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(54) **Title:**
CAPPING-PRONE RNA POLYMERASE ENZYMES AND THEIR APPLICATIONS

(57) **Abstract:**
The invention provides a chimeric enzyme comprising at least one catalytic domain of a RNA triphosphatase, at least one catalytic domain of a guanylyltransferase, at least one catalytic domain of a N7-guanine methyltransferase, and at least one catalytic domain of a DNA- dependant RNA polymerase. The invention also provides pharmaceutical composition comprising said chimeric enzyme and uses of said chimeric enzyme.

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(54) Title: CAPPING-PRONE RNA POLYMERASE ENZYMES AND THEIR APPLICATIONS

(57) Abstract: The invention provides a chimeric enzyme comprising at least one catalytic domain of a RNA triphosphatase, at least one catalytic domain of a guanylyltransferase, at least one catalytic domain of a N^7-guanine methyltransferase, and at least one catalytic domain of a DNA- dependant RNA polymerase. The invention also provides pharmaceutical composition comprising said chimeric enzyme and uses of said chimeric enzyme.

CAPPING-PRONE RNA POLYMERASE ENZYMES AND THEIR APPLICATIONS

The present invention relates to the field of transgenesis, particularly in eukaryotic cells.

In particular, the invention relates to a chimeric enzyme useful for the production of RNA molecules with 5'-terminal m^7GpppN cap structures.

Eukaryotic expression is very widely used in the life sciences, biotechnology and medicine. Thus, many methods for efficient transgenesis in eukaryotic cells have been developed. Common DNA sources and delivery mechanisms are viruses (e.g. baculovirus, retrovirus, or adenovirus) or non-viral vectors including plasmids and artificial chromosomes.

Because of their simplicity, the non-viral plasmids are commonly used as expression vectors for gene transfer into eukaryotic cells both *in vitro* and *in vivo* applications. However, the levels of transgene expression achieved by non-viral methods are usually modest and declines rapidly. A common explanation for this modest efficacy is the fact that DNA molecules, which are over approximately 40,000 Daltons, are too large to pass through the nuclear pores and enter the nucleus, where they are transcribed by the nuclear RNA polymerase II (Lang, Scholz et al. 1986; Zabner, Fasbender et al. 1995). In fact, only a very small amount (< 0.1-0.001 %) of large DNA molecules is actively transferred from the cytoplasm to the nucleus of eukaryotic cells. The mechanisms by which expression rapidly declines are also possibly nuclear-specific and related to the silencing of transgene expression by various epigenetic mechanisms (Loser, Jennings et al. 1998; Gill, Smyth et al. 2001; Miao, Thompson et al. 2001; Nicol, Wong et al. 2002; Miao, Ye et al. 2003).

Other drawbacks of transgenesis methods using endogenous RNA transcription system of eukaryotic cells also restrain their use. Firstly, the weak processivity of nuclear eukaryotic RNA polymerases (e.g. 10-20 nucleotides/second for RNA polymerase II) (Fire, Samuels et al. 1984; Ucker and Yamamoto 1984; Bengal, Flores et al. 1991; Izban and Luse 1992). Secondly, the competition between endogenous gene transcription and transgene transcription. Thirdly, the extreme complexity of eukaryotic RNA polymerases, which are made of several subunits (e.g. 12 subunits for RNA polymerase II and regulated by multiple transcription factors (Lodish, Berk et al. 2008).

In view of these disadvantages, some transgenesis methods based on bacteriophage DNA-dependent RNA polymerases have been developed. These methods have notably the advantage of not using the endogenous RNA transcription system of eukaryotic cells but some bacteriophage DNA-dependent RNA polymerases, which have a higher processivity than the eukaryotic RNA polymerases.

The pET expression system is a popular method for gene expression in prokaryotes (Studier, Rosenberg et al. 1990). It relies on the expression of the bacteriophage single-subunit T7 DNA-dependent RNA polymerase (T7 RNA polymerase, T7RNAP), the product of T7 gene 1, to transcribe genes of interest engineered to be expressed under the control of a T7 promoter. The pET expression system has been adapted to eukaryotic cells and is usually designated as the hybrid RNA polymerase. However, in an eukaryotic environment, the high enzymatic activity of the T7 DNA dependent RNA polymerase remarkably contrasts with very weak translation yields of the T7 transcripts (Fuerst, Niles et al. 1986). The absence of maturation of the transcripts in eukaryotic cells, which are neither modified by the addition of cap structures at their 5'- terminal (Benton, Eng et al. 1990; Dower and Rosbash 2002), nor strongly polyadenylated at their 3'-terminal (Mifflin and Kellems 1991; Dower and Rosbash 2002), provides an explanation for this discrepancy.

Methods for improving the translatability of uncapped transcripts produced by the hybrid system have thus been developed, like the vaccinia virus/bacteriophage RNAP hybrid expression system. This eukaryotic expression system is based on a recombinant vaccinia virus that synthesizes the bacteriophage T7 DNA dependent RNA polymerase in the cytoplasm of infected mammalian cells (Fuerst, Niles et al. 1986; Fuerst, Earl et al. 1987; Elroy-Stein, Fuerst et al. 1989; Fuerst, Fernandez et al. 1989; Fuerst and Moss 1989; Elroy-Stein and Moss 1990). The target gene for the bacteriophage RNA polymerase, flanked by T7 promoter and termination sequences, is introduced into infected cells either by transfection of a recombinant plasmid or by infection with a second recombinant vaccinia virus (Fuerst, Niles et al. 1986; Elroy-Stein, Fuerst et al. 1989; Elroy-Stein and Moss 1990). It was expected that the vaccinia virus-encoded cytoplasmic enzymes for mRNA capping would act on the T7 transcripts to improve their translatability. However, the capping of T7 transcripts remains infra-optimal (Fuerst and Moss 1989). For instance, using this expression system, it was found that T7 transcripts can comprise up to 30% of total cytoplasmic RNA after a 24 hour period, but only 5%-10% of T7 transcripts contained 5'-terminal cap structures (Fuerst and Moss 1989). Although rather efficient, technical drawbacks of the vaccinia virus/bacteriophage RNAP hybrid expression system clearly restrain its generalization and use at large-scale. Firstly, this system is based on recombinant vaccinia viruses, which are infectious for humans. Therefore, handling these recombinant viruses require specific laboratory facilities and practices. An attenuated avian host-range-restricted strain, i.e. the modified vaccinia Ankara (MVA), which aborts its replicative cycle at a late-stage packaging step in human cells, can be used to better control this hazard (Wyatt, Moss et al. 1995; Engleka, Lewis et al. 1998). Secondly, the recombinant vaccinia or MVA viruses are cytotoxic. Therefore, the vaccinia virus/bacteriophage RNAP hybrid expression system can only be used for transient

transgenesis (Elroy-Stein, Fuerst et al. 1989; Elroy-Stein and Moss 1990). Thirdly, the vaccinia virus/bacteriophage RNAP hybrid expression system can be readily used in some cellular models that are permissive to vaccinia infection (e.g. BSC-1), whereas some are not (e.g. CHO). The insertion of the CP77 gene of cowpox virus into the genome of the recombinant vaccinia virus can overcome the vaccinia virus/bacteriophage RNAP hybrid expression system host range restriction of Chinese hamster ovary (CHO) cells by enabling the vaccinia virus to productively infect these cells (Spehner, Gillard et al. 1988; Ramsey-Ewing and Moss 1996). Fourthly, due to the complexity of the system, significant variability in its efficacy can be expected, even in the same cellular model. Fifthly, the vaccinia virus/bacteriophage RNAP hybrid expression system is a cost and labor-consuming technology, which is therefore poorly appropriate for large-scale assays and protein production.

In an attempt to couple capping to transcription and thus to improve the translatability of uncapped transcripts produced by the T7 RNA polymerase, this enzyme has been fused to the carboxyl-terminal domain (CTD) of the largest subunit of the RNA polymerase II (POLR2A), (Natalizio, Robson-Dixon et al. 2009). The CTD comprises 25-52 heptapeptide repeats of the consensus sequence ^{1}YSPTSPS^{7}, which is highly conserved throughout evolution and subject to reversible phosphorylation during the transcription cycle (Palancade and Bensaude 2003). When phosphorylated, the CTD is thought to mediate the coupling of transcription and capping of nascent transcripts, by binding one or more subunits of the mRNA capping enzymes in yeast (Cho, Takagi et al. 1997; McCracken, Fong et al. 1997) and mammals (McCracken, Fong et al. 1997; Yue, Maldonado et al. 1997). Noticeably, RNA polymerase II with Ser^{5}-phosphorylated CTD repeats undergoes promoter proximal pausing which is coincident with the co-transcriptional capping of the nascent transcripts (Komarnitsky, Cho et al. 2000; Schroeder, Schwer et al. 2000). However, in contrast to what could be expected intuitively, the fusion of the CTD to the single-unit T7 RNA polymerase is not sufficient to enhance the capping of both constitutively and alternatively spliced substrates *in vivo* (Natalizio, Robson-Dixon et al. 2009).

The capping is a specialized structure found at the 5'-end of nearly all eukaryotic messenger RNAs. The simplest cap structure, cap0, results of the addition of a guanine nucleoside methylated at N^{7} that is joined by 5'-5' triphosphate bound to the end of primary RNA (i.e. m^{7}GpppN where N is any base, p denotes a phosphate and m a methyl group). The formation of the cap0 involves a series of three enzymatic reactions: RNA triphosphatase (RTPase) removes the γ phosphate residue of 5' triphosphate end of nascent pre-mRNA to diphosphate, RNA guanylyltransferase (GTase) transfers GMP from GTP to the diphosphate nascent RNA terminus, and RNA N7-guanine methyltransferase (N7-MTase) adds a methyl residue on azote 7 of guanine to the GpppN cap (Furuichi and Shatkin 2000). In higher

eukaryotes and some viruses, the 2'-hydroxyl group of the ribose of the first (i.e. cap1 structures; $m^7GpppNm^{2'-O}pN$) and second (i.e. cap2 structures; $m^7GpppNm^{2'-O}pNm^{2'-O}$) transcribed nucleotides can be methylated by two separate ribose-2'-O MTases, respectively named cap1- and cap2-specific MTases (Langberg and Moss 1981). However, In contrast to the cellular N7-MTase activity that is exclusively nuclear, cap1 ribose-2'-O MTase activity has been detected in both the cytoplasm and nucleus of HeLa cells, whereas cap2 MTase activity is exclusively found in their cytoplasm (Langberg and Moss 1981).

The formation of the 5'-terminal m^7GpppN cap is the first step of pre-mRNA processing. The m^7GpppN cap plays important roles in mRNA stability and its transport from the nucleus to the cytoplasm (Huang and Steitz 2005; Kohler and Hurt 2007). In addition, the 5'-terminal m^7GpppN cap is important for the translation of mRNA to protein by anchoring the eukaryotic translation initiation factor 4F (eIF4F) complex, which mediates the recruitment of the 16S portion of the small ribosomal subunit to mRNA (Furuichi, LaFiandra et al. 1977; Gingras, Raught et al. 1999; Rhoads 1999). The 5'-terminal m^7GpppN cap therefore enhances drastically the translation of mRNA both in vitro (Lo, Huang et al. 1998), and in vivo (Malone, Felgner et al. 1989; Gallie 1991; Lo, Huang et al. 1998; Kozak 2005). In contrast, the effects of 2'-O-methylation on mRNA translation appear to depend on the type of cells and the conditions of the experimentation (Epicentre Biotechnologies website ; Drummond, Armstrong et al. 1985; Kuge, Brownlee et al. 1998).

