	8880
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<210> SEQ ID NO 78
<211> LENGTH: 1720
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: pPNic602 insert
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<400> SEQUENCE: 78

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ctcatgttgg tatttgtaaa tagacgcaga tcggaaacac tgaaaaataa cagttattat 180
tgcgttcagg caaccagagc tacatagtga gatctgtct caaaaaaaaaaaaataatct 240
aaggcttcaa agggttcaat ctcttagtga gctaaatatg aacaaaattt gggaatgtg 300
accttttctt tagtgacagt cagatagaac cttoctcgagt gcaaggacac caagtgc当地 360
caggctcaag aacagectgg aaaggcttag tgctatgggg cttaggtcg aatgccaact 420
gttttcaaga actgtgttggg ttttctgcc tgtaacgaat tcagattcat ttttcaaaaac 480
tcggggagag ttttccccct ttataattt ttttttaaat ttattaaact ttgttgc当地 540
ccccctgttt tgagaattgc agagtcatcc accctgtcac agtgc当地 caggaggg 600
tggggccagg ggcctggccg ggctgaaggg gctggggaaag cgagggtcc aaagggaccc 660
cagtggtggca ggagccaaag cccttagtcc cttagaacgca gagggccaccg ggacccccc当地 720
gacgggtaa ggggggtgggt gtctgggccc cgaagccgc当地 ctgc当地 catgc gccc当地 gaggtcc 780
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gtcttagcta gttccgtccg agaccccgagt tcagtc当地 cc当地 tctgtg aggactgtg 1140
ccggccgc当地 tggtgaggag aagccgc当地 gcttggc当地 gctgagagac ggggaggggg 1200
cgccggacacg aggggc当地 gagcc cggcc当地 acgttctgtt tccgtggccc gcgaggaaagg 1260
cgactgtccctt gaggcggagg accccagc当地 gggcc当地 aagatggccg ccaagtgaa gcttggggg 1320
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gcctccgaag tgagagccgt gggcgttga ctgtcggag aggtcggtcg gatttcatc 1500
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 tgaacctgga ctaaggcgca catgacgacg cggttctat gggctataa tgggtggtga 1620
 ggacattcc ctagatctaa catccaaaga cgaagggtt aatgaaacct ttttgccatc 1680
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<210> SEQ ID NO 79
 <211> LENGTH: 455
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: EF1a promoter

<400> SEQUENCE: 79
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 gcctccccgt caccacccccc cccaaacccgc cccgaccggg gctgagagta attcatacaa 120
 aaggactcgc ccctgcctt gggaatccca gggaccgtcg ttaaactccc actaacgtag 180
 aacccagaga tcgctgcgtt cccgccccct caccggccg ctctcgcat cactgaggtg 240
 gagaagagca tgcgtgaggc tccggtgccc gtcagtgggc agagcgcaca tcgcccacag 300
 tccccgagaa gttgggggaa ggggtcgca attgaaccgg tgcctagaga aggtggcgcg 360
 gggtaaaactg ggaaagtgtat gtcgtgtact ggctccgcct ttttcccgag ggtggggag 420
 aaccgtatat aagtgcagta gtcggcgtga acgtt 455

<210> SEQ ID NO 80
 <211> LENGTH: 2250
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: EEE1-A1

