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(54) Title: A METHOD TO INCREASE RNA STABILITY

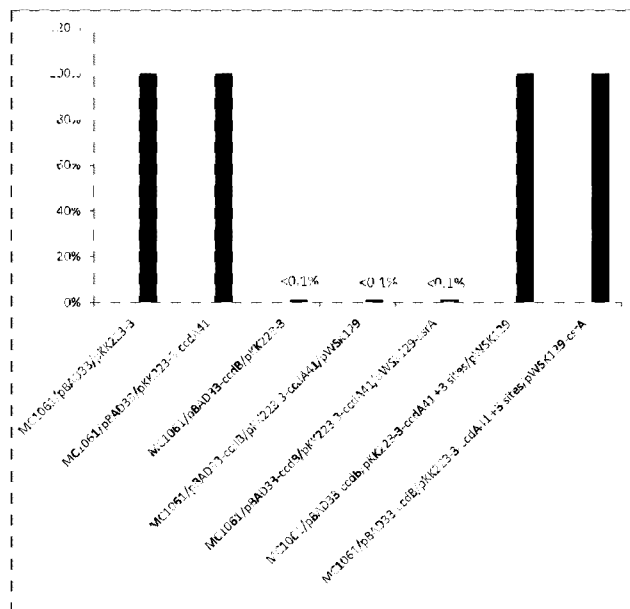


Fig. 1

(57) Abstract: The present invention is related to a method and tools to increase the stability of a RNA molecule by the addition of at least one box sequence at its 3'end, this box sequence being able to specifically bind a RNA-binding protein, this RNA-binding protein being able to stabilize this RNA molecule.

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A METHOD TO INCREASE RNA STABILITY**Field of the invention**

[0001] The present invention is related to a method
10 and tools to increase the stability of a RNA molecule,
preferably a RNA molecule being a mRNA sequence, to this
obtained recombinant RNA molecule, as well as to its
corresponding DNA sequence and to the cells expressing this
RNA molecule.

15

Background of the invention and state of the art

[0002] Recombinant micro-organisms, especially
E.coli are used for the production of a variety of
molecules, such as polypeptides, in a cheap and flexible
20 way.

[0003] However, a better yield of production of a
target protein and/or of a polypeptide requires increased
concentrations of the corresponding mRNA.

[0004] Similarly, the industrial production of
25 regulatory RNA relies on high cytosolic concentrations.

[0005] (m)RNA production can be controlled by an
adequate selection of promoters.

[0006] It is however more difficult to obtain an
inhibition of (m)RNA degradation.

30 [0007] Chen et al., 2007, (BBRC 357, 56-61)
discloses a mRNA stabilized in an ATP-dependent manner by
the pentamer ARE (nucleotide sequence) able to bind the
mammalian HuD protein upon ATP exposure.

[0008] Wei et al., 2001, (Mol. microbial., 40, 245-256) discloses the binding of CsrA protein at the 5'-end of a RNA allowing its stabilization.

[0009] WO 01/49838 discloses that a 26-nucleotide
5 sequence in the 3' terminal region of the parathyroid hormone mRNA increases this mRNA stability through the recruitment of 50 to 60 kDa mammalian proteins, including one AUF1 isoform.

10 Summary of the invention

[0010] The present invention provides a new method and new tools that do not present the drawbacks of the state of the art, especially a new method and new tools that allow reduced (m)RNA degradation into a cell,
15 especially new recombinant nucleotide sequences and recombinant cells that allow a reduced (m)RNA degradation.

[0011] More particularly, the present invention discloses (m)RNA molecules that are advantageously and efficiently stabilized and/or that present an increased
20 half-live.

[0012] The present invention relates to a recombinant (m)RNA molecule (or sequence) comprising a first RNA sequence encoding a peptide and/or (being) a nucleotide of interest (such as a regulatory RNA, which
25 sequence is preferably a viral or eukaryote sequence) and a second (non coding) RNA sequence located at the 3' part of this first RNA sequence, this second RNA sequence comprising a box sequence (nucleotide box or a box) able to specifically (non covalently) bind (fix) a (RNA-)binding
30 protein stabilizing this (m)RNA molecule (sequence).

[0013] Preferably, the second (non coding) RNA sequence (being part of the (m)RNA molecule) consists of at least two (or three) repeating units of the same (identical

or almost identical) box (able to bind this binding protein).

[0014] Possibly, in this recombinant (m)RNA, (comprising these first and second RNA sequences) the
5 box(es) able to bind a (RNA-)binding protein has (have) a size (a length) comprised between about 8 and about 25 nucleotides.

[0015] Possibly, in this recombinant (m)RNA molecule, the second RNA sequence comprises one (1, 2, 3,
10 4, 5, at least one, at least two, at least 3) box(es), each box (box sequence(s)) presenting at least (about) 50% (preferably, at least 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100%) of sequence
15 identity with the sequence SEQ.ID.NO.1 or with the sequence SEQ.ID.NO.2.

[0016] Preferably, this first RNA sequence is a sequence encoding a peptide of interest and/or the RNA molecule is a messenger RNA (mRNA) molecule (or sequence).

20 [0017] Alternatively, this first RNA sequence is a sequence encoding a (regulatory) non-coding RNA sequence (such as a siRNA) and, preferably, the sequence of this non-coding RNA is exogenous and more preferably is a viral sequence or an eukaryote sequence (a sequence deduced from
25 the genome of a eukaryote or from the genome of a virus).

[0018] The present invention also relates to a recombinant DNA molecule (or sequence) encoding this recombinant (m)RNA molecule (or sequence), this recombinant DNA molecule being preferably in the form of a vector,
30 preferably a self replicating vector, such as plasmid or a viral nucleic acid construct.

[0019] The present invention relates also to a DNA vector (preferably a self replicating vector with an origin of replication (ORI)) comprising a promoter (sequence), a

site of insertion of a first DNA sequence able to be transcribed into a first RNA sequence and encoding a peptide of interest; and a second DNA sequence located at the 3'-end of this site of insertion and able to be transcribed into a second RNA sequence (these first and second RNA sequences being on the same RNA molecule), this promoter being able to induce, from these first and second DNA sequences, the transcription of a (one) RNA molecule comprising this second transcribed RNA sequence being a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box(es) (box sequence(s)) able to specifically (non covalently) bind a binding protein able to stabilize this transcribed RNA molecule (or sequence).