There remains therefore a significant need in the art for new and improved systems for efficient transgenesis in eukaryotic cells, which are appropriate for gene therapy and large-scale protein production without cytotoxicity or induced-cytotoxicity. The present inventor has made a significant step forward with the invention disclosed herein.

The purpose of the invention is to fulfill this need by providing new chimeric enzymes, which make it possible to solve in whole or part the problems mentioned-above.

Unexpectedly, the inventor has demonstrated that chimeric enzymes comprising a catalytic domain of a RNA triphosphatase, a catalytic domain of a guanylyltransferase, a catalytic domain of a N^7-guanine methyltransferase, and a catalytic domain of a DNA-dependant RNA polymerase are able to synthesize RNA molecules with 5'-terminal m^7GpppN caps, which are highly translatable by the eukaryotic translational machinery, without cytotoxicity and while not inducing apoptosis.

These results are surprising since the capping of T7 transcripts remains infra-optimal with the vaccinia virus/bacteriophage RNAP hybrid expression system, and cannot be achieved by the fusion enzyme CTD-T7 RNA polymerase.

Thus, in one aspect, the invention relates to a chimeric enzyme comprising:

- at least one, in particular a catalytic domain of a RNA triphosphatase,
- at least one, in particular a catalytic domain of a guanylyltransferase,
- at least one, in particular a catalytic domain of a N^7-guanine methyltransferase, and,
- at least one, in particular a catalytic domain of a DNA-dependant RNA polymerase.

In particular the chimeric enzyme according to the invention is able to synthesize RNA molecules with 5'-terminal m^7GpppN caps.

The chimeric enzyme according to the invention has in particular the following advantages:

- There is no competition between the endogenous gene transcription and the transgene transcription;
- It is not expensive, quick and easy to implement and thus appropriate for large-scale assays and protein production;
- In contrast to the vaccinia virus/bacteriophage RNAP hybrid expression system, it has no obvious cytotoxicity or pro-apoptotic activities;
- It allows the production of RNA transcripts in any eukaryotic species (e.g. yeast, plants, rodents, dairy ruminants, primates, and humans).

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one skilled in the relevant art.

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and claims are provided.

As used herein, the term "chimeric enzyme" refers to enzyme that is not a native enzyme that is found in nature. Accordingly, a chimeric enzyme may comprises catalytic domains that are derived from different sources (e.g. from different enzymes) or catalytic domains derived from the same source (e.g. from the same enzyme), but arranged in a different manner than that found in nature.

The term "chimeric enzyme" encompasses monomeric (i.e. single-unit) enzyme but also oligomeric (i.e. multi-unit) enzyme, in particular hetero-oligomeric enzyme.

As used herein, the term "monomeric enzyme" relates to a single-unit enzyme that consists of only one polypeptide chain.

As used herein, the term "oligomeric enzyme" refers to a multi-units enzyme that consists of at least two polypeptides chains, linked together covalently or noncovalently. The term "oligomeric enzyme" encompasses a multi-units enzyme, wherein at least two units of said enzyme are linked together covalently or noncovalently. The term "oligomeric enzyme" encompasses homo-oligomeric enzyme that is a multi-unit enzyme consisting of only one type of monomers (subunit) and hetero-oligomeric enzyme consisting of different types of monomers (subunits).

In particular, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase and of a DNA-dependant RNA polymerase are linked together covalently and/or noncovalently.

In particular, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase and of a DNA-dependant RNA polymerase are operatively linked together to synthesize RNA molecules with 5'-terminal m^7GpppN caps.

In particular, the chimeric enzyme according to the invention comprising:

- at least one, particularly a catalytic domain of a RNA triphosphatase,
- at least one, particularly a catalytic domain of a guanylyltransferase,
- at least one, particularly a catalytic domain of a N^7-guanine methyltransferase, and
- at least one, particularly a catalytic domain of a DNA-dependant RNA polymerase;

wherein at least two of said catalytic domains are linked together, preferably at their extremity (N or C terminal end), covalently or non-covalently, more particularly wherein at least one of the catalytic domain chosen in the group consisting of:

- said at least one, particularly a catalytic domain of a RNA triphosphatase,
- said at least one, particularly a catalytic domain of a guanylyltransferase, and
- said at least one, particularly a catalytic domain of a N^7-guanine methyltransferase

is linked, preferably at its extremity (N or C terminal end), covalently or non-covalently with

- said at least one, particularly a catalytic domain of a DNA-dependant RNA polymerase, preferably at its extremity (N or C terminal end).

In particular, the invention relates to the chimeric enzyme according to the invention comprising:

- at least one, particularly a catalytic domain of a RNA triphosphatase,
- at least one, particularly a catalytic domain of a guanylyltransferase,
- at least one, particularly a catalytic domain of a N^7-guanine methyltransferase, and
- at least one, particularly a catalytic domain of a DNA-dependant RNA polymerase;

with the exception of chimeric enzyme comprising:

- a catalytic domain of a RNA triphosphatase,
- a catalytic domain of a guanylyltransferase,
- a catalytic domain of a N^7-guanine methyltransferase, and
- only catalytic domains of nuclear eukaryotic DNA-dependant RNA polymerase I, II and/or III; and more particularly, only catalytic domain(s) of the DNA-dependant RNA polymerase II.

In particular, the invention relates to the chimeric enzyme according to the invention comprising:

- at least one, particularly a catalytic domain of a RNA triphosphatase,
- at least one, particularly a catalytic domain of a guanylyltransferase,
- at least one, particularly a catalytic domain of a N^7-guanine methyltransferase, and
- at least one, particularly a catalytic domain of a DNA-dependant RNA polymerase;

with the exception of chimeric enzyme comprising:

- a catalytic domain of a RNA triphosphatase,
- a catalytic domain of a guanylyltransferase,
- a catalytic domain of a N^7-guanine methyltransferase, and
- catalytic domains of nuclear eukaryotic DNA-dependant RNA polymerase I, II and/or III, more particularly, at least a catalytic domain of the DNA-dependant RNA polymerase II.

In particular, upon expression in a eukaryotic host cell, said chimeric enzyme according to the invention is able to synthesize RNA molecules with 5'-terminal m^7GpppN cap, which are preferably translatable by the eukaryotic translational machinery.

Particularly, upon expression in a eukaryotic host cell said catalytic domains of a RNA triphosphatase, of a guanylyltransferase and of a N^7-guanine methyltransferase are able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules synthesized by said catalytic domain of a DNA-dependant RNA polymerase and preferably said RNA molecules with 5'-terminal m^7GpppN cap are translatable by the eukaryotic translational machinery.

Particularly, upon expression in a eukaryotic host cell, when said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacteriophage DNA-dependant RNA polymerase, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase and of a N^7-guanine methyltransferase are able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules that have a guanosine ribonucleotide at their 5' terminal end.

Particularly, upon expression in a eukaryotic host cell, when said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacterial DNA-dependant RNA polymerase, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase and of a N^7-guanine methyltransferase are able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules that have a guanosine or a adenosine ribonucleotide at their 5' terminal end.

Particularly, upon expression in a eukaryotic host cell, when said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a human or mouse mitochondrial DNA-dependant RNA polymerase, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase and of a N^7-guanine methyltransferase are able to add a m^7GpppN cap at the

5'-terminal end of RNA molecules that have a adenosine or a thymidine ribonucleotide at their 5' terminal end.

A used herein the term "catalytic domain" of an enzyme relates to domain, which is necessary and sufficient, in particular in its three-dimensional structure, to assure the enzymatic function. For example, a catalytic domain of a RNA triphosphatase is the domain, which is necessary and sufficient to assure the RNA triphosphatase function. The term "catalytic domain" encompasses catalytic domain of wild type or mutant enzyme.

The chimeric enzyme according to the invention comprises at least said catalytic domains but can further comprise the whole or part of the enzymes containing said catalytic domains. In fact, according to one embodiment of the chimeric enzyme according to the invention, said catalytic domain of a DNA-dependant RNA polymerase can be included in the whole or part of a DNA-dependant RNA polymerase, preferably of a monomeric DNA-dependant RNA polymerase. Said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase and said catalytic domain of a N^7-guanine methyltransferase can also be included in the whole or part of a capping enzyme, preferably of a monomeric capping enzyme.

The chimeric enzyme according to the invention can be a nuclear enzyme, a subcellular compartment enzyme or a cytoplasmic enzyme. Thus, the chimeric enzyme according to the invention can comprise a signal peptide or a marker-signal well known by one skilled in the art, which directs the transport of the enzyme in cells. For example, the chimeric enzyme according to the invention can comprise a nuclear localization signal (NLS), which directs the enzyme to the nucleus. Such NLS is often a unit consisting of five basic, plus-charged amino acids. The NLS can be located anywhere on the peptide chain.

Preferably, the chimeric enzyme according to the invention is a cytoplasmic chimeric enzyme. In particular, it does not comprise signal peptide or marker-signal that directs the transport of the enzyme, except to the cytoplasm.

The cytoplasmic localisation of the chimeric enzyme according to the invention has the advantage that it optimizes the levels of transgene expression by avoiding the active transfer of large DNA molecules (i.e. transgene) from the cytoplasm to the nucleus of eukaryotic cells and the export of RNA molecules from the nucleus to the cytoplasm.

These cytoplasmic chimeric enzymes according to the invention can thus be useful to generate a host-independent, eukaryotic gene expression system that is able to work in the cytoplasm in which significantly higher amounts of transfected DNA are usually found as compared to the nucleus.

These cytoplasmic chimeric enzymes according to the invention are able to synthesize RNA molecules with 5'-terminal m^7GpppN caps, which are highly translatable by the eukaryotic cytoplasmic translational machinery, without cytotoxicity and while not inducing apoptosis.

There is also no competition between the endogenous gene transcription and the transgene transcription, since the endogenous gene transcription occurs in the nucleus of eukaryotic cells in contrast to the transgene transcription, which occurs in the cytoplasm.

The cytoplasmic chimeric enzyme according to the invention is thus notably appropriate for large-scale assays and protein production.

In one embodiment of the chimeric enzyme according to the invention, said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, said catalytic domain of a N^7-guanine methyltransferase, are included in a monomer, i.e. in one polypeptide. For example, said monomer can be a monomeric capping enzyme or a monomeric chimeric enzyme according to the invention.

In particular, said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, and said catalytic domain of a N^7-guanine methyltransferase are included in a monomeric capping enzyme. In this case, the chimeric enzyme according to the invention comprise a monomeric capping enzyme, which includes said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, and said catalytic domain of a N^7-guanine methyltransferase. Said monomeric capping enzyme can be a monomeric virus capping enzyme, in particular chosen in the group consisting of the wild type bluetongue virus capping enzyme, the wild type bamboo mosaic virus capping enzyme, the wild type African swine fever virus capping enzyme, the wild type acanthamoeba polyphaga mimivirus capping enzyme and mutants and derivatives thereof which are able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules and, more particularly of the wild type African swine fever virus capping enzyme and mutants and derivatives thereof which are able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules, and even more particularly the wild type African swine fever virus capping enzyme.

In particular, said catalytic domain of a DNA-dependant RNA polymerase can also be included in a monomer, i.e. in one polypeptide. For example, said monomer can be a monomeric DNA-dependent RNA polymerase or a monomeric chimeric enzyme according to the invention.