<400> SEQUENCE: 80
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 tcccaccata ccttaaatat aatttaaaag agagagtgtg agaaaactcaa agtgaacaag 120
 actaaaaagag actaagacac aaggcagaat aatacactcc ctcatggtgc tgactttgcc 180
 caatttcatc aggcaagtctt gggtaagcc taatcatcac tggatatacag catgatttg 240
 gtccacatcc aggtcacccca agacacagta cagggctggg ttttatatacatacata 300
 gagggaaatat gtctttggc ctgggtctgc agtatggagt gtgttttct tgcagggt 360
 aggccagcacc atgcctttcc tcctccttgc gcctcagcat tggagggtta gtggagagg 420
 agctctagaa ccggtagaa ggagattaca taatcctttt ttatcctatt atatgaatga 480
 aagatttggc ctatgttctt ggctttggg ataatgactt acaataactac ttatggtgt 540
 ggcccttacc agcgcgggtgg gtggtgccgt gcttttagtc ctgatattgg ggaggcagag 600
 gaaggaggat cactctcact acgtggcac ctggtgtttt acgagactac cacctctggc 660
 tgtgttacag tgaatagcac agaccctacc aatgcatata acaacaacta gatctattgt 720
 gccacgaagg gtagctctcc agcctctt atagtttagga cctcatggaa ggaagaggc 780
 aggaagctgt tctttggtag atacacctaa ctagagttca ttagtgagtg catgcaccta 840
 atataaaat ttgatctaag gaggcatagcc tactaatctc ttaggtgggtt atttcactt 900
 ctatatttgg gaaagagtc tataccctag ctaaagttac aaagaacctt ctgcactcct 960
 aggtctggaa gtgaaaagg gtgaaaagac agctgctatg ctgttgaggt ttggccctt 1020

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ccctcgtaa ggctagtcta tactacatca gagagcaata tacaggcaga atcgtaatga	1080
catacttata cctatacacg ggcacactat accccataaa attatactt agagcaataa	1140
attatcatag atacgatggg catctatagg taattctatg accatccacc ctgcacatgt	1200
ggctgcctgg tgaggcaagc ccccaggggg cagccgggcc agtagccccct ggggtaccgt	1260
ggggtgctat gcctccccac tctgggaggt ggctatgggg ttgctgttt gtacggacag	1320
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cggaagcgcc gtggtgccgt ggcggcgcc tgatcctacc cgctatgtcg cgccgtctg	1440
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agtccctaac cgcatattccg cggagggatc gtccggaggg cgtggcttct gaggattata	1680
taaggcgact cccgggggggt cttagctagt tccgtcgagg acccgagttc agtcggcgct	1740
tctctgtgag gactgtgtcc gcccggctg gtgaggagaa ggcggccggc ttggcgtagc	1800
tgagagacgg ggagggggcg cggacacgag gggcagcccg cggcctggac gttctgttc	1860
cgtggcccgcc gaggaaggcg actgtctga ggccggaggac ccagcgccaa gatggcgcc	1920
aagtggaaagc ctgaggggat aggegagcg ccctgaggcg ctgcacgggg ttggggggga	1980
agcagggcccg cgaggcagct gcagccggga acgtgcggcc aaccccttat ttttttgac	2040
gggttggggg ccgttaggtgc ctccgaagtg agagccgtg gcgtttact gtcggagag	2100
gtcggtegga ttttcatccg ttgctaaaga cggaaagtgcg actgagacgg gaaggggggg	2160
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<210> SEQ ID NO 81
 <211> LENGTH: 1449
 <212> TYPE: DNA
 <213> ORGANISM: Artificial sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: EEE1-A2

<400> SEQUENCE: 81

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tttcccttag tgacagtcag atagaacctt ctgcagtgca aggacaccaa gtgcaaacag	180
gctcaagaac acgcctggaaa ggtctagtgc tatgggctt caggtcgaat gccaactgtt	240
ttcaagaact gtgtggattt ttctgcctgt aacgaattca gattcattt tcaaaactcg	300
gggagagttt tccccctta taatttttt tttaattta ttaaactttg ttctgttccc	360
cttggtttga gaattgcaga gtcatccacc ctgtcacagt gccaggggagc tcagggatgg	420
gcccaggggc ctggcgccgc tgaaggggct ggggaagcga gggctccaaa gggacccag	480
tgtggcgaga gccaaagccc taggtcccta gaacgcagag gccacccggga ccccccagac	540
ggggtaagcg ggtgggtgtc tggggcgccga agccgcactg cgcatgcggc gaggtccgct	600
ccggccgcgc tgatccaagc cgggttctcg cgccgacctg gtcgtgatg acaagtacaca	660
cacgctgatc cctccgcggg gccgcacagg gtcacagcct ttccccctccc cacaagccc	720
cctactgtct ggccatctct ctcgtagaat gcatcacccg gtcgttagatg ccacaactga	780