[0020] This recombinant DNA molecule (in the form of a vector), preferably further comprises a third nucleotide sequence encoding an antidote protein belonging to the poison-antidote systems, being more preferably selected from the group consisting of CcdA, Kis, ParD, Phd, MazE and RelB (including their variants or equivalents having the same antidote activity) and being still more preferably CcdA sequence.

[0021] The present invention also relates to a recombinant prokaryote cell, (preferably *E. coli*) over-expressing a RNA-binding protein being able to (non covalently) bind to a (target) (m)RNA and to stabilize this (m)RNA, this cell further having incorporated in its genome a DNA sequence encoding a poison protein from a poison-antidote system under the control of an inducible promoter (and possibly not the corresponding antidote), wherein this poison protein from a poison-antidote system is preferably selected from the group consisting of CcdB, KiD, ParE, Doc, MazF and RelE (including their variants or equivalents having the same poisonous activity) and being more preferably CcdB.

[0022] Preferably, this recombinant (*E. coli*) cell further comprises the tldD sequence and/or tldE sequence that is (are) submitted to a partial or total deletion of the tldD sequence and/or tldE sequence or to one or more
5 mutation(s) inactivating the activity of the TldD protein and/or TldE protein.

[0023] Alternatively, the present invention relates to a recombinant prokaryote (*E. coli*) cell overexpressing a RNA-binding protein being able to bind to a RNA and to
10 stabilize the said RNA and this recombinant prokaryote (*E. coli*) cell further comprises the tldD sequence and/or tldE sequence that is (are) submitted to a partial or total deletion or wherein the tldD sequence and/or tldE sequence is (are) submitted to one more mutations inactivating the
15 corresponding protein.

[0024] Preferably, this (these) recombinant cell(s) according to the present invention further expresses the recombinant (m)RNA molecule according to the present invention (comprising the second RNA sequence being a box
20 (box sequence) able to specifically bind the (RNA-)binding protein (over-) expressed in this recombinant cell).

[0025] Alternatively (or in addition), this recombinant cell(s) according to the present invention further comprises the recombinant DNA molecule and/or DNA
25 vector according to the present invention encoding the first and second RNA sequences, this second RNA sequence comprising at least a box (box sequence) able to specifically bind the (RNA-)binding protein (over-)expressed in the recombinant cell and/or wherein the
30 antidote (possibly) expressed by the recombinant DNA sequence is the specific antidote of the poison protein (possibly) encoded by this recombinant cell.

[0026] Preferably, in this recombinant cell, the (over-expressed) RNA-binding protein has a molecular weight

comprised between about 5 kDa and about 50 kDa more preferably between about 7 kDa and about 30 kDa, still more preferably between about 8 kDa and about 13 kDa.

[0027] Possibly, in this recombinant cell, the
5 (over-expressed) RNA-binding protein is CsrA (SEQ.ID.NO.5), or comprises an active CsrA homolog or a (peptidic) sequence sharing a structural homology with the active CsrA.

[0028] Possibly, this recombinant prokaryote (*E.*
10 *coli*) cell (over-expressing a RNA binding protein and further having TldD and/or TldE mutation) further comprises the nucleotide sequence encoding Dam protein (SEQ.ID.NO.7) being submitted to a partial or total deletion and/or to one or more mutation(s) inactivating the activity of the
15 this Dam protein, being preferably Dam 1-59 polypeptide (SEQ.ID.NO.8).

[0029] Possibly, this recombinant prokaryote (*E.*
coli) cell (having Dam and TldD and/or TldE mutation and overexpressing a RNA-binding protein) does not overexpress
20 CsrA or, preferably, does not overexpress a partially active form of (mutated) CsrA, such as CsrA 1-50.

[0030] Another aspect of the present invention is a kit of part comprising:

- the recombinant cell of the present invention
25 (overexpressing the RNA-binding protein) and
- the recombinant DNA molecule (or sequence) or the DNA vector of the present invention.

[0031] A last aspect of the present invention is related to a method for stabilizing a (m)RNA molecule (or
30 sequence) in a cell, preferably *E. coli* cell, comprising a first RNA sequence encoding a peptide of interest and/or a first sequence being a nucleotide of interest, this method comprising the step of (recombinantly) adding (binding, fixing or inserting) at the (locus corresponding to the)

3'-end of the DNA encoding this first sequence, a second DNA sequence comprising a box (box sequence) able to specifically bind an RNA-binding protein on the transcribed (m)RNA molecule, this RNA-binding protein stabilizing this (m)RNA molecule (or sequence).

[0032] A related aspect of the present invention is a method for increasing the synthesis of a selected peptide in a cell, this method comprising the step of (recombinantly) adding (binding, fixing or inserting) at the 3'-end of a (locus corresponding to the) DNA sequence coding for this selected peptide, a second DNA sequence comprising a box (box sequence) able to specifically bind a (RNA-)binding protein on the transcribed mRNA molecule, this (RNA-)binding protein stabilizing this mRNA molecule (or sequence).

[0033] Preferably, in this (these) method(s), the second DNA sequence consists of at least two (substantially) identical boxes (box sequence) able to specifically bind a (RNA-)binding protein on the transcribed (m)RNA molecule.

[0034] Possibly, in this (these) method(s), the second RNA sequence comprise a box able to bind the RNA-binding protein presenting at least 85% (more preferably at least 90%, 91%, 92%, 93%, 94%, at least 95%, 96%, 97%, 98%, 99% or even 100%) sequence identity with the sequence SEQ.ID.NO.1 or with the sequence SEQ.ID.NO.2.

[0035] Preferably, this method further comprises the step of over-expressing the RNA-binding protein in the cell, being preferably a protein having a molecular weight comprised between about 5 kDa and about 50 kDa, more preferably between about 6 kDa and about 30 kDa, still more preferably between about 8 kDa and about 13 kDa.

[0036] More preferably, the cell used in this (these) method(s) is the recombinant cell of the present

invention and/or is an (*E. coli*) cell further comprising the tldD sequence and/or tldE sequence that is (are) submitted to a partial or total deletion of the tldD sequence and/or tldE sequence or to one or more mutation(s) inactivating the activity of the TldD protein and/or TldE protein.