In particular, said catalytic domain of a DNA-dependant RNA polymerase is included in a monomeric DNA-dependent RNA polymerase. In this case, the chimeric enzyme according to the invention comprises a monomeric DNA-dependent RNA polymerase, which includes said catalytic domain of a DNA-dependant RNA polymerase. Said monomeric DNA-dependent RNA polymerase can be a monomeric phage DNA-dependent RNA polymerase, in particular chosen

in the group consisting of T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase and mutants or derivatives thereof, which are able to synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, more particularly of T7 RNA polymerase and mutants or derivatives thereof, which are able to synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction.

Said catalytic domain of a DNA-dependant RNA polymerase and at least one, preferably at least two and more preferably the whole catalytic domain chosen in the group consisting of:

- said catalytic domain of a RNA triphosphatase;
- said catalytic domain of a guanylyltransferase; and
- said catalytic domain of a N^7-guanine methyltransferase;

can be included in a monomer.

The chimeric enzyme according to the invention can be monomeric or oligomeric. In fact, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase and of a DNA-dependant RNA polymerase can be included in one or several polypeptides.

Preferably, the chimeric enzyme according to the invention is monomeric.

In fact, the inventor has demonstrated that a monomeric chimeric enzyme comprising a catalytic domain of a RNA triphosphatase, a catalytic domain of a guanylyltransferase, a catalytic domain of a N^7-guanine methyltransferase, and a catalytic domain of a DNA-dependant RNA polymerase are able to synthesize RNA molecules with 5'-terminal m^7GpppN caps, which are highly translatable by the eukaryotic translational machinery, without cytotoxicity and while not inducing apoptosis.

It was not obvious that the capping of transcripts well occurred with a monomeric enzyme, due to steric hindrance and components and the enzyme, which have to remain in their native conformation. In fact, the capping of T7 transcripts cannot be achieved by the fusion enzyme CTD-T7 RNA polymerase, although it was supposed to trigger m^7GpppN capping at the 5'-terminal end of nascent RNA molecules.

The monomeric chimeric enzyme according to the invention has in particular the advantages that it is not expensive, quick and easy to implement and thus appropriate notably for large-scale assays and protein production. In fact, the production of a monomeric enzyme is easier than of oligomeric enzyme. There is also no problem of unit assembly, since there is only a single-unit. The monomeric enzyme is also easier to manipulate than multimeric enzyme.

As used herein, the term "DNA-dependent RNA polymerase" (RNAPs) relates to nucleotidyl transferases that synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction.

Preferably, said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of an enzyme, which have a relatively simple structure and more preferably, which have characterized genomic enzymatic regulation elements (i.e. promoter, transcription termination signal and concatemer junction). Thus, in particular, said catalytic domain of a DNA-dependant RNA polymerase can be a catalytic domain of a bacteriophage DNA-dependant RNA polymerase, of a bacterial DNA-dependant RNA polymerase or of a DNA-dependant RNA polymerase of various eukaryotic organelles (e.g. mitochondria, chloroplast and proplastids).

In one embodiment, said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacteriophage DNA-dependant RNA polymerase.

The bacteriophage DNA-dependant RNA polymerases have notably the advantage that they optimize the levels of transgene expression, in particular by having a higher processivity than the eukaryotic RNA polymerases. The bacteriophage DNA-dependant RNA polymerases have also a much simpler structure than most nuclear eukaryotic polymerases, which have complex structure with multiple subunits (e.g. RNA polymerase II) (Chen and Schneider 2005). Most of the bacteriophage DNA-dependant RNA polymerases characterized so far are single-subunit enzymes, which require no accessory proteins for initiation, elongation, or termination of transcription (Chen and Schneider 2005). Several of these enzymes, which are named for the bacteriophages from which they have been cloned, have also well-characterized regulation genomic elements (i.e. promoter, termination signals, transcriptional pausing sequences), which are important for transgenesis.

There is also no competition between the endogenous gene transcription and the transgene transcription. The chimeric enzymes according to the invention, which comprise bacteriophage DNA dependant RNA-polymerase moieties allow the production of RNA transcripts in any eukaryotic species (e.g. yeast, rodents, and humans). They are not expensive, quick and easy to implement and thus appropriate for large-scale assays and protein productions; it allows the production of RNA transcripts in any biological system (e.g. acellular reaction mix, cultured cells, and living organisms), since in contrast to eukaryotic RNA polymerase such as RNA polymerase II, most of bacteriophage DNA dependant RNA polymerases do not require associated factors for initiation, elongation or termination of transcription.

Said catalytic domain of a bacteriophage DNA-dependant RNA polymerase can be a catalytic domain of a bacteriophage DNA-dependant RNA polymerase, in particular chosen in the group consisting of the wild type of the T7 RNA polymerase, the wild type of the T3 RNA

polymerase (NCBI genomic sequence ID# NC_003298; GeneID# 927437; UniProtKB/Swiss-Prot ID# Q778M8), the wild type of the K11 RNA polymerase (NCBI genomic K11 RNAP sequence ID# NC_004665; GeneID# 1258850; UniProtKB/Swiss-Prot ID# Q859H5), the wild type of the φA1122 RNA polymerase (NCBI genomic sequence ID# NC_004777; GeneID# 1733944; UniProtKB/Swiss-Prot protein ID# Q858N4), the wild type of the φYeo3-12 RNA polymerase (NCBI genomic sequence ID# NC_001271; GeneID# 1262422; UniProtKB/Swiss-Prot ID# Q9T145) and the wild type of the gh-1 RNA polymerase (NCBI genomic sequence ID# NC_004665; GeneID# 1258850; UniProtKB/Swiss-Prot protein ID# Q859H5), the wild type of the K1-5 RNAP RNA polymerase (NCBI genomic sequence ID# NC_008152; GeneID# 5075932; UniProtKB/Swiss-Prot protein ID# Q8SCG8) and the wild type of the SP6 RNA polymerase (NCBI genomic sequence ID# NC_004831; GeneID# 1481778; UniProtKB/Swiss-Prot protein ID# Q7Y5R1), and mutants or derivatives thereof, which are able to synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, more particularly of the wild type of T7 RNA polymerase.

As used herein, the term "T7 RNA polymerase" relates to the bacteriophage T7 DNA-dependant RNA polymerase. Preferably, the T7 RNA polymerase have the amino acid sequence of SEQ ID N°1 (NCBI genomic sequence ID# NC_001604; GeneID# 1261050; UniProtKB/Swiss-Prot ID# P00573) and is a 883 amino-acid protein with a molecular weight of 98.8 kDa (Davanloo, Rosenberg et al. 1984; Moffatt, Dunn et al. 1984).

The T7 RNA polymerase has in particular the advantage that, *in vitro*, the enzyme is extremely processive and elongates 240-250 nucleotides/s at 37°C in the 5' → 3' direction (Golomb and Chamberlin 1974; Lyakhov, He et al. 1997; Zhang and Studier 1997; Finn, MacLachlan et al. 2005). Moreover, when expressed in eukaryotic cells, the T7 RNA polymerase, remains largely in the cytoplasm (Elroy-Stein and Moss 1990; Gao and Huang 1993; Brisson, He et al. 1999), and thus optimizes the levels of transgene expression by avoiding the active transfer of large DNA molecules (i.e. transgene) from the cytoplasm to the nucleus of eukaryotic cells and the export of RNA molecules from the nucleus to the cytoplasm.

The catalytic domain of a DNA-dependant RNA polymerase can be the one of the wild-type of the T7 RNA polymerase but also of mutants of the T7 RNA polymerase, which are able to synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, even with reduced processivity. For example, said mutants can be chosen in the group comprising R551S, F644A, Q649S, G645A, R627S, I810S, and D812E (Makarova, Makarov et al. 1995), and K631M (Osumi-Davis, de Aguilera et al. 1992; Osumi-Davis, Sreerama et al. 1994).

In one embodiment of the chimeric enzyme according to the invention, said catalytic domain of a DNA-dependant RNA polymerase is located C-terminally with respect to said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, and said catalytic domain of a N^7-guanine methyltransferase.

In fact, when the catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacteriophage DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, said catalytic domain preferably conserves its native carboxyl-terminal end. In particular, the C-terminal end of said catalytic domain of a DNA-dependant RNA polymerase, particularly of a bacteriophage DNA-dependant RNA polymerase corresponds to the C-terminal end of said chimeric enzyme. In particular, when the chimeric enzyme comprises a whole bacteriophage DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, said polymerase preferably conserves its native carboxyl-terminal end. In particular, the C-terminal end of said catalytic domain of a DNA-dependant RNA polymerase, particularly of a bacteriophage DNA-dependant RNA polymerase corresponds to the C-terminal end of said chimeric enzyme. Particularly, said catalytic domain of a DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, is included in the whole or part of a bacteriophage DNA-dependant RNA polymerase and wherein the C-terminal end of said bacteriophage DNA-dependant RNA polymerase corresponds to the C-terminal end of said chimeric enzyme.

In one embodiment of the chimeric enzyme according to the invention, said catalytic domain of a DNA-dependant RNA polymerase, particularly said catalytic domain of a bacteriophage DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, is located C-terminally with respect to said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, and said catalytic domain of a N^7-guanine methyltransferase.

In another embodiment, said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacterial DNA-dependant RNA polymerase.

Preferably said bacterial DNA-dependant RNA polymerase has a moderate structure complexity.

For example, said bacterial DNA-dependant RNA polymerase can be the E. coli DNA-dependent RNA polymerase (NCBI genomic sequence of K-12 substrain DH10B ID#

NC_010473), which consists of four different subunits (α subunit: rpoA GeneID# 6060938, UniProtKB/Swiss-Prot ID# B1X6E7; β subunit: rpoB GeneID# 6058462, UniProtKB/Swiss-Prot ID# B1XBY9; β' subunit: rpoC GeneID# 6058956, UniProtKB/Swiss-Prot ID# B1XBZ0; σ subunit: rpoE GeneID# 6060683, UniProtKB/Swiss-Prot ID# B1XBQ0), which are assembled in a five ααββ'σ subunit complex (Lodish, Berk et al. 2008). The genomic elements involved in the regulation of the enzymatic activity are well-characterized, including E. coli RNA polymerase promoters (Lisser and Margalit 1993), termination signals including rho-dependant and -independent terminators (Platt 1986; Uptain and Chamberlin 1997), and transcriptional pausing sequences (Lee, Phung et al. 1990).

In another embodiment, said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a DNA-dependant RNA polymerase of a eukaryotic organelle, like mitochondria, chloroplast and proplastids. In fact, these polymerases can also have relatively simple structure.

In particular, said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a mitochondrial DNA-dependant RNA polymerase.