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cccgccctccg	ctcctgtgtc	agcataggcc	ttgggaagta	gtcctttagc	ggaataccgc	840
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taacctagtt	ccgtcgacca	gccgagagta	cccgccgtt	ctcagagtgc	agtccagcg	960
ccgcgcgtgg	tgaggagaag	ccgcgcgtt	tggcgttagct	gagagacggg	gagggggcgc	1020
ggacacgagg	ggcageccgc	ggcctggacg	tctgtttcc	gtggccccgc	aggaaggcga	1080
ctgtccttag	gcccgggacc	cageggcaag	atggcgccca	agtggaaagcc	tgaggggata	1140
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tgctaaagac	ggaagtgcga	ctgagacggg	aagggggggg	agtcgggttg	tggcggtga	1380
acctggacta	aggcgacat	gacgtcgccg	tttctatggg	ctcataatgg	gtggtgagga	1440
cattccct						1449

<210> SEQ ID NO 82

<211> LENGTH: 1449

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: EEE1-A3

<400> SEQUENCE: 82

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tttccttag	tgacagtcag	atagaacctt	ctcgagtgc	aggacaccaa	gtgcaaacag	180
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ttcaagaact	gtgtggattt	ttctgcctgt	aacgaattca	gattcatttt	tcaaaactcg	300
gggagagttt	tcccccttta	taatttttt	ttaaaattta	ttaaactttt	tttcgttccc	360
cttgggttga	gaattgcaga	gtcatccacc	ctgtcacagt	gccagggagc	tcagggatgg	420
gcccaggggc	ctggcgccgc	tgaaggggct	ggggaaagcga	gggctccaaa	gggacccag	480
tgtggcagga	gccaagccc	tagtcccta	gaacgcagag	gccacccgg	ccccccagac	540
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ccggccgcgc	tgatccaagc	cgggttctcg	cgccgacactg	gtcgtgattt	acaagtccaca	660
cacgctgtac	cctccgcggg	gccgcacagg	gtcacagctt	ttccccctcc	cacaaagccc	720
cctactctct	gggcaccaca	cacgaacatt	ccttgagctg	gaccttgcgt	gtcttagtca	780
ggcgccctcg	gtgcagagac	tggAACggcc	ttgggaagta	gtccctaacc	gcattccgc	840
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ccggccgtgg	tgaggtaat	ccggccgtt	agccgattt	ctctctcg	gaggccgcgc	1020
gtgtcgatcc	cctggggccgc	ggggacctcg	aagacaaagc	gacctgcgc	aggaaggcga	1080
ctgtccttag	gcccgggtgc	ctccggcaag	atggcgctgt	tcaccttgg	actccccatt	1140
cccgccgggg	ggactcccg	acgtcgccca	accccccctt	cgccggccgc	gtccctccac	1200
tgtcgccct	cgaccgcct	tggggatattt	tttttctcg	ggttgccccgc	cgatccaccc	1260
agcggttact	ctcccggtgg	cgtttgtac	acgcctctcc	acggctcgct	aaagtagcgt	1320
tgctaaagac	gtttcacccgt	gactgacggg	aagggggggg	agtcgcaacc	acccgggtct	1380

tggacctgaa aggcggtgta ctcgtcgca aaagataacc gactggggtt ttggtgagga 1440
catttcctt 1449