Detailed description of the invention

[0037] The present invention is based on a recombinant DNA molecule (or sequence) (preferably in the form of a plasmid) encoding a recombinant RNA (preferably a messenger RNA (mRNA)) comprising a first (upstream) sequence able to be translated into a peptide (of interest)), or encoding a nucleotide of interest (such as a regulatory RNA sequence); and, located at the 3' part of this first sequence (and/or at the 3' part of the transcribed RNA sequence), a second (downstream) sequence encoding at least one (non coding RNA) nucleotide sequence being a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box(es) (box sequence(s)) able to specifically (non covalently) bind an RNA-binding protein on this (transcribed) (m)RNA, this binding protein being able to stabilize this (transcribed) (m)RNA.

[0038] Preferably, this recombinant DNA molecule (or sequence) (more preferably in the form of a plasmid) encodes a recombinant RNA in the form of a messenger RNA (mRNA) and/or comprises a first (upstream) nucleotide sequence able to be translated into a peptide (of interest) and, located at the 3' part of this first sequence (and/or at the 3' part of the transcribed RNA sequence), a second (downstream) sequence encoding at least one (RNA) nucleotide sequence being a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box(es) (box sequence(s)) able to specifically (non covalently) bind a RNA-binding protein on

this transcribed mRNA, this binding protein being able to stabilize this transcribed mRNA.

[0039] Alternatively, this recombinant DNA molecule (or sequence) (preferably in the form of a plasmid) encodes
5 the recombinant RNA molecule comprising a first (upstream) RNA sequence being a nucleotide of interest (such as a regulatory RNA, for instance a siRNA) and, located at the 3' part of this first RNA sequence (and/or at the 3' part of the transcribed RNA sequence), a second (downstream)
10 sequence comprising (consisting of, or consisting essentially of) at least one (RNA) nucleotide sequence being a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box(es) (box sequence(s)) able to specifically (non covalently) bind a RNA-binding protein on this transcribed (regulatory)
15 RNA, this RNA-binding protein being able to stabilize this transcribed (regulatory) RNA.

[0040] Alternatively, the recombinant DNA molecule (preferably in the form of a plasmid) comprises a promoter (for the production of a (m)RNA from a gene of interest), a
20 site of insertion of a first DNA (nucleotide) sequence (encoding the gene of interest) and a second DNA sequence located at the 3'-end (downstream) of this site of insertion, this promoter being able to induce, from this first and second DNA sequences, the transcription of one
25 RNA molecule comprising this second transcribed RNA sequence (present on this transcribed RNA molecule) comprising (consisting of, or consisting essentially of) a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box (sequence) able to specifically (non covalently) bind a RNA-binding
30 protein able to stabilize this transcribed RNA sequence.

[0041] Possibly, these first (upstream) and second (downstream) DNA sequences are separated by less than 100 nucleotides, preferably by less than 50 nucleotides, more preferably by less than 20 nucleotides (from the "stop

codon" of the gene of interest and/or from the site of insertion of this gene of interest or from the 5'-end of the nucleotide sequence of interest, to the first nucleotide of the second sequence).

5 [0042] Preferably, in this recombinant DNA molecule, the second (downstream) nucleotide sequence comprises (consists of, or consists essentially of) at least 2 boxes (at last 2 box sequences) able to specifically bind a (more preferably identical) RNA-binding protein on the
10 (transcribed) RNA and/or mRNA.

[0043] More preferably these at least 2 boxes are substantially identical. Still more preferably, these box sequences are (fully) identical.

[0044] Preferably, these at least 2 (substantially
15 or fully) identical boxes have a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16 nucleotides.

[0045] Possibly, this second (downstream) nucleotide
20 sequence consists (essentially) of these 2 or 3 (substantially or fully) identical boxes having a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16
25 nucleotides.

[0046] In the context of the present invention, "substantially identical (DNA) nucleotide sequences being a box" and/or "substantially identical boxes" preferably refers

30 to boxes sharing at least 85%, more preferably at least 90% of sequence identity

and/or

to boxes that, when transcribed into (m)RNA, have a comparable (similar) affinity for the (a) same RNA-binding

protein (i.e. the affinity, when expressed as a base 10 logarithm, of one box for the RNA-binding protein is comprised within the range of affinity of the other box(es) for the same RNA-binding protein plus or minus 1 log, and
5 more preferably the affinity of one box is comprised within the range of affinity of the other box(es) plus or minus 0.5 log).

[0047] Possibly, this box sequence (able to specifically bind a RNA-binding protein on this transcribed
10 (m)RNA molecule) comprises the GGA trinucleotide of SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4 sequence and further shares at least 50%, at least 60%, at least 70%, preferably at least 80%, 85%, 90%, 95% or even 100% of sequence identity with the remaining part of the
15 corresponding SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4 sequence.

[0048] Possibly (or in addition), this box (sequence) (able to specifically bind a RNA-binding protein on this transcribed (m)RNA) is at least 50% (preferably at
20 least 55%, 60%, 65%, 70%, 75%, 80%, 85% 90%, more preferably at least 95% or even 100%) identical to 5'-GUGUUGCACACGGAUU-3' or 5'-RUACARGGAUGU-3' (SEQ.ID.NO.1 sequence or SEQ.ID.NO.2 sequence) and/or this box (sequence) (able to specifically bind a RNA-binding protein
25 on this transcribed mRNA) is at least 50% (preferably at least 55%, 60%, 65%, 70%, 75%, 80%, 85% 90%, more preferably at least 95% or even 100%) identical to 5'-GTGTTGCACACGGATT-3' or 5'-RTACARGGATGT-3' (SEQ.ID.NO.3 sequence or SEQ.ID.NO.4 sequence).

30 [0049] Advantageously, this recombinant DNA molecule (or sequence) encoding this (these first and) second nucleotide(s) sequence(s), further comprises a (third) nucleotide sequence encoding an antidote protein belonging to the poison-antidote systems, being preferably selected

from the group consisting of CcdA, Kis, ParD, Phd, MazE and RelB, and being more preferably CcdA.

[0050] Preferably, this recombinant DNA molecule (or sequence) further comprising this (third) nucleotide
5 sequence encoding an antidote protein is in the form of a plasmid and/or of a vector.

[0051] Possibly, this third DNA sequence, when transcribed, is carried by another RNA molecule (than the RNA molecule of the present invention comprising the first
10 and second RNA sequences) and/or is under the control of another promoter (than the promoter for the transcription of RNA molecule of the present invention comprising the first and second RNA sequences).