Particularly, said catalytic domain of a DNA-dependant RNA polymerase can be the catalytic domain of the mammalian mouse mitochondrial RNA polymerase, which is a single-unit 120 kDa protein (GeneID# 216151, UniProtKB/Swiss-Prot ID# Q8BKF1), which shares homology with the bacteriophage RNA polymerases (Tiranti, Savoia et al. 1997). Several transcription factors are required for transcription initiation, elongation, or termination: TFB1M (mitochondrial transcription factor B1; mouse GeneID# 224481, UniProtKB/Swiss-Prot ID# Q8JZM0) or TFB2M (mitochondrial transcription factor B2; mouse GeneID# 15278, UniProtKB/Swiss-Prot ID# Q3TL26), TFAM (mitochondrial transcription factor A; mouse GeneID# 21780, UniProtKB/Swiss-Prot ID# P40630), and mTERF (mitochondrial transcription termination factor; mouse GeneID# 545725, UniProtKB/Swiss-Prot ID# Q8CHZ9) for termination of transcription (Fisher and Clayton 1985; Fisher, Topper et al. 1987; Fisher and Clayton 1988; Topper and Clayton 1989; Fernandez-Silva, Martinez-Azorin et al. 1997; Prieto-Martin, Montoya et al. 2001; McCulloch, Seidel-Rogol et al. 2002). The genomic elements involved in the regulation of the enzymatic activity of the mitochondrial RNA polymerase are well-characterized, including two promoters at light- and heavy-strands of the mitochondrial genome (Ojala, Montoya et al. 1981; Clayton 1991), as well as transcriptional termination signals (Kruse, Narasimhan et al. 1989).

As used herein, the term "RNA triphosphatase" (RTPase) relates to the enzyme, which removes the γ phosphate residue of 5' triphosphate end of nascent pre-mRNA to diphosphate (Furuichi and Shatkin 2000).

As used herein, the term "RNA guanylyltransferase" (GTase) refers to the enzyme, which transfers GMP from GTP to the diphosphate nascent RNA terminus (Furuichi and Shatkin 2000).

As used herein, the term "N7-guanine methyltransferase" (N7-MTase) relates to the enzyme, which adds a methyl residue on azote 7 of guanine to the GpppN cap (Furuichi and Shatkin 2000).

Said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase, can be of the same or of different capping enzymes. If said catalytic domains are of the same enzyme, said catalytic domain of a DNA-dependant RNA polymerase is of a different enzyme.

Preferably, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase are from one or several cytoplasmic enzymes, which have advantageously relatively simple structure and well-characterized enzymatic activities. Thus, in particular, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase can be catalytic domains of one or several virus capping enzymes, or of capping enzymes of cytoplasmic episomes.

In one embodiment, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase are from one or several virus capping enzymes, in particular chosen in the group consisting of the wild type bluetongue virus capping enzyme, the wild type bamboo mosaic virus capping enzyme, the wild type African swine fever virus capping enzyme, the wild type acanthamoeba polyphaga mimivirus capping enzyme, and mutants or derivatives thereof which are able respectively to remove the γ phosphate residue of 5' triphosphate end of nascent pre-mRNA to diphosphate or transfer GMP from GTP to the diphosphate nascent RNA terminus or add a methyl residue on azote 7 of guanine to the GpppN cap, more particularly of the wild type African swine fever virus capping enzyme.

As used herein the term "bluetongue virus capping enzyme" relates to the single-unit VP4 capping enzyme of Bluetongue virus (BTV), which is a 76 kDa protein (644 amino-acids; for sequence, see for instance NCBI BTV serotype 10 genomic sequence ID# Y00421; GeneID# 2943157; UniProtKB/Swiss-Prot ID# P07132, D0UZ45, Q5J7C0, Q65751, Q8BA65, P33428, P33429, P33427, C3TUP7, Q8BAD5, C5IWW0, B4E551, Q3KVQ2, Q3KVQ1, Q65732, Q3KVP8, Q3KVP9, Q3KVQ0). This capping enzyme is likely able to homodimerize through the leucine zipper located near its carboxyl-terminus (Ramadevi, Rodriguez et al. 1998). VP4 catalyze all three enzymatic steps required for mRNA m^7GpppN capping synthesis: RTPase (Martinez-Costas, Sutton et al. 1998), GTase (Martinez-Costas, Sutton et al. 1998; Ramadevi, Burroughs et al. 1998) and N7-MTase (Ramadevi, Burroughs et al. 1998).

As used herein, the term "bamboo mosaic virus capping enzyme" relates to ORF1, the Bamboo Mosaic Virus (BMV) mRNA capping enzyme, which is a single-unit 155-kDa protein (1365-amino acids; NCBI BMV isolate BaMV-O genomic sequence ID# NC_001642; GeneID# 1497253; UniProtKB/Swiss-Prot ID# Q65005). ORF1 protein has all the enzymatic activities required to generate m^7GpppN mRNA capping, i.e. RTPase (Li, Shih et al. 2001; Han, Tsai et al. 2007), GTase and N7-MTase (Li, Chen et al. 2001; Li, Shih et al. 2001). In addition, ORF1 has RNA-dependent RNA-polymerase activity, which is not mandatory for chimeric enzymatic activities according to the invention and can be abolished by deletion of $Asp^{1229}Asp^{1230}$ residues of the mRNA capping enzyme (Li, Cheng et al. 1998). As used herein, the term "African swine fever virus capping enzyme " relates to the NP868R capping enzyme (G4R), (ASFV), which is a single-unit 100 kDa protein (868 amino-acids; NCBI ASFV genomic sequence strain BA71V ID# NC_001659; GeneID# 1488865; UniProtKB/Swiss-Prot ID# P32094).

As used herein, the term "acanthamoeba polyphaga mimivirus capping enzyme " relates to R382, (APMV), which is another single-unit 136.5 kDa protein (1170 amino-acids; NCBI APMV genomic sequence ID# NC_006450; GeneID# 3162607; UniProtKB/Swiss-Prot ID# Q5UQX1).

In one embodiment, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase are from one or several capping enzymes of cytoplasmic episomes, like pGKL2. In particular, said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase are included in the whole or part of the capping enzyme of the yeast linear extra-chromosomal episome pGKL2.

Cytoplasmic linear episomes are double-stranded DNA structures, which stably replicate in the cytoplasm of various yeast strains (Cong, Yarrow et al. 1994). One prototype of yeast linear extra-chromosomal episome, pGKL2 (13,457 bp; also named pGK12), has been entirely sequenced from various yeast strains, including Kluyveromyces lactis CB 2359 and Saccharomyces cerevisiae F102-2 (Tommasino, Ricci et al. 1988). The capping enzyme encoded by the ORF3 gene of *Kluyveromyces lactis* pGKL2 (NCBI *Kluyveromyces lactis* CB 2359 pGKL2 genomic sequence ID# NC_010187; UniProtKB/Swiss-Prot ID# P05469) is a 594 amino-acid protein (70.6 kDa protein).

In one embodiment of the chimeric enzyme according to the invention, at least two, in particularly at least three and more particularly the whole catalytic domains can be assembled, fused, or bound directly or indirectly by a linking peptide.

In particular at least two, particularly at least three and more particularly the whole catalytic domains chosen in the group consisting of:

- a catalytic domain of a RNA triphosphatase,

- a catalytic domain of a guanylyltransferase,
- a catalytic domain of a N^7-guanine methyltransferase, and
- a catalytic domain of a DNA-dependant RNA polymerase.

are bound directly or by a linking peptide.

Linking peptide has the advantage of generating fusion proteins in which steric hindrance is minimizes and enough space is provided for the components of the fusion protein to remain in their native conformation.

Preferably, at least said catalytic domain of a DNA-dependant RNA polymerase is bound by a linking peptide to at least one of the catalytic domain chosen in the group consisting of:

- said catalytic domain of a RNA triphosphatase;
- said catalytic domain of a guanylyltransferase; and
- said catalytic domain of a N^7-guanine methyltransferase.

Particularly, the linking peptide can be located N-terminally with respect to said catalytic domain of a DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, and C-terminally with respect to said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, and said catalytic domain of a N^7-guanine methyltransferase.

In particular, the N-terminal end of said catalytic domain of a DNA-dependant RNA polymerase, in particular of a bacteriophage DNA-dependant RNA polymerase chosen in the group consisting of T7, T3 and SP6-RNA polymerases, is linked by covalent linkage, in particular by a linking peptide, to the C-terminal end of one of the catalytic domain chosen in the group consisting of:

- said catalytic domain of a RNA triphosphatase,
- said catalytic domain of a guanylyltransferase, and
- said catalytic domain of a N^7-guanine methyltransferase.

Said linking peptide can be chosen from the group consisting of:

- peptides of formula $(Gly_mSer_p)_n$, in which:
  - m represents an integer from 0 to 12, in particular from 1 to 8, and more particularly from 3 to 6 and even more particularly 4;
  - p represents an integer from 0 to 6, in particular from 0 to 5, more particularly from 0 to 3 and more particularly 1; and
  - n represents an integer from 0 to 30, in particular from 0 to 12, more particularly from 0 to 8 and even more particularly between 1 and 6 inclusive;
- peptides consisting of an amino acid sequence selected from the group consisting of SEQ ID N°2, SEQ ID N°3, SEQ ID N°4,.SEQ ID N°5, SEQ ID N6, SEQ ID N°7, SEQ ID

N°8, SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°16,SEQ ID N°17, SEQ ID N°18.

The flexible linker peptides of formula $(Gly_mSer_p)_n$ have the advantages that the glycine residues confer peptide flexibility, while the serine provide some solubility (Huston, Levinson et al. 1988). Furthermore, the absence of sensitive sites for chymotrypsin I, factor Xa, papain, plasmin, thrombin and trypsin in the $(Gly_mSer_p)_n$ linker sequences is supposed to increase the overall stability of the resulting fusion proteins (Crasto and Feng 2000).

$(Gly_mSer_p)_n$ linkers of variable lengths are commonly used to engineer single-chain Fv fragment (sFv) antibodies (Huston, Levinson et al. 1988). In addition, $(Gly_mSer_p)_n$ linkers have been used to generate various fusion proteins, which frequently retain the biological activities of each of their components (Newton, Xue et al. 1996; Lieschke, Rao et al. 1997; Shao, Zhang et al. 2000; Hu, Li et al. 2004).

Other types of peptide linkers can be also considered to generate chimeric enzymes according to the invention, such as GGGGIAPSMVGGGGS (SEQ ID N°2) (Turner, Ritter et al. 1997), SPNGASNSGSAPDTSSAPGSQ (SEQ ID N°3) (Hennecke, Krebber et al. 1998), EGKSSGSGSESKSTE (SEQ ID N°4) (Bird, Hardman et al. 1988), EGKSSGSGSESKEF (SEQ ID N°5) (Newton, Xue et al. 1996), GGGSGGGSGGGTGGGSGGG (SEQ ID N°6) (Robinson and Sauer 1998), GSTSGSGKSSEGKG (SEQ ID N°7) (Bedzyk, Weidner et al. 1990), YPRSIYIRRRHPSPSLTT (SEQ ID N°8) (Tang, Jiang et al. 1996), GSTSGSGKPGSGEGSTKG (SEQ ID N°9) (Whitlow, Bell et al. 1993), GSTSGSGKPGSGEGS (SEQ ID N°10) (Ting, Kain et al. 2001), SSADDAKKDAAKKDDAKKDDAKKDA (SEQ ID N°11) (Pantoliano, Bird et al. 1991), GSADDAXXDAAXKDDAKKDDAKKDGS (SEQ ID N°12) (Gregoire, Lin et al. 1996), LSADDAKKDAAKKDDAKKDDAKKDL (SEQ ID N°13) (Pavlinkova, Beresford et al. 1999), AEAAAKEAAAKEAAAKA (SEQ ID N°14) (Wickham, Carrion et al. 1995), GSHSGSGKP (SEQ ID N°15) (Ting, Kain et al. 2001), GSTSGSGKPGSGEGSTGAGGAGSTSGSGKPSGEG (SEQ ID N°16) (Ting 2003), LSLEVAEEIARLEAEV (SEQ ID N°17) (Liu, Jian-Bo et al. 2005), and GTPTPTPTPTGEF (SEQ ID N°18) (Gustavsson, Lehtio et al. 2001).