<210> SEQ ID NO 83
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-B1

<400> SEQUENCE: 83
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tttccttag tgacagtcag ataactgcat tccgagtgcg aggacaccaa gtgcaaacag 180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtcgaat gccaaactgtt 240
ttcaagaact gtgtggattt ttctgcctgt aacgaattca gattcattt tcaaaactcg 300
gggagagttt tccccctta taattttttt tttaaattta tttaactttt tttcgttccc 360
cttggggta gaattgcaga gtcatccacc ctgtcacagt gccaggagc tcagggatgg 420
gcgcaggggc ctgggggggc tgaaggggct gggaaagcga gggctccaaa gggacccag 480
tgtggcagga gccaaagccc taggtcccta gaacgcagag gccacccggc ccccccagac 540
ggggtaagcg ggtgggtgtc tggggcgcga agccgcactg cgcatgcgcc gaggtccgct 600
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cacgcttgcgca cttccgcggg gccgcacagg gtcacagect ttccccctccc cacaaggccc 720
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ggcgcctccg gtgcagagac tggAACGGCC ttgggcttagt atacctaacc gcatttcgc 840
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ttagctagtt ccgtcgagaa cccgagttca gtgcgcgtt ctctgtgagg actgtgcgc 960
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tccgaagtga gagccgtggg cgtttgcactg tcgggagagg tcggcggat tttcatccgt 1320
tgctaaagac ggaagtgcga ctgagacggg aagggggggg agtcgggttg tggcggtga 1380
acctggacta aggccacat gacgtcgccg ttctatggg ctcataatgg gagggtgtga 1440
catttcctt 1449

<210> SEQ ID NO 84
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
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gcttcaaagg gttcaatctc ttaggttagct aaatatgaac aaaatttggg aaatgtgacc 120

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157

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tttccttag tgacagtcag atagaacatt ctcgagtgcaggacaccaa gtgcaaacag	180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtccat gccaactgtt	240
ttcaagaact gtgtggattt ttctgcctgt aaccattca gattcat taaaacttg	300
gggagagttt tccccctta taattttttt tttaaattta ttaaactttt tttccttccc	360
cttggggta gaattgcaga gtcatccacc ctgtcacagt gccaggagc tcagggatgg	420
gcccgaggc ctggggggc tgaaggggctt gggaaagcga gggctccaaa gggacccag	480
tgtggcagga gccaaagccc taggtcccta gaaccagag gccacccggga ccccccagac	540
ggggtaaggg ggtgggtgtc tggggccca agccccactt cccatggcc caggtccctt	600
ccggccgcgc tgatccaagc cgggttctgg ccccccacctg gtgggtatg acaagtca	660
cacgctgatc cttccgcgg gccgcacagg gtcacagcc tttccctccc cacaagccc	720
cctactctct gggcaccaca caggaacatt ctttgagcc tttttttttt gctctatgtca	780
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cagctggaa cctgcggccca accccattt ttttttgc acgtttttttt cgttagtgcc	1260
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tgctaaagac ggaagtgcga ctgagacggg aagggggggg agtcgggtgg tgggggttga	1380
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<210> SEQ ID NO 85

<211> LENGTH: 1449

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: EEE1-B3

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tttccttag tgacagtcag atagaacatt ctcgagtgcaggacaccaa gtgcaaacag	180
gctcaagaac agcctggaaa ggtctagtgc tatggggctt caggtcgaat gccaactgtt	240
ttcaagaact gtgtggattt ttctgcctgt aaccattca gattcat taaaacttg	300
gggagagttt tccccctta taattttttt tttaaattta ttaaactttt tttcgttccc	360
cttggggta gaattgcaga gtcatccacc ctgtcacagt gccaggagc tcagggatgg	420
gcccgaggc ctggggggc tgaaggggctt gggaaagcga gggctccaaa gggacccag	480
tgtggcagga gccaaagccc taggtcccta gaaccagag gccacccggga ccccccagac	540
ggggtaaggc ggtgggtgtc tggggccca agccccactt cccatgcggcc gaggtccctt	600
cctgcgcgc tgatccaagc cgggttctgg cggccacccgt gtcgtatgt acaagtca	660
cacgctgatc cttccgcgg gccgcacagg gtcacagcc tttccctccc cacaagccc	720

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159

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cctactctct gggcaccaca cacgaacatt ccttgagcgt gaccttgtg gctctagtc      780
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ggagggatcg tcggggaggcgt gggtttctg aggattataat aaggcgactc cgggggggtc      900
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ccggcgctgg tgaggagaag ccggcgccgt tggcgtagct gagagacggg gagggggcgc      1020
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cagccggaa cgtcgccca accccttatt tttttgacg ggttggggc cgttagtgcc      1260
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tgctaaagac ggaagtgcga ctgagacggg aaggggggggg agtcgggttg tggcggtga      1380
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<210> SEQ ID NO 86
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-B4