[0052] A related aspect of the present invention is
15 a recombinant (m)RNA molecule (or sequence) comprising a first RNA sequence encoding a peptide of interest or a nucleotide of interest and a second RNA sequence located at the 3' part of this first RNA sequence (and/or at the 3' part of this (m)RNA), this second RNA sequence comprising a
20 (1, 2, 3, 4, 5 or more, preferably 2 or 3) box sequence(s) able to specifically bind a RNA-binding protein being able to stabilize this (m)RNA.

[0053] Preferably, this recombinant (m)RNA comprises (consists of, or consists essentially of) at least 2
25 (preferably identical) nucleotide sequences being a box able to specifically bind a RNA-binding protein on this (m)RNA.

[0054] More preferably these at least 2 boxes are substantially identical. Still more preferably, these at
30 least 2 boxes (box sequences) are (fully) identical.

[0055] Preferably, these at least 2 (substantially or fully) identical boxes (able to fix a binding protein) have a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20

nucleotides, more preferably between (about) 12 and (about) 16 nucleotides.

[0056] Possibly, this second (RNA) nucleotide sequence consists (essentially) of these 2 or 3
5 (substantially or fully) identical boxes (able to bind a RNA-binding protein) having a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16 nucleotides.

10 [0057] In the context of the present invention, "substantially identical (RNA) nucleotide sequences being a box (able to bind a RNA-binding protein)" and/or "substantially identical boxes" preferably refers
15 to boxes sharing at least 85%, more preferably at least 90% of sequence identity and/or
to RNA boxes that have a comparable affinity for the same RNA-binding protein (i.e. the affinity, when expressed as a base 10 logarithm, of one box is comprised within the range of affinity of the other box(es) plus or minus 1 log, and
20 more preferably, the affinity of one box is comprised within the range of affinity of the other box(es) plus or minus 0.5 log).

[0058] Possibly, this box (sequence) (able to specifically bind a RNA-binding protein on this (m)RNA)
25 comprises (the) GGA trinucleotide of SEQ.ID.NO.1 or of SEQ.ID.NO.2 and further shares at least 50%, at least 60%, at least 70%, preferably at least 80%, 85%, 90%, 95% or even 100% of identity with the remaining part of the corresponding SEQ.ID.NO.1 or SEQ.ID.NO.2.

30 [0059] Possibly, this box (sequence) (able to specifically bind a RNA-binding protein on this (m)RNA) presents at least 50 (preferably, at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100%) of sequence

identity with the sequence SEQ.ID.NO.1 or with the sequence SEQ.ID.NO.2.

[0060] Preferably, this box (sequence) able to specifically bind a RNA-binding protein is (are) sequence(s) able to fix a (m)RNA-binding protein having a molecular weight between (about) 5 kDa and (about) 50 kDa, more preferably between (about) 7 kDa and (about) 30 kDa, still more preferably between (about) 8 and (about) 13 kDa.

[0061] Preferably, this (m)RNA-binding protein is (are) devoid of nuclease and/or of helicase activity.

[0062] Possibly, this (m)RNA-binding protein is (are) originating from another species than the sequence encoding the peptide of interest.

[0063] Possibly, this (m)RNA-binding protein is (are) originating from a eukaryote.

[0064] Alternatively, this (m)RNA-binding protein is (are) originating from a prokaryote.

[0065] Preferably, this (m)RNA-binding protein is not a partially active or an inactive fragment of CsrA, such as CsrA1-50; SEQ.ID.NO.6, or an homolog thereof.

[0066] Alternatively (or in addition), this (m)RNA-binding protein is a fusion protein, preferably a (naturally-occurring) (m)RNA-binding protein fused to a tag (i.e. HA, FLAG, myc,...).

[0067] Possibly, this (m)RNA-binding protein is the full CsrA protein (SEQ.ID.NO.5) (or an active fragment thereof), or a CsrA-tag (i.e. CsrA-HA, CsrA-FLAG, CsrA-myc,...) or an active fragment thereof.

[0068] Preferably, the RNA of the present invention is a messenger RNA (mRNA) and/or the first RNA sequence is a sequence encoding a peptide of interest.

[0069] Advantageously, the encoded peptide (of interest) and/or this first nucleotide sequence is (are) selected from the group consisting of an antigen, an

antibody or a fragment thereof (including humanized fab fragment, nano-bodies or alphabodyTM), (human) hormones, enzymes and any protein that may be recovered for any industrial, medical, cosmetic, chemical, food (feed) or
5 beverage-related or environmental application.

[0070] Alternatively, this first RNA sequence is a sequence encoding a regulatory RNA, such as a siRNA, preferably, the sequence of this siRNA is exogenous and
10 more preferably is a (at least 20, 21, 22, 23, 40, 42, 44 or 100, 200, 300 nucleotide) sequence being a viral or eukaryote sequence or being identical to a sequence obtained from the genome of a eukaryote or from the genome of a virus.

15 [0071] Another aspect of the present invention is a (prokaryote) recombinant cell (preferably *E. coli*) (over-) expressing a RNA-binding protein being able to bind to and stabilize a mRNA molecule.

[0072] Advantageously, this recombinant (prokaryote)
20 cell has further incorporated a DNA molecule (and/or sequence) encoding a poison from a poison-antidote system (but preferably not (yet) the corresponding antidote) under the control of an inducible promoter.

[0073] Preferably, this poison from the poison-
25 antidote system is selected from the group consisting of CcdB, KiD, ParE, Doc, MazF and RelE and is more preferably CcdB.

[0074] Alternatively (or in addition), this recombinant (prokaryote) cell has further been submitted to
30 a partial or total deletion of its tldD gene and/or tldE gene or to one or more mutation(s) inactivating the corresponding TldD protein and/or TldE protein.

[0075] Preferably, this recombinant cell (over-) expressing a RNA-binding protein being able to bind

(specifically) to and stabilize a RNA molecule has further incorporated the recombinant DNA molecule (possibly in the form of a vector) of the present invention, which possibly encodes a (m)RNA molecule comprising a first (upstream) nucleotide sequence encoding a peptide of interest or being non-coding RNA of interest and a second (downstream) nucleotide sequence being a (at least one) box able to (non covalently) specifically bind a RNA-binding protein being (selectively) recognized by the (exogenous or recombinant) RNA-binding protein (over-expressed) by this recombinant cell.

[0076] More preferably, this recombinant cell, having incorporated a DNA molecule (and/or sequence) encoding a poison from a poison-antidote system, has incorporated the recombinant DNA molecule (and/or nucleotide sequence) of the present invention further comprising a (third) nucleotide sequence encoding an antidote protein belonging to poison-antidote systems and this antidote protein being the specific antidote of this poison protein (possibly) expressed by this recombinant cell (and/or belong to the same poison-antidote system).