Other types of covalent linkage include but are not limited to disulfide bounds (Mantile, Fuchs et al. 2000), transglutamination (Paguirigan and Beebe 2007), as well as protein trans-linking by chemical and/or physical agents, e.g. cross-linking by tris(bipyridine)ruthenium(II)-dichloride and ultraviolet light illumination (Fancy and Kodadek 1999).

Said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase, and of a DNA-dependant RNA polymerase can also be assembled by specific protein elements, like leucine zippers, like biotinylation domain to one of the catalytic domain (e.g. Avi-tag II (Cronan 1990) or PFB-tag (Wu, Yeung et al. 2002)) and a biotin binding domain

to one of the other catalytic domain (e.g. Strep-tag II (Schmidt and Skerra 1993) or Nano-tag (Lamla and Erdmann 2004)) in the chimeric enzyme according to the invention.

In one embodiment of the chimeric enzyme according to the invention, at least two of said catalytic domains can be assembled, by non-covalent linkage, in particular by leucine zippers.

Preferably, at least said catalytic domain of a DNA-dependant RNA polymerase is assembled by non-covalent linkage, in particular by leucine zippers, to at least one of the catalytic domain chosen in the group consisting of:

- said catalytic domain of a RNA triphosphatase;
- said catalytic domain of a guanylyltransferase; and
- said catalytic domain of a N^7-guanine methyltransferase.

The leucine zippers, which are dimeric coiled-coil protein structures composed of two amphipathic α-helices that interact with each other, are commonly used to homo- or hetero-dimerize proteins (O'Shea, Klemm et al. 1991). Each helices consist of repeats of seven amino acids, in which the first amino-acid (residue a) is hydrophobic, the fourth (residue d) is usually a Leucine, while the other residues are polar. The leucine zippers VELCRO ACID-p1 and BASE-p1, which form a parallel heterodimeric two-stranded coiled coil structures, have high propensity to form parallel protein hetero-dimers (O'Shea, Lumb et al. 1993). They have been used to heterodimerize membrane proteins (Chang, Bao et al. 1994; Pashine, Busch et al. 2003), as well as several soluble proteins (Busch, Reich et al. 1998; Busch, Pashine et al. 2002).

Other types of oligomerisation peptide domains can be also considered to generate chimeric enzyme according to the invention, to assemble at least two of said catalytic domains of the chimeric enzyme according to the invention, especially leucine zippers that form antiparallel heteromeric structures, such as the ACID-a1/BASE-a1 (Oakley and Kim 1998), ACID-Kg/BASE-Eg (McClain, Woods et al. 2001), NZ/CZ (Ghosh, Hamilton et al. 2000), ACID-pLL/ BASE-pLL (Lumb and Kim 1995), and $EE_{1234}L$ and $RR_{1234}L$ (Moll, Ruvinov et al. 2001) leucine zippers. Disulfide-linked versions of leucine zippers can be also used to generate disulfide coiled coil-bound heterodimeric chimeric enzyme according to the invention (O'Shea, Lumb et al. 1993), as well as interchain disulfide bridges between cysteine residues under oxidizing conditions (Wells and Powers 1986).

At least two of said catalytic domains of a RNA triphosphatase, of a guanylyltransferase, of a N^7-guanine methyltransferase, and of a DNA-dependant RNA polymerase can thus be assembled by leucine zippers, in particular leucine zippers that form antiparallel heteromeric structures, such as the ACID-a1/BASE-a1 (Oakley and Kim 1998), ACID-Kg/BASE-Eg (McClain, Woods et al. 2001), NZ/CZ (Ghosh, Hamilton et al. 2000), and ACID-pLL/ BASE-

pLL leucine zippers, disulfide coiled coil-bound (O'Shea, Lumb et al. 1993), as well as disulfide bridges between cysteine residues (Wells and Powers 1986).

In one embodiment, the chimeric enzyme according to the invention comprises:

- the wild type mRNA capping enzyme of the African Swine Fever virus or a mutant or a derivative thereof, which is able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules, in particular the wild type African swine fever virus capping enzyme, fused to
- the amino-terminal end of the wild type T7 RNA polymerase or mutant or derivative thereof which is able to synthesize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, in particular the wild type of T7 RNA polymerase,

in particular via a linker, and more particularly via a $(Gly_3Ser)_4$ linker.

In another embodiment, the chimeric enzyme according to the invention comprises:

- the wild type mRNA capping enzyme of the African Swine Fever virus or a mutant or a derivative thereof, which is able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules, in particular the wild type African swine fever virus capping enzyme, fused to
- the amino-terminal end of the wild type T3 RNA polymerase or mutant or derivative thereof which is able to synthetize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, in particular the wild type of T3 RNA polymerase,

in particular via a linker, and more particularly via a $(Gly_3Ser)_4$ linker.

In another embodiment, the chimeric enzyme according to the invention comprises:

- the wild type mRNA capping enzyme of the African Swine Fever virus or a mutant or a derivative thereof, which is able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules, in particular the wild type African swine fever virus capping enzyme, fused to
- the amino-terminal end of the wild type SP6 RNA polymerase or mutant or derivative thereof which is able to synthetize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, in particular the wild type of SP6 RNA polymerase,

in particular via a linker, and more particularly via a $(Gly_3Ser)_4$ linker.

In another embodiment, the chimeric enzyme according to the invention comprises

- the wild type mRNA capping enzyme of the African Swine Fever virus or a mutant or a derivative thereof, which is able to add a m^7GpppN cap at the 5'-terminal end of RNA molecules, in particular the wild type African swine fever virus capping enzyme, and
- the amino-terminal end of the wild type T7 RNA polymerase or mutant or derivative thereof which is able to synthetize single-stranded RNA complementary in sequence to the double-stranded template DNA in the 5' → 3' direction, in particular the wild type of T7 RNA polymerase,

assembled by leucine zippers.

The chimeric enzyme according to the invention can also further comprise a domain, which enhance the activity of at least one catalytic domain of the chimeric enzyme of the invention, in particular of at least one catalytic domain chosen in the group consisting of a catalytic domain of a RNA triphosphatase, a catalytic domain of a guanylyltransferase, a catalytic domain of a N^7-guanine methyltransferase and a catalytic domain of a DNA-dependant RNA polymerase.

For example said domain, which enhance the activity of at least one catalytic domain of the chimeric enzyme of the invention, can be a 31-kDa subunit encoded by the vaccinia virus D12L gene (genomic sequence ID# NC_006998.1; GeneID#3707515; UniProtKB/Swiss-Prot ID#YP_232999.1), which has no intrinsic enzymatic activity, but enhances drastically the RNA N7-guanine methyltransferase activity of the D1R subunit of the vaccinia mRNA capping enzyme (Higman, Bourgeois et al. 1992; Higman, Christen et al. 1994; Mao and Shuman 1994).

In one embodiment, the chimeric enzyme of the invention comprises:

- at least one catalytic domain of the vaccinia mRNA capping enzyme, in particular the 95 kDa subunit encoded by the vaccinia virus D1R gene (genomic sequence ID# NC_006998.1; GeneID# 3707562; UniProtKB/Swiss-Prot ID# YP_232988.1), which has RNA-triphosphatase, RNA guanylyltransferase and RNA N7-guanine methyltransferase enzymatic activities (Cong and Shuman 1993; Niles and Christen 1993; Higman and Niles 1994; Mao and Shuman 1994; Gong and Shuman 2003);
- at least one catalytic domain of a DNA-dependant RNA polymerase, in particular chosen in the group consisting of T7, T3 and SP6-RNA polymerases and more particularly the T7 RNA polymerase; and
- a 31-kDa subunit encoded by the vaccinia virus D12L gene,

in particular assembled in whole or part via a linker, and more particularly via a $(Gly_3Ser)_4$ linker and/or by leucine zippers.

The invention also relates to an isolated nucleic acid molecule or a group of isolated nucleic acid molecules, said nucleic acid molecule(s) encoding a chimeric enzyme according to the invention.

Said group of isolated nucleic molecules encoding a chimeric enzyme according to the invention comprises or consists of all the nucleic acid molecules which are necessary and sufficient to obtain a chimeric enzyme according to the invention by their expression.

In one embodiment, said group of isolated nucleic acid molecules encoding a chimeric enzyme according to the invention comprises or consists of:

- a nucleic acid molecule encoding at least one catalytic domain of a RNA triphosphatase, at least one catalytic domain of a guanylyltransferase and at least one catalytic domain of a N^7-guanine methyltransferase; and

- a nucleic acid molecule encoding at least one catalytic domain of a DNA-dependant RNA polymerase.

In another embodiment, said group of isolated nucleic acid molecules encoding a chimeric enzyme according to the invention comprises or consists of:

- a nucleic acid molecule encoding at least one catalytic domain of a RNA triphosphatase,

- a nucleic acid molecule encoding at least one catalytic domain of a guanylyltransferase ,

- a nucleic acid molecule encoding at least one catalytic domain of a N^7-guanine methyltransferase; and

- a nucleic acid molecule encoding at least one catalytic domain of a DNA-dependant RNA polymerase.

In particular, the nucleic acid molecule according to the invention can be operatively linked to at least one, preferably the whole promoter(s) chosen from the group consisting of:

- a promoter for an eukaryotic DNA dependant RNA polymerase, preferably for RNA polymerase II; and

- a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

The link of the nucleic acid to a promoter for a eukaryotic DNA dependant RNA polymerase, preferably for RNA polymerase II has notably the advantage that when the chimeric enzyme of the invention is expressed in an eukaryotic host cell, the expression of the chimeric enzymes is driven by the eukaryotic RNA polymerase, preferably the RNA polymerase II. These chimeric enzymes, in turn, can initiate transcription of the transgene. If tissue-specific RNA polymerase II promoters are used, the chimeric enzyme of the invention can be selectively expressed in the targeted tissues/cells.

Said promoter can be a constitutive promoter or an inducible promoter well known by one skilled in the art. The promoter can be developmentally regulated, inducible or tissue specific.

The invention also relates to a vector comprising a nucleic acid molecule according to the invention. Said vector can be appropriated for semi-stable or stable expression.

The invention also relates to a group of vectors comprising said group of isolated nucleic acid molecules according to the invention.

Particularly said vector according to the invention is a cloning or an expression vector.

The vectors can be viral vectors such as bacteriophages, or non-viral, such as plasmid vectors.

In one embodiment, said vector according to the invention is a bicistronic vector, in particular comprising a nucleic acid molecule according to the invention and a promoter for said catalytic domain of a DNA-dependant RNA polymerase and/or at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

Said vector according to the invention can also comprise a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

The invention also relates to a host cell comprising an isolated nucleic acid molecule according to the invention or a group of isolated nucleic acid molecules according to the invention or a vector according to the invention or a group of vectors according to the invention.

The host cell according to the invention can be useful for large-scale protein production.

Preferably, said catalytic domains of the DNA-polymerase RNA polymerase chimeric enzyme according to the invention are from different enzymes than those of the host cell to prevent the competition between the endogenous gene transcription and the transgene transcription.