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gctcaagaac acgctggaaa ggtctagtgc tatgggggtt caggtccaat gccaactgtt      240
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ggccaggggc ctgggggggc tgaagggggtt ggggaaggggg gggctccaaa gggaccccg      480
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<210> SEQ ID NO 87
<211> LENGTH: 1449
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: EEE1-B5

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gctcaagaac agcctggaaa ggtctagtgc tatgggctt caggtagaat gccaaactgtt	240
ttcaagaact gtgtggattt ttctgcctgt aacgaattca gattcattt tcaaaactcg	300
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cttggtttga gaattgcaga gtcatccacc ctgtcacagt gccagggggc tcagggatgg	420
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cacgctgatc cttcccgccc gccgcacagg gtcacagccct tttccctccc cacaaagccc	720
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tagttacaga cggaaagtgcg actgaggcgg ggaggaggag agtcgggtgg tggcggtga	1380
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cattcacct	1449

<210> SEQ ID NO 88
<211> LENGTH: 2617
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Transcription regulating sequence

<400> SEQUENCE: 88

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gcccaggggc ctggggggc tgaaggggct ggggaagcga gggctccaaa gggacccag	480
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The invention claimed is:

1. A nucleic acid construct comprising a first promoter, a second promoter, and a single heterologous nucleotide sequence of interest, wherein said first promoter and second promoter are both operably linked to said single heterologous nucleotide sequence of interest, and wherein said second promoter is flanked by a first intronic sequence located upstream of said second promoter, and wherein said single heterologous nucleotide sequence of interest is under the control of said first promoter and said second promoter,

wherein said first promoter and said first intronic sequence comprise a sequence having at least 96% identity to SEQ ID NO: 1 over its whole length.

2. The nucleic acid construct according to claim 1, wherein said nucleic acid construct further comprises an additional expression regulating sequence, and wherein said additional expression regulating sequence, said first promoter and said second promoter are all operably linked to said nucleotide sequence of interest.

3. The nucleic acid construct according to claim 1, wherein said single heterologous nucleotide sequence of interest encodes a protein or polypeptide of interest.

4. An expression vector comprising the nucleic acid construct according to claim 1.

5. An in vitro cell comprising the nucleic acid construct according to claim 1.

6. A non-human cell comprising the nucleic acid construct according to claim 1.

7. The nucleic acid construct according to claim 2, wherein said additional expression regulating sequence comprises or consists of an intron.

15 8. The nucleic acid construct according to claim 1, wherein said first promoter and said first intronic sequence comprise a sequence having at least 97% identity to SEQ ID NO: 1 over its whole length.

9. The nucleic acid construct according to claim 1, wherein said first promoter and said first intronic sequence comprise a sequence having at least 98% identity to SEQ ID NO: 1 over its whole length.

10. The nucleic acid construct according to claim 1, wherein said first promoter and said first intronic sequence comprise a sequence having at least 99% identity to SEQ ID NO: 1 over its whole length.

11. The nucleic acid construct according to claim 1, wherein said first promoter and said first intronic sequence comprise a sequence having 100% identity to SEQ ID NO: 1 over its whole length.

12. The nucleic acid construct according to claim 1, wherein said second promoter comprises a sequence having at least 95% identity to SEQ ID NO: 57 over its whole length.

13. The nucleic acid construct according to claim 1, wherein said second promoter is a human or murine cytomegalovirus (CMV) promoter.

14. A method for transcription and optionally purifying the produced transcript comprising the steps of:

- a) providing the nucleic acid construct as defined in claim 1; and,
- b) contacting a cell with said nucleic acid construct to obtain a transformed cell; and,
- c) allowing said transformed cell to produce a transcript of the single heterologous nucleotide sequence of interest; and optionally,
- d) purifying said produced transcript.

* * * * *