[0077] Possibly, in this recombinant cell, the (over-expressed) RNA-binding protein is exogenous and/or recombinant.

[0078] Preferably, in this recombinant cell, this (exogenous or recombinant) over-expressed RNA-binding protein has a molecular weight between (about) 5 kDa and (about) 50 kDa, more preferably between (about) 7 kDa and (about) 30 kDa, still more preferably between (about) 8 and (about) 13 kDa.

[0079] Preferably, in this recombinant cell, this (exogenous or recombinant) over-expressed RNA-binding protein is devoid of nuclease and/or of helicase activity.

[0080] Alternatively (or in addition) in this recombinant cell, this (exogenous) recombinant over-expressed RNA-binding protein is a fusion protein, preferably a wild-type (m)RNA-binding protein fused to a tag (i.e. HA, FLAG, myc,...).

[0081] Possibly in this recombinant cell, this (exogenous or recombinant) (over-expressed) RNA-binding protein is the full CsrA protein (SEQ.ID.NO.5) (or an homolog thereof or an active fragment thereof, or a CsrA-tag (i.e. CsrA-HA, CsrA-FLAG, CsrA-myc,...)).

[0082] Preferably in this recombinant cell, this (exogenous or recombinant) over-expressed RNA-binding protein is not a partially active or an inactive CsrA protein, such as CsrA1-50 (SEQ.ID.NO.6) and homologs thereof.

[0083] Possibly, in this recombinant cell, this (exogenous or recombinant) over-expressed RNA-binding protein is originating from a prokaryote.

[0084] Alternatively, in this recombinant cell, this (exogenous or recombinant) (over-expressed) RNA-binding protein is originating from a eukaryote.

[0085] Preferably, this recombinant cell is a prokaryote cell, most preferably being selected from the group consisting of *Clostridium* sp., *Sphingomonas* sp., *Bacillus* sp., *Lactobacillus* sp., *Bifidobacterium* sp., *Lactococcus* sp and *Escherichia coli*, still more preferably being *E. coli*.

[0086] Advantageously, the DNA sequence(s) encoding the poison protein and/or the (exogenous and/or recombinant and/or over-expressed) RNA-binding protein is (are) present on the chromosome of this (prokaryote) cell.

[0087] Preferably, this recombinant prokaryote cell overexpressing a RNA-binding protein is an *E. coli* cell further comprising the tldD sequence and/or tldE sequence

that is (are) submitted to a partial or total deletion of the of the tldD sequence and/or tldE sequence or to one or more mutation(s) inactivating the activity of the TldD protein and/or TldE protein.

- 5 [0088] Advantageously, this recombinant prokaryote (being preferably *E. coli*) cell overexpressing a RNA-binding protein further comprises Dam (SEQ.ID.NO.7), and tldD sequence and/or tldE sequence that are submitted either
- 10 to a partial or total deletion of this (these) tldD sequence and/or tldE nucleotide sequence, and to a partial or total deletion of this dam nucleotide sequence or
- to one or more mutation(s) inactivating the activity of the produced TldD protein and/or TldE protein, and possibly of
- 15 the (translated) Dam protein.

[0089] Possibly, in this recombinant prokaryote cell, the produced Dam protein is Dam 1-59 polypeptide and/or SEQ.ID.NO.8.

- 20 [0090] Most preferably, in this recombinant prokaryote cell, the tldD gene and/or tldE gene is (are) fully deleted.

- [0091] Advantageously, this recombinant cell further encodes the RNA molecule of the present invention and/or
- 25 comprises the DNA vector of the present invention.

[0092] A related aspect of the invention is a kit of part comprising:

- the recombinant cell of the present invention (over-) expressing an (exogenous or recombinant) RNA-binding protein being able to (non covalently) bind to
- 30 and stabilize a (m)RNA and further comprising
- a sequence encoding a poison protein from a poison-antidote system under the control of an inducible promoter,

and/or a partial or total deletion of tldD gene and/or tldE gene),

- and the recombinant DNA molecule (or sequence) of the present invention encoding a second (downstream) nucleotide sequence comprising a (1, 2, 3, 4, 5 or more, preferably 2 or 3) box(es) able to specifically (non covalently) bind a RNA-binding protein on the (transcribed) (m)RNA, this binding protein being able to stabilize this (transcribed) (m)RNA, wherein the (exogenous or recombinant) RNA-binding protein (over-) expressed by this recombinant cell is able to be specifically bound to the second (downstream) nucleotide sequence (when transcribed in RNA);
- this recombinant DNA sequence possibly further comprising a third sequence encoding the antidote protein corresponding to this poison protein (possibly expressed by this recombinant cell).

[0093] Preferably, this recombinant DNA molecule present in the kit of the present invention comprises this second (downstream) nucleotide sequence consisting (essentially) of at least 2 boxes (more preferably identical) able to specifically bind a RNA-binding protein on this (transcribed) RNA and/or mRNA.

[0094] More preferably these at least 2 boxes are substantially identical. Still more preferably, these boxes (box sequences) are (fully) identical.

[0095] Preferably, these at least 2 (substantially or fully) identical boxes have a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16 nucleotides.

[0096] Possibly, this second (downstream) nucleotide sequence consists (essentially) of these 2 or 3

(substantially or fully) identical boxes having a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16
5 nucleotides.

[0097] Possibly, the DNA sequence of the present kit comprises at least one (downstream) nucleotide sequence being at least 50% (preferably, at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%,
10 94%, 95%, 96%, 97%, 98%, 99% or even 100%) identical to (SEQ.ID.NO.3 or SEQ.ID.NO.4).

[0098] Possibly, this (downstream) box sequence (able to specifically bind a RNA-binding protein on this (m)RNA) comprises (the) GGA trinucleotide of SEQ.ID.NO.3 or
15 SEQ.ID.NO.4 and further shares at least 80%, 85%, 90%, 95% or even 100% homology with the remaining part of SEQ.ID.NO.3 or SEQ.ID.NO.4.