The invention also relates to a genetically engineered non-human eukaryotic organism, which expresses a chimeric enzyme according to invention. Said non-human eukaryotic organism can be any non-human animals, plants.

The invention also relates to the use, particularly *in vitro* or *ex vivo*, of a chimeric enzyme according to the invention or an isolated nucleic acid molecule according to the invention or a group of isolated nucleic acid molecules according to the invention, for the production of RNA molecule with 5'-terminal m^7GpppN cap.

The invention also relates to the *in vitro* or *ex vivo* use of a chimeric enzyme according to the invention or an isolated nucleic acid molecule according to the invention or a group of isolated nucleic acid molecules according to the invention for the production of protein, in particular protein of therapeutic interest like antibody, particularly in eukaryotic systems, such as in vitro synthesized protein assay or cultured cells.

The invention also relates to a method, particularly *in vitro* or *ex vivo*, for producing a RNA molecule with 5'-terminal m^7GpppN cap encoded by a DNA sequence, in a host cell, said method comprising the step of expressing in the host cell a nucleic acid molecule or a group of

nucleic acid molecules according to the invention, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase, particularly said promoter being effective in said host cell.

Preferably, said catalytic domain of the DNA-dependent RNA-polymerase of the chimeric enzyme according to the invention is from different enzymes than those of the host cell to prevent the competition between the endogenous gene transcription and said DNA sequence transcription.

In particular, said method according to the invention can further comprise the step of introducing in the host cell said DNA sequence and/or the nucleic acid according to the invention, using well-known methods by one skilled in the art like by transfection using calcium phosphate, by electroporation or by mixing a cationic lipid with DNA to produce liposomes.

In one embodiment, said method according to the invention further comprises the step of inhibiting, in particular silencing (preferably by siRNA) the cellular transcription and post-transcriptional machineries of said host cell.

In one embodiment, said method according to the invention further comprises the step of inhibiting the expression of at least one of the subunits of the endogenous DNA-dependent RNA polymerase and/or of the endogenous capping enzyme in said host cell.

Said additional steps (i.e. inhibiting, in particular silencing (preferably by siRNA or shRNA) the cellular transcription and post-transcriptional machineries of said host cell and/or inhibiting the expression of one or several subunits of the endogenous DNA-dependent RNA polymerase and/or of the endogenous capping enzyme in said host cell) allow the optimization of RNA molecules with 5'-terminal m^7GpppN caps synthesis.

As used herein the term "endogenous DNA-dependent RNA polymerase" relates to the endogenous DNA-dependent RNA polymerase of said host cell. When the host cell is a eukaryotic cell, said endogenous DNA-dependent RNA polymerase is the RNA polymerase II.

As used herein the term "endogenous capping enzyme" refers to the endogenous capping enzyme of said host cell.

As used herein the term "inhibiting the expression of a protein" relates to a decrease of at least 20%, particularly at least 35%, at least 50% and more particularly at least 65%, at least 80%, at least 90% of expression of said protein. Inhibition of protein expression can be determined by techniques well known to one skilled in the art, including but not limiting to Northern-Blot, Western-Blot, RT-PCR.

The step of inhibiting the expression of the endogenous DNA-dependent RNA polymerase and/or the endogenous capping enzyme in said host cell can be implemented by any techniques well known to one skilled in the art, including but not limiting to siRNA (small interfering RNA)

techniques that target said endogenous DNA-dependent RNA polymerase and/or the endogenous capping enzyme, anti-sens RNA techniques that target said endogenous DNA-dependent RNA polymerase and/or the endogenous capping enzyme, shRNA (short hairpin RNA) techniques that target said endogenous DNA-dependent RNA polymerase and/or the endogenous capping enzyme.

In addition to siRNA (or shRNA; short hairpin RNA), other inhibitory sequences might be also considered for the same purpose including DNA or RNA antisense (Liu and Carmichael 1994; Dias and Stein 2002), hammerhead ribozyme (Salehi-Ashtiani and Szostak 2001), hairpin ribozyme (Lian, De Young et al. 1999) or chimeric snRNA U1-antisense targeting sequence (Fortes, Cuevas et al. 2003). In addition, other cellular target genes might be considered for inhibition, including other genes involved in the cellular transcription (e.g. other subunits of the RNA polymerase II or transcription factors), post-transcriptional processing (e.g. other subunit of the capping enzyme, as well as polyadenylation or spliceosome factors), and mRNA nuclear export pathway.

In one embodiment of the method according to the invention, said RNA molecule can encode a polypeptide of therapeutic interest.

In another embodiment, said RNA molecule can be a non-coding RNA molecule chosen in the group comprising siRNA, ribozyme, shRNA and antisense RNA. In particular, said DNA sequence can encode a RNA molecule chosen in the group consisting of mRNA, non-coding RNA, particularly siRNA, ribozyme, shRNA and antisense RNA.

The invention also relates to the use of a chimeric enzyme according to the invention as a capping enzyme and DNA-dependent RNA polymerase.

The invention also relates to a kit for the production of a RNA molecule with 5'-terminal m^7GpppN cap, comprising at least one chimeric enzyme according to the invention, and/or an isolated nucleic acid molecule and/or a group of nucleic acid molecules according to the invention, and/or a vector according to the invention and/or a group of vectors according to the invention.

In one embodiment the kit of the invention comprises a vector according to the invention and/or a group of vectors according to the invention, wherein said vector(s) comprising:

- a promoter for said catalytic domain of a DNA-dependant RNA polymerase, and/or
- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

The kit according to the invention can further comprise:

- a vector comprising a promoter for said catalytic domain of a DNA-dependant RNA polymerase; and/or

- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

The invention also relates to a chimeric enzyme according to the invention, an isolated nucleic acid molecule according to the invention, a group of nucleic acid molecules according to the invention or a vector according to the invention, for its use as a medicament, in particular for the prevention and/or treatment of human or animal pathologies, preferably by means of gene therapy.

The invention also relates to a pharmaceutical composition comprising a chimeric enzyme according to the invention, and/or an isolated nucleic acid molecule according to the invention and/or a group of nucleic acid molecules according to the invention, and/or a vector according to the invention. Preferably, said pharmaceutical composition according to the invention is formulated in a pharmaceutical acceptable carrier.

Pharmaceutical acceptable carriers are well known by one skilled in the art.

The pharmaceutical composition according to the invention can further comprise:

- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

Such components (in particular chosen in the group consisting of a chimeric enzyme according to the invention, an isolated nucleic acid molecule according to the invention, a group of isolated nucleic acid molecules according to the invention, a vector according to the invention, a group of vector(s) according to the invention and at least one DNA sequence of interest) can be present in the pharmaceutical composition or medicament according to the invention in a therapeutically amount (active and non-toxic amount).

Such therapeutically amount can be determined by one skilled in the art by routine tests including assessment of the effect of administration of said components on the pathologies and/or disorders which are sought to be prevent and/or to be treated by the administration of said pharmaceutical composition or medicament according to the invention.

For example, such tests can be implemented by analyzing both quantitative and qualitative effect of the administration of different amounts of said aforementioned components (in particular chosen in the group consisting of a chimeric enzyme according to the invention, an isolated nucleic acid molecule according to the invention, a group of isolated molecules according to the invention, a vector according to the invention, a group of vectors according to the invention and at least one DNA sequence of interest) on a set of markers (biological and/or clinical) characteristics of said pathologies and/or of said disorders, in particular from a biological sample of a subject.

The invention also relates to a therapeutic method comprising the administration of a chimeric enzyme according to the invention, and/or an isolated nucleic acid molecule according to the invention, and/or a group of nucleic acid molecules according to the invention and/or a vector according to the invention and/or a group of vectors according to the invention in a therapeutically amount to a subject in need thereof. The therapeutic method according to the invention can further comprise the administration of at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase, in a therapeutically amount to a subject in need thereof.

Said chimeric enzyme, nucleic acid molecule and/or said vector according to the invention can be administrated simultaneously, separately or sequentially of said DNA sequence of interest, in particular before said DNA sequence of interest.

The invention also relates to a pharmaceutical composition according to the invention for its use for the prevention and/or treatment of human or animal pathologies, in particular by means of gene therapy.

Said pathologies can be chosen from the group consisting of pathologies, which can be improved by the administration of said at least one DNA sequence of interest.

The invention also relates to the use of a chimeric enzyme according to the invention, and/or an isolated nucleic acid molecule according to the invention, and/or a group of nucleic acid molecules according to the invention and/or a vector according to the invention, and/or a group of vectors according to the invention for the preparation of a medicament for the prevention and/or treatment of human or animal pathologies, in particular by means of gene therapy.

The invention also relates to a combination product, which comprises:

- at least one enzyme according to the invention and/or at least one nucleic acid molecule according to the invention and/or a group of nucleic acid molecules according to the invention and/or a at least one vector comprising and/or expressing a nucleic acid molecule according to the invention and/or a group of nucleic acid molecules according to the invention; and
- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase;

for simultaneous, separate or sequential administration.

Said DNA sequence of interest can be an anti-oncogene (a tumor suppressor gene).

Said DNA sequence of interest can encode a polypeptide of therapeutic interest or a non-coding RNA chosen in the group comprising siRNA, ribozyme, shRNA and antisense RNA.

Said polypeptide of therapeutic interest can be selected from, a cytokine, a cell or nuclear receptor, a ligand, a coagulation factor, the CFTR protein, insulin, dystrophin, a growth

hormone, an enzyme, an enzyme inhibitor, a polypeptide which has an antineoplastic effect, a polypeptide which is capable of inhibiting a bacterial, parasitic or viral, in particular HIV, infection, an antibody, a toxin, an immunotoxin, a subunit of RNA polymerase II (in particular the Rpb1subunit of RNA polymerase II, which can be inhibited by the alpha-amanitin toxin) and a marker.

Preferably, the combination product according to the invention can be formulated in a pharmaceutical acceptable carrier.

In one embodiment of the combination product according to the invention, said vector is administrated before said DNA sequence of interest.

The invention also relates to a combination product according to the invention, for its use for the prevention and/or treatment of human or animal pathologies, in particular by means of gene therapy.

Said pathologies can be chosen from the group consisting of pathologies, which can be improved by the administration of at least one DNA sequence of interest, as described above.

For example, said pathologies, as well as their clinical, biological or genetic subtypes, can be chosen from the group comprising cancers and their predisposition (especially breast and colorectal cancers, melanoma), malignant hemopathies (in particular leukemias, Hodgkin's and non-Hodgkin's lymphomas, myeloma), coagulation and primary hemostasis disorders, hemoglobinopathies (especially sickle cell anemia and thalassemias), autoimmune disorders (including systemic lupus erythematosus and scleroderma), cardiovascular pathologies (in particular cardiac rhythm and conduction disorders, and hypertrophic cardiomyopathy), metabolic disorders (especially type I and type II diabetes mellitus and their complications, dsylipidemia, atherosclerosis and their complications, mucopolysaccharidoses, glycogen storage diseases, phenylketonuria), infectious disorders (including AIDS, viral hepatitis B, viral hepatitis C, influenza flu and other viral diseases; botulism, tetanus and other bacterial disorders; malaria and other parasitic disorders), muscular disorders (including Duchenne muscular dystrophy and Steinert myotonic muscular dystrophy), respiratory diseases (especially cystic fibrosis and alpha-1 antitrypsin deficiency), renal disease (especially polycystic kidney disease), liver diseases (including cirrhosis, Wilson disease, hepatotoxicity due to the alpha-amanitin, drug-related hepatotoxicity), colorectal disorders (including Crohn's disease and ulcerative colitis), ocular disorders especially retinal diseases (especially Leber's amaurosis, retinitis pigmentosa, age related macular degeneration), central nervous system disorders (especially Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, neurofibromatosis, adrenoleukodystrophy, bipolar disease, schizophrenia and autism), and skin and connective tissue disorders (especially Marfan syndrome and psoriasis).