[0099] Another aspect of the present invention is a method for stabilizing a RNA-binding molecule in a cell
20 comprising a first (upstream) sequence possibly encoding a peptide of interest, this method comprising the step of adding (binding, fixing or inserting) at the 3'-end of the DNA encoding this first sequence a second (downstream) DNA sequence comprising at least one (1, 2, 3, 4, 5 or more,
25 preferably 2 or 3) nucleotide sequence being a box sequence able to specifically bind a RNA-binding protein on the transcribed mRNA molecule.

[0100] A related aspect of the present invention is a method for increasing the production of a peptide of
30 interest in a cell comprising the step of adding (inserting) at the 3'-end of a locus corresponding to a first (upstream) DNA sequence encoding the said peptide of interest a second (downstream) DNA sequence comprising at least one (1, 2, 3, 4, 5 or more, preferably 2 or 3)

nucleotide sequence being a box sequence able to specifically bind a RNA-binding protein on the (transcribed) RNA molecule, this binding protein being able to stabilize this (recombinant) RNA.

- 5 [0101] Preferably, in this method (for stabilizing a RNA molecule in a cell and/or in this method for increasing the production of a peptide of interest in a cell), this second (downstream) DNA sequence comprises (consists of, or consists essentially of) at least 2 boxes (box sequences)
10 (more preferably identical) able to specifically bind to a binding protein on this (transcribed) RNA and/or mRNA.

[0102] More preferably these at least 2 (downstream) boxes (box sequences) are substantially identical. Still more preferably, these box sequences are (fully) identical.

- 15 [0103] Preferably, these at least 2 (substantially or fully) identical (downstream) boxes have a size comprised between (about) 8 and (about) 25 nucleotides, preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16
20 nucleotides.

- [0104] Possibly, this second (downstream) nucleotide sequence consists (essentially) of these 2 or 3 (substantially or fully) identical boxes having a size comprised between (about) 8 and (about) 25 nucleotides,
25 preferably between (about) 10 and (about) 20 nucleotides, more preferably between (about) 12 and (about) 16 nucleotides.

- [0105] Possibly, in this method (for stabilizing a RNA molecule in a cell and/or in this method for increasing
30 the production of a peptide of interest in a cell), this (downstream) box sequence (able to specifically bind a RNA-binding protein on this transcribed (m)RNA) comprises the GGA trinucleotide of SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4 and further shares at least 80%, 85%, 90%,

95% or even 100% homology with the remaining part of the corresponding SEQ.ID.NO.1, SEQ.ID.NO.2, SEQ.ID.NO.3 or SEQ.ID.NO.4.

[0106] Possibly, in this method (for stabilizing a
5 RNA molecule in a cell and/or in this method for increasing the production of a peptide of interest in a cell), the (at least one) box sequence(s) able to fix a binding protein is at least 50% (preferably, at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,
10 96%, 97%, 98%, 99% or even 100%) identical to (SEQ.ID.NO.3 or SEQ.ID.NO.4).

[0107] Preferably, in this (these) method(s), the RNA-binding protein has a size comprised between (about) 5 kDa and (about) 50 kDa, preferably of (about) 7 kDa and
15 (about) 30 kDa, more preferably of (about) 8 and (about) 13 kDa.

[0108] Preferably, this method further comprises the step of over-expressing this RNA-binding protein (in the cell).

20 [0109] Preferably, in this method, the RNA-binding protein is devoid of nuclease and/or of helicase activity.

[0110] Possibly, in this method, the RNA-binding protein is originating from a eukaryote.

[0111] Possibly, in this method, the RNA-binding
25 protein is originating from a prokaryote.

[0112] Possibly, in this method, the RNA-binding protein is the full CsrA protein (SEQ.ID.NO.5) or CsrA-tag (i.e. CsrA-HA, CsrA-FLAG, CsrA-myc,...), or an active homolog thereof or an active fragment thereof.

30 [0113] Preferably, in this method, the RNA-binding protein is not a partially active or an inactive CsrA fragment such as CsrA1-50 (SEQ.ID.NO.6) or homolog thereof.

[0114] Advantageously, in this method, the cell is a prokaryote cell.

[0115] Advantageously, in this method, the RNA is a messenger RNA (mRNA), which preferably encodes a peptide of interest.

[0116] A closely related aspect is the use of the
5 recombinant cell of the present invention for increasing the stability of the recombinant RNA of the present invention.

[0117] Another closely related aspect is the use of the recombinant cell of the present invention for increased
10 production of a peptide of interest, this peptide of interest being encoded by the recombinant mRNA molecule of the present invention (comprising a first RNA sequence encoding this peptide of interest end a second RNA sequence at the 3' part of the first RNA sequence and comprising a
15 box to bind a specific RNA-binding protein stabilizing this mRNA).

[0118] In the context of the present invention, "RNA-binding protein(s)" relates to peptides that specifically bind to a consensus sequence (such as the
20 downstream box sequences of the present invention) of a recombinant (m)RNA and stabilize it, preferably, these peptide have a size smaller than 100 kDa.

[0119] Preferably, these RNA-binding proteins have no direct or indirect RNase activity, possibly including
25 helicase activity and/or specific function in RNA splicing and/or in RNA interference events.

[0120] Preferably, these RNA-binding proteins are over-expressed.

[0121] Possibly, these over-expressed RNA-binding
30 proteins are exogenous and/or recombinant.

[0122] Preferably, in the context of the present invention (and more particularly in the context of the recombinant cell according to the present invention) the term "over-expressed" points to any increase in the content

of the RNA-binding protein and more preferably to an increase of at least 10%, more preferably of at least 50%, still more preferably to at least 100% and/or to an increase resulting into the stabilization of the corresponding (m)RNA and/or to an increase able to stabilize the corresponding (m)RNA when it is transcribed by this recombinant cell.

[0123] Preferably, in the context of the present invention (and more particularly in the context of the recombinant DNA and/or RNA according to the present invention), the "3' part of the RNA" points, in the case of this RNA is a messenger RNA, to the portion of this mRNA downstream the gene of interest (i.e. at 1 to 20, 50 or 100 nucleotide after the stop codon and/or in the untranslated locus of this mRNA) or downstream of the site of insertion of the gene of interest i.e. at 1 to 20, 50 or 100 nucleotide after this site of insertion) and/or downstream to the nucleotide sequence of interest.

[0124] Preferably, in the context of the present invention, poison-antidote systems point to a protein mechanism whereby an antidote protein is produced to neutralize the activity of a corresponding toxic protein. Possibly, in these poison-antidote systems, the poison-antidote complex that is formed into a cell further represses the promoter controlling the transcription of the poison.