In one embodiment, the combination product of the invention comprises:

- at least one vector comprising and expressing a nucleic acid molecule according to the invention and/or a group of nucleic acid molecules according to the invention, wherein said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacteriophage DNA-dependant RNA polymerase, particularly of a T7 bacteriophage DNA-dependant RNA polymerase; and
- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a bacteriophage DNA-dependant RNA polymerase, particularly of a T7 bacteriophage DNA-dependant RNA polymerase, wherein said DNA sequence of interest encodes the Rpb1 subunit of RNA polymerase II, which can be inhibited by the alpha-amanitin toxin.

The invention also relates to this combination product for its use for the prevention and/or treatment of human or animal hepatotoxicity due to the alpha-amanitin, by means of gene therapy.

The invention also relates to a method for producing the chimeric enzyme according to the invention comprising the step of expressing in at least one host cell said nucleic acid molecule or said group of nucleic acid molecules encoding the chimeric enzyme of the invention in conditions allowing the expression of said nucleic acid molecule(s) in said host cell.

The invention also relates to a method for producing the chimeric enzyme according to the invention comprising the steps of:

- expressing a part of said group of nucleic acid molecules encoding a chimeric enzyme of the invention in a first host cell in conditions allowing the expression of said nucleic acid molecules in said host cell, to obtain a first part of the chimeric enzyme of the invention;
- expressing the other part of said group of nucleic acid molecules encoding the chimeric enzyme of the invention in a second host cell in conditions allowing the expression of said nucleic acid molecules in said host cell to obtain a second part of the chimeric enzyme of the invention; and
- assembling said first part and said second part to obtain the chimeric enzyme of the invention.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 represents a firefly luciferase gene reporter expression assay, which was used to monitor the translation yields triggered by a chimeric enzyme according to the invention the NP868R-T7RNAP. The pNP868R-T7RNAP or the pT7RNAP plasmids were co-transfected with the pT7p-Luciferase plasmid in the human HEK-293 cultured cells. The expression of NP868R-

T7RNAP and T7RNAP enzymes is driven by the RNA polymerase II-dependent CMV promoter of the corresponding plasmids. The NP868R-T7RNAP and T7RNAP enzymes, in turn, are expected to initiate transcription at the T7 promoter of the pT7p-Luciferase gene reporter plasmid. If both the mRNA capping and DNA-dependent RNAP enzymatic activities of the NP868R-T7RNAP enzymes are retained, luciferase mRNA having a m^7GpppGm cap structures are to be synthesized, which can be translated into firefly luciferase protein and detected by cell luminescence assay. In contrast, the T7RNAP enzyme is expected to synthesize RNA molecules without 5'-terminal m^7GpppN cap, which are therefore poorly translated.

Figure 2 (A–B) represents the physical maps of the pNP868R-T7RNAP and pT7RNAP plasmids. Physical maps of *(A)* pT7RNAP plasmid, which encodes for the wild-type phage DNA-dependent T7 RNA polymerase, *(B)* pNP868R-T7RNAP plasmids, which encodes for a fusion between the NP868R mRNA capping enzymes (African Swine Fever Virus) and the wild-type phage DNA-dependent T7 RNA polymerase (bacteriophage T7), via a flexible $(Gly_3Ser)_4$ linker. These two plasmids have the same design: CMV promoter, Kozak sequence followed by the NP868R-T7RNAP or T7RNAP open-reading frames (ORFs), poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal.

Figure 3 (A-C) represents the physical maps of the firefly luciferase gene reporter plasmids. *(A)* pT7p-Luciferase: was designed to assay the activity of the NP868R-T7RNAP and the T7RNAP enzymes. It consist of an array of RNA polymerase promoters (T7, T3 and SP6 phage RNAP promoters, followed by the E. coli ribosomal rrnD1 promoter), a Lac operator sequence, the entire ORF of the firefly luciferase, a poly[A]-track, a hepatitis-D ribozyme encoding sequence for RNA auto-cleavage and the TΦ terminator in pET-22b(+) backbone, *(B)* BamHI-digested pT7p-Luciferase: in which the physical connection between the luciferase ORF and the promoter array is disrupted by the restriction enzyme digestion. This plasmid is used as a negative control. Arrows indicate the sites of digestion. *(C)* pCMV-Luciferase: in which the entire ORF of the firefly luciferase is under control of the CMV promoter. This plasmid is used as a positive comparator.

Figure 4 (A-C) shows the Firefly luciferase gene reporter expression after plasmid transfection in HEK-293 cells. HEK-293 cells were cultured and transfected as described above. Cells were transfected with either the pNP868R-T7RNAP or the pT7RNAP plasmids (0.4μg DNA/well and 1μL/well Lipofectamine 2000), and/or the pT7p-Luciferase, BamHI-digested pT7p-Luciferase, or pCMV-T7RNAP (0.4μg DNA/well and 1μL/well Lipofectamine 2000), or none. The firefly luciferase luminescence was assayed at selected time points using the Luciferase Assay System (Promega, Madison WI USA). To normalize for transfection efficiency, cells were also transfected with the pORF-eSEAP plasmid, which encodes for the

secreted placental alkaline phosphatase (SEAP) that was assayed in cell culture medium using the Quanti-Blue colorimetric enzyme assay kit (InvivoGen, San Diego, CA). Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU; relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Two independent repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM). Statistical analyses were performed using Student's t two-tailed test. *(A)* Firefly luciferase gene reporter expression in pNP868R-T7RNAP/pT7p-Luciferase, pT7RNAP/pT7p-Luciferase and pCMV-Luciferase transfected cells. Cells transfected with the pNP868R-T7RNAP/pT7p-Luciferase and the pCMV-Luciferase plasmids display 23- and 33-fold fold higher signal than the cells co-transfected with pT7RNAP/pT7p-Luciferase, respectively (*$p<0.05$). *(B)* Firefly luciferase gene reporter expression for cells transfected with pT7RNAP/pT7p-Luciferase, pT7RNAP/BamHI-digested pT7p-Luciferase (*$p<0.05$) and other control conditions (pT7RNAP alone, pT7p-Luciferase digested or not alone, or transfection reagent only). *(C)* Firefly luciferase gene reporter expression for the pNP868R-T7RNAP/pT7p-Luciferase, pNP868R-T7RNAP/BamHI-digested pT7p-Luciferase (*$p<0.05$) and other control conditions (pNP868R-T7RNAP alone, pT7p-Luciferase digested or not alone, or transfection reagent only).

Figure 5 (A-C) represents the Firefly luciferase gene reporter expression of HEK-293 transfected cells treated with α-amanitin. *(A)* Schematic diagram of the assay. For the pCMV-Luciferase plasmid (expression of luciferase is driven by the RNA polymerase II-dependent CMV promoter), α-amanitin was added to cell medium (at 0 or 20µg/ml) simultaneously to cell transfection. For the pNP868R-T7RNAP/pT7p-Luciferase plasmids, a first transfection with the pNP868R-T7RNAP plasmid (expression of NP868R-T7RNAP is driven by the RNA polymerase II-dependent CMV promoter) was performed 24 hours before addition of α-amanitin to the cell medium (at concentrations ranging from 0 to 20µg/ml) and a second transfection with the pT7p-Luciferase plasmid. Two repetitions of these experiments were performed. Errors bars represent standard error of the mean (SEM). Statistical analysis was performed as described above. *(B)* α-amanitin nearly completely abolished luciferase gene reporter expression of cells transfected with the pCMV-Luciferase plasmid; *$p<0.05$) *(C)* α-amanitin triggered only a mild decrease of luciferase expression signal in cells transfected with the pNP868R-T7RNAP/pT7p-Luciferase plasmids (*$p<0.05$), which therefore suggest that the transcription by the NP868R-T7RNAP enzyme is dependent of its phage DNA-dependent T7 RNA polymerase moiety.

Figure 6 (A-C) represents the cell viability, cytotoxicity and apoptosis assays of HEK-293 transfected cells. Cells were cultured and transfected as above with the pNP868R-T7RNAP or pT7RNAP plasmids. Cell viability, cytotoxicity and apoptosis were assessed at selected time

points using the ApoTox-Glo Triplex Assay. Two repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM). Cell viability, cytotoxicity and apoptosis levels were expressed as the luminescence/fluorescence signal in studied cells subtracted for luminescence/fluorescence in untreated cells. Statistical analysis was performed as above. The transfection reagent (i.e. Lipofectamine 2000), with or without plasmid DNA, impairs cell viability, cytotoxicity and apoptosis. However, no statistically significant difference was observed between cells transfected with the pNP868R-T7RNAP plasmid and the pT7RNAP plasmid for *(A)* cell viability levels, at all time points, except at day 1 (two-tailed Student's t test, *$p<0.05$), *(B)* cytotoxicity levels at all time points, or (C) apoptosis levels at all time points.

Figure 7 (A-D) represents the physical maps of the plasmids used for the monomeric NP868R-T3RNAP and NP868R-SP6RNAP chimeric enzymes. Physical maps of: (A) pT3RNAP plasmid, which encodes for the phage DNA-dependent T3 RNA polymerase, (B) pSP6 RNAP plasmid, which encodes for the phage DNA-dependent SP6 RNA polymerase, (C) pNP868R-T3RNAP plasmid, which encodes for a fusion between the NP868R African Swine Fever Virus mRNA capping enzyme and the phage DNA-dependent T3 RNA polymerase, via the flexible $(Gly_3Ser)_4$ linker, (D) pNP868R-SP6RNAP plasmid, which encodes for a fusion between the NP868R African Swine Fever Virus mRNA capping enzyme and the phage DNA-dependent SP6 RNA polymerase, via the flexible $(Gly_3Ser)_4$ linker. These two plasmids have the same design: CMV promoter, Kozak sequence followed by the ORFs, poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal.

Figure 8 represents the expression of the luciferase reporter gene by the monomeric NP868R-T3RNAP and NP868R-SP6RNAP chimeric enzymes. Transfection and luciferase assay were performed as previously described. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Four repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

Figure 9 (A-C) represents the schematic structure of heterodimeric and heterotrimeric chimeric enzymes. (A) Heterodimeric RR_{1234}L-NP868R/EE_{1234}L-T7RNAP enzyme. The leucine zippers EE_{1234}L (acid) and RR_{1234}L (basic) were added to the amino-terminal ends of NP868R and T7 RNA polymerase, respectively, which interact to form an heterodimer; (B) Heterodimeric D12L/D1R-T7RNAP CCPP enzyme. The D1R subunit of the vaccinia virus mRNA capping enzyme is fused to the amino-terminal end of the single-unit T7 RNA polymerase. When co-expressed with the D12L subunit of the mRNA capping enzyme, D1R-T7RNAP forms a heterodimer designated D12L/D1R-T7RNAP. For simplification, the other construction, i.e.