[0125] Most preferred poison antidote systems are CcdB/CcdA, Kid/Kis, ParE/ParD, Doc/Phd, MazF/MazE and RelE/RelB. The still most preferred poison antidote system is CcdB/CcdA.

Short description of the figures

[0126] Figure 1 represents *Escherichia coli* cells transformed with plasmids expressing CcdA41 and/or CcdB. In the colonies transformed with a plasmid encoding CcdB, only those expressing ccdA41 mRNA stabilized by the specific binding of CsrA at the 3'-part of this mRNA were able to survive.

Example 1: establishment and characterization of the strain according to the invention

[0127] The inventors have selected the ccdA41 gene (of *E. coli*) as model system and have added three CsrA-binding boxes to it.

[0128] More particularly, they preferred the addition of the CsrA-binding boxes at the untranslated 3'-end of the mRNA transcribed by this ccdA41 gene.

[0129] This ccdA41 (the 41 C-terminal amino acids of CcdA: SEQ.ID.NO.10) gene encodes a labile antidote to the CcdB poison protein, more particularly, this antidote is less potent than the corresponding full-length CcdA protein (SEQ.ID.NO.9).

[0130] The inventors then induced the production by the host cell of the corresponding poison CcdB protein. Since the poor stability and/or potency of this CcdA41 antidote protein, this test is very stringent and robust and reflects, at the cell viability level, any increase of the content of CcdA41 protein (thus any increase of the corresponding messenger).

[0131] The inventors observed that more cells are viable in the conditions in which the mRNA is modified by the boxes addition showing that more antidote protein is produced because of the increased stability of the corresponding mRNA (Fig. 1).

[0132] Furthermore, in another test run in parallel, the inventors concomitantly over-expressed the RNA-binding CsrA protein.

[0133] In this condition, having both the over-expression of the CsrA protein and the CsrA-binding sites added to the target mRNA (at the 3'-end), the inventors observed that the resulting colonies are of bigger size and thus conclude that this double modification results into an even more stabilized target mRNA.

10 [0134] The inventors then over-expressed a CsrA-FLAG protein (being the FLAG peptide added at the 3'-end of the CsrA protein), instead of CsrA, and observed even improved results.

[0135] The inventors replaced with success the FLAG tag with other small peptides also known as tags that help at identifying and/or at quantifying a target protein.

[0136] These tags are added (preferably at one end of the RNA-binding protein) to the RNA-binding protein using common recombinant technology.

20 [0137] The preferred tags are selected from the group consisting of HA, FLAG, His₆, Myc and isopeptag (ATTVHGETVVNGAKLTVTKNLDLVNSNA; SEQ.ID.NO.11).

[0138] The inventors conclude that the binding of proteins of a suitable size to the untranslated 3'-end of a mRNA increases its stability, preferably with the proviso that the protein does not per se affect mRNA metabolism (for instance that the protein is not a factor directly or indirectly degrading mRNA, such as a (ribo)nuclease, a helicase or a factor recruiting them).

CLAIMS

1. A recombinant RNA molecule comprising
- a first RNA sequence encoding a peptide of interest
and/or being a non-coding regulatory RNA sequence,
5 and,
- a second RNA sequence located at the 3' part of the
said first RNA sequence, the said second RNA sequence
comprising a box sequence able to specifically bind a
specific RNA-binding protein stabilizing the said RNA
10 molecule.
2. The recombinant RNA molecule of claim
1, wherein the second RNA sequence comprises at least
two substantially identical boxes, the said boxes
having a size comprised between 8 and 25 nucleotides.
- 15 3. The recombinant RNA molecule of claims
1 or 2, wherein the second RNA sequence comprises at
least two boxes presenting at least 50% sequence
identity with the sequence SEQ.ID.NO.1 or with the
sequence SEQ.ID.NO.2.
- 20 4. The recombinant RNA molecule according
to any of the preceding claims, wherein the specific
RNA-binding protein has a molecular weight comprised
between 5 kDa and 50 kDa.
- 25 5. The recombinant RNA molecule of claim
4, wherein the specific RNA-binding protein has a
molecular weight comprised between 5 kDa and 30 kDa.
6. The recombinant RNA molecule according
to any of the preceding claims, which is a messenger
RNA (mRNA).
- 30 7. A recombinant DNA molecule encoding
the recombinant RNA molecule according to any of the
preceding claims 1 to 6, preferably in the form of a
vector.
8. A DNA vector comprising

- a promoter,
- a site of insertion for a first DNA sequence able to be transcribed into a first RNA sequence and encoding a peptide or RNA of interest and
5 - a second DNA sequence located at the 3'-end of the said site of insertion and able to be transcribed into a second RNA sequence,
the said promoter being able to induce, from the said first and second DNA sequences, the transcription of
10 one RNA molecule, wherein the said second transcribed RNA sequence comprises a box sequence able to specifically bind a RNA-binding protein able to stabilize the transcribed RNA molecule.

9. The DNA vector of claim 7 or 8,
15 further comprising a third nucleotide sequence encoding an antidote protein belonging to the poison-antidote systems, being preferably selected from the group consisting of CcdA, Kis, ParD, Phd, MazE and RelB, being more preferably CcdA.

20 10. The DNA vector according to any of the preceding claims 7 to 9, wherein the RNA-binding protein able to stabilize the transcribed RNA molecule has a molecular weight comprised between 5 kDa and 50 kDa.

25 11. The DNA vector of claim 10, wherein the RNA-binding protein able to stabilize the transcribed RNA molecule has a molecular weight comprised between 5 kDa and 30 kDa.

30 12. A recombinant prokaryote cell, being preferably *E. coli*, overexpressing a RNA-binding protein being able to bind to a RNA and stabilize the said RNA, the said cell having further incorporated in its genome,

a DNA sequence encoding a poison protein from a poison-antidote system under the control of an inducible promoter; and/or

5 a partial or total deletion of the tldD sequence and/or tldE sequence and/or the said cell comprising one or more mutations inactivating the corresponding tldD protein and/or tldE protein.

10 13. The recombinant cell of claim 12, wherein the RNA-binding protein has a molecular weight comprised between 5 kDa and 50 kDa and preferably wherein the RNA-binding protein is SEQ.ID.NO.5.

14. The recombinant cell of claim 13, wherein the RNA-binding protein has a molecular weight comprised between 5 kDa and 30 kDa

15 15. The recombinant cell according to any of the preceding claims 12 to 14, wherein further the DNA sequence encoding the Dam protein is submitted to a mutation being preferably Dam 1-59 polypeptide.

20 16. The recombinant cell according to any of the preceding Claims 12 to 15 further encoding the recombinant RNA according to any of the preceding claims 1 to 6 or comprising the DNA vector according to any of the preceding claims 7 to 11.

25 17. Use of the recombinant cell of claim 16 for increasing the stability of the recombinant RNA according to any of the preceding claims 1 to 6.

30 18. A kit of part comprising the recombinant cell according to any of the preceding claims 12 to 15 and either the recombinant DNA sequence of claim 7 or the DNA vector according to any of the preceding claims 8 to 11.

19. A method for stabilizing into a cell a RNA molecule comprising a first RNA sequence

encoding a peptide of interest and/or a first RNA sequence being a nucleotide sequence of interest, the said method comprising the step of adding, at the site corresponding to the 3'-end of the first DNA sequence encoding the said first RNA sequence, a second DNA sequence comprising a box sequence able to specifically bind a RNA-binding protein on the transcribed RNA molecule, the said binding protein stabilizing the said RNA molecule.

20. The method of claim 19, wherein the second DNA sequence comprises at least 2 substantially identical boxes specifically binding a RNA-binding protein on the transcribed RNA molecule, preferably wherein the said RNA molecule is a messenger RNA sequence.

21. The method of claims 19 or 20, wherein the box sequence(s) comprises at least 50% sequence identity with the sequence SEQ.ID.NO.1 or with the sequence SEQ.ID.NO.2.

22. The method according to any of the preceding claims 19 to 21, which further comprises the step of over-expressing in the cell the RNA-binding protein, being preferably a protein having a molecular weight comprised between 5 kDa and 50 kDa.

23. The method according to any of the preceding claims 19 to 22, wherein the RNA-binding protein, has a molecular weight comprised between 5 kDa and 30 kDa.

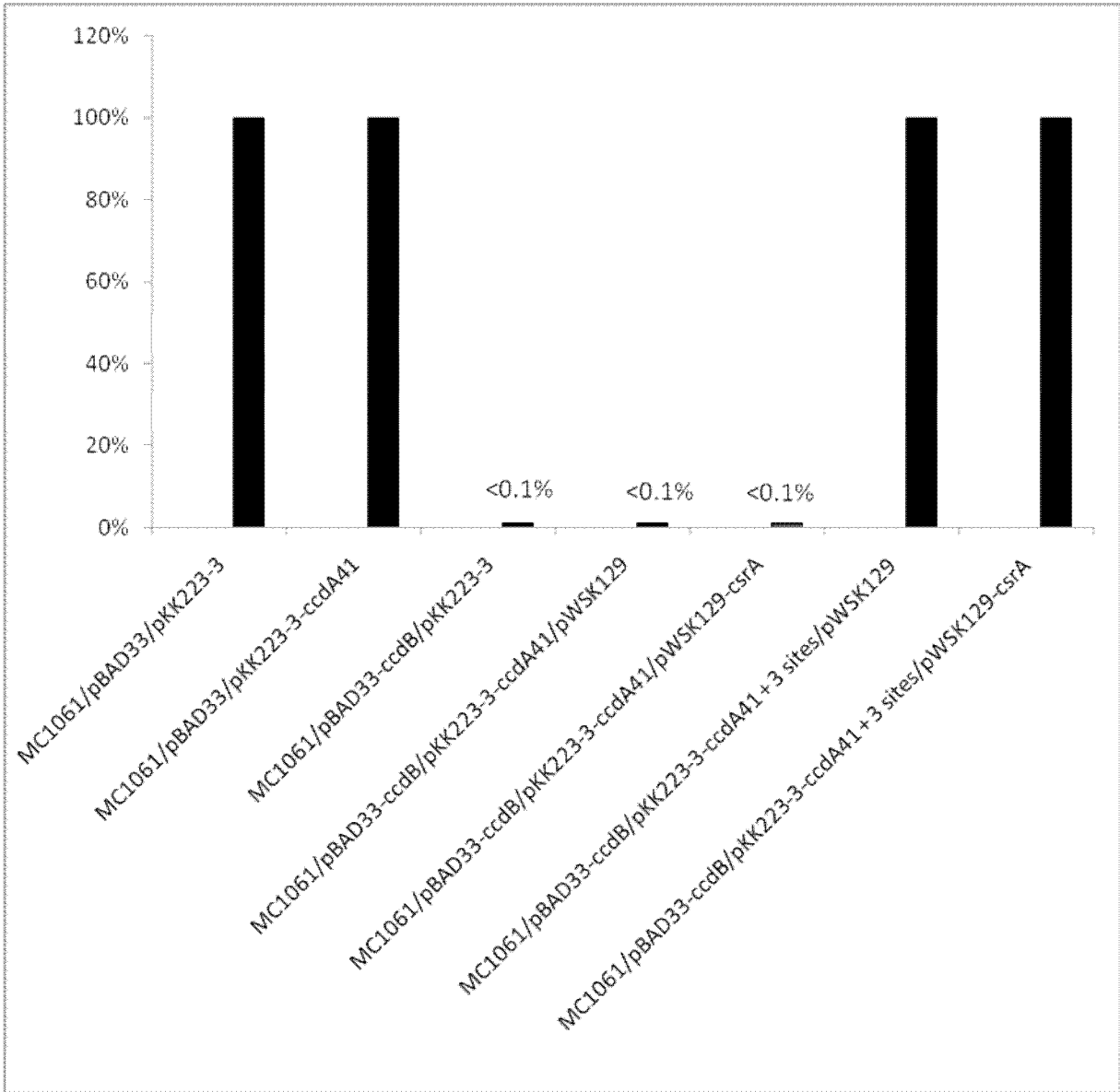


Fig. 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/056248

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/67
ADD. C12N15/68 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	CHEN H H ET AL: "LM04 mRNA stability is regulated by extracellular ATP in F11 cells", BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 357, no. 1, 25 May 2007 (2007-05-25), pages 56-61, XP026422374, ISSN: 0006-291X	1,4,6-8, 19,22,23
Y	the whole document ----- -/--	2-5,10, 11,20, 21,23

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

4 July 2012

Date of mailing of the international search report

10/07/2012

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Andres, Serge

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/056248

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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Information on patent family members

International application No

PCT/EP2012/056248

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