D1R/D12L-T7RNAP chimeric enzyme, is not shown; (C) Heterotrimeric D12L/RR1234L-D1R/EE1234L-T7RNAP chimeric enzyme. The leucine zippers EE1234L (acid) and RR1234L (basic) were added to the amino-terminal ends of D1R and T7 RNA polymerase, respectively. The co-expression of RR1234L-D1R together with EE1234L-T7RNAP and the D12L subunit of the vaccinia virus mRNA capping enzyme form a heterotrimer. For simplification, the other construction, i.e. D1R/RR1234L-D12L/EE1234L-T7RNAP chimeric enzyme, is not shown. Open arrows indicate leucine-zipper binding in antiparallel orientation. Black arrows indicate other types of protein interaction.

Figure 10 (A-H) represents physical maps of the plasmids used for heterodimeric and heterotrimeric chimeric enzymes. Physical maps of (A) pD1R plasmid, which encodes for D1R, the large subunit of the vaccinia mRNA capping enzyme, (B) pD12L plasmid, which encodes for the D12L, the small subunit of the vaccinia mRNA capping enzyme, (C) pRR1234L-NP868R plasmid, which encodes for the RR1234L leucine-zipper fused to the amino-terminal end of NP868R, the African Swine Fever Virus mRNA capping enzyme, (D) pRR1234L-D1R plasmid, which encodes for the RR1234L leucine-zipper fused to the amino-terminal end of D1R, the large subunit of the vaccinia mRNA capping enzyme, (E) pRR1234L-D12L plasmid, which encodes for the RR1234L leucine-zipper fused to the amino-terminal end of D12L, the small subunit of the vaccinia mRNA capping enzyme, (F) pEE1234L-T7RNA plasmid, which encodes for the pEE1234L leucine-zipper fused to the phage DNA-dependent T7 RNA polymerase, (G) pD1R-T7RNAP plasmid, which encodes for a fusion between the large subunit of the vaccinia mRNA capping enzyme and the phage DNA-dependent T7 RNA polymerase, via the flexible $(Gly_3Ser)_4$ linker, (H) pD12L-T7RNAP plasmid, which encodes for a fusion between the small subunit of the vaccinia mRNA capping enzyme and the phage DNA-dependent T7 RNA polymerase, via the flexible $(Gly_3Ser)_4$ linker. All these plasmids have the same design: CMV promoter, Kozak sequence followed by the ORFs, poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal.

Figure 11 represents the expression of the luciferase reporter gene by the heterodimeric RR1234L-NP868R/EE1234L-T7RNAP chimeric enzymes. HEK-293 cells were cultured and transfected as described above. Cells were transfected with either the corresponding plasmids (0.4μg DNA/well and 1μL/well lipofectamine 2000) and pT7p-Luciferase, or pCMV-T7RNAP (0.4μg DNA/well and 1μL/well lipofectamine 2000). Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Four repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

Figure 12 represents the expression of the luciferase reporter gene by the heterodimeric D1R/D12L-T7RNAP and D12L/D1R-T7RNAP chimeric enzymes. Transfection and luciferase assay were performed as previously described. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Two repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

Figure 13 represents the expression of the luciferase reporter gene by the heterotrimeric D1R/RR1234L-D12L/EE1234L-T7RNAP and D12L/RR1234L-D1R/EE1234L-T7RNAP chimeric enzymes. Transfection and luciferase assay were performed as previously described. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Two repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

Figure 14 represents the expression of the luciferase reporter gene by the monomeric NP868R-SP6RNAP chimeric enzymes in presence of siRNAs targeting the large subunit of RNA polymerase II (POL2AR) or the human capping enzyme (RNGTT). Transfection and luciferase assay were performed as previously described, except that siRNA at 25 nM final concentration were added to the transfection reagent. The siRNA SI04364381, SI04369344, SI04250162 and SI04354420 target the POLR2A gene, whereas the siRNA SI00055986, SI03021508, SI00055972, and SI00055979 target RNGTT. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Two repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

Figure 15 represents the dose effect activity of siRNAs targeting the large subunit of RNA polymerase II (POL2AR) and the human capping enzyme (RNGTT). Transfection and luciferase assay were performed as previously described, except that siRNA were added at concentration ranging from 0 to 100 nM to the transfection reagent. The siRNA SI04369344 and SI00055972 target the POLR2A and RNGTT genes, respectively. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Three repetitions of this experiment were performed. Errors bars represent standard error of the mean (SEM).

The present invention will be explained in detail with examples in the following, but the technical scope of the present invention is not limited to these examples.

EXAMPLES

EXAMPLE 1 – EXAMPLE OF ACTIVE MONOMERIC CHIMERIC ENZYME NP868R-T7RNAP

I. Plasmids

One plasmid has been synthesized, which encode for fusions between NP868R, the mRNA capping enzyme of the African Swine Fever Virus, and the wild-type phage DNA-dependent RNA polymerase of the bacteriophage T7. The capping enzyme was fused to the amino-terminal end of the T7 RNA polymerase via a $(Gly_3Ser)_4$ linker.

The pNP868R-T7RNAP plasmid was used to assess the activity of the encoded enzyme by a firefly luciferase gene reporter expression assay (Figure 1). In brief, pNP868R-T7RNAP and pT7p-Luciferase plasmids were co-transfected in the human HEK-293 cultured cells. The expression of the NP868R-T7RNAP enzyme is driven by the RNA polymerase II-dependent CMV promoter of the corresponding plasmid. NP868R-T7RNAP enzyme, in turn, is expected to initiate the transcription of the pT7p-Luciferase plasmid at its T7 promoter. If both the mRNA capping and DNA-dependent RNAP enzymatic activities of the NP868R-T7RNAP enzyme are retained, luciferase mRNA having $m^7GpppGm$ cap structures are to be synthesized, which in turn can be translated into firefly luciferase protein and detected by cell luminescence assay. In addition, the pNP868R-T7RNAP plasmid was used to investigate the cellular distribution of the enzyme, as well as the cell viability, cytotoxicity and apoptosis related with the expression of the enzyme in the HEK-293 cells.

The plasmid encoding for the NP868R-T7RNAP and T7RNAP (T7 RNA polymerase) enzymes were synthesized in four steps by GeneArt AG (Regensburg, Germany). The protein sequence encoded by pT7RNA plasmid corresponds to SEQ ID N°19. The protein sequence encoded by pNP868R-T7RNAP plasmid corresponds to SEQ ID N°20. Firstly, a DNA fragment containing the T7 RNA polymerase promoter and the multiple cloning site (MCS) was removed from the pCMV-Script plasmid (Stratagene, La Jolla, CA USA). Secondly, a cassette was introduced in the pCMV-Script plasmid between its CMV promoter and its SV40 polyadenylation signal. This cassette consisted of the Lac operator stem-loop (Gilbert and Maxam 1973), a MCS, a poly[A]-tract, and a TΦ class-I hairpin terminator signal (Lyakhov, He et al. 1997). Thirdly, the Kozak consensus sequence for initiation of translation (Kozak 1987), followed by the entire open-reading frame (ORF) of the NP868R-T7RNAP or T7RNAP enzymes were assembled from synthetic oligonucleotides using a PCR-based method, cloned

and fully sequence verified. The ORF of the NP868R-T7RNAP (SEQ ID N°21) and of the ORF of the T7RNAP (SEQ ID N°22) were optimized by altering for preferred codon usage, removing of cis-acting elements such as cryptic splice sites and poly(A) signals, as well as improving mRNA stability by removal of direct repeats and secondary structure elements. Fourthly, the entire ORFs of each NP868R-T7RNAP or T7RNAP were subcloned in the MCS of the cassette, resulting in the pNP868R-T7RNAP plasmid and the pT7RNAP plasmid. As a consequence of the construction strategy, two additional amino-acids (Glu, Phe) were added immediately downstream to the ATG of the Kozak sequence, two other were added immediately upstream to the $(Gly_3Ser)_4$ linker (Gly, Pro), and two immediately downstream to this linker (Leu, Glu) of the NP868R-T7RNAP enzyme. Finally, the pNP868R-T7RNAP and pT7RNAP plasmids had the following design (Figures 2A and 2B): CMV promoter, Kozak sequence followed by the NP868R-T7RNAP or T7RNAP ORFs, poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal.

Two plasmids encoding for the firefly luciferase reporter gene were synthesized by Eurofins/MWG/Operon (Ebersberg, Germany). The pET-22b(+)RNAPp-Luciferase plasmid (named pT7p-Luciferase thereafter) was designed to assay the activity of the chimeric enzyme according to the invention. A test sequence was introduced in the pET-22b(+) backbone (Novagen, San Diego, CA USA), which consisted of an array of RNA polymerase promoters (T7, T3 and SP6 phage RNAP promoters, followed by the E. coli ribosomal rrnD1 promoter), a Lac operator stem-loop sequence, the entire ORF of the firefly luciferase, a poly[A]-track, a hepatitis-D ribozyme encoding sequence for RNA auto-cleavage (Conzelmann and Schnell 1994; Garcin, Pelet et al. 1995; Bridgen and Elliott 1996; Schurer, Lang et al. 2002; Walker, Avis et al. 2003) and the TΦ terminator for phage RNA polymerase transcription (Figure 3A). A BamHI-digested version of the pT7p-Luciferase plasmid, which disrupts the physical connection between the luciferase ORF and the T7 promoter, was also used as negative control (Figure 3B). Moreover, the pCMV-Luciferase plasmid, which was used as an active comparator, contained the firefly luciferase downstream to the RNA polymerase II-dependent CMV promoter of the pCMV-Script plasmid (Figure 3C).

II. Cell culture and transfection

The Human Embryonic Kidney 293 cells (HEK-293, ATCC CRL 1573) were grown at 37°C with 5% CO_2. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 3.97mM L-alanyl-L-glutamine (substituted on a molar equivalent basis for L-glutamine), 10% fetal bovine serum (FBS), 1% non-essential amino-acids, 1% penicillin and streptomycin, and 0.2% fungizone.

The day before transfection, HEK-293 cells were plated in 24 well plates at densities of approximately 8 x 10^4 cells per well. One hour prior to transfection, the medium was changed to fresh complete medium without antibiotics. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA USA) according to manufacturer's recommendations. In brief, plasmid DNA diluted in Opti-MEM I reduced serum medium (Invitrogen, Carlsbad, CA USA) and mixed with Lipofectamine 2000, were added to the cell medium. Following transfection, cells were incubated up to 120 hours prior to testing for luciferase and SEAP gene reporter expression.

Cells were co-transfected with the pT7RNAP or pNP868R-T7RNAP (0.4μg DNA/well and 1μL/well Lipofectamine 2000), together with the pT7p-Luciferase reporter plasmid (0.4μg DNA/well and 1μL/well Lipofectamine 2000). A series of other transfection conditions were used as negative controls and included: *(a)* the same co-transfection as before, except that the pT7p-Luciferase was digested by the BamHI restriction enzyme, which disrupts the physical connection between the luciferase ORF and the T7 promoter, *(b)* the pNP868R-T7RNAP or pT7RNAP plasmids alone, *(c)* the pT7p-Luciferase reporter plasmid digested or not alone, (d) the transfection reagent alone (i.e. Lipofectamine 2000). Cells were also transfected with the pORF-eSEAP plasmid (InvivoGen, San Diego, CA; used to normalize for transfection efficacy), as well as with the pCMV-T7RNAP plasmid (used as an active comparator).

III. <u>Firefly luciferase luminescence and SEAP colorimetric assays</u>

The firefly luciferase luminescence was assayed with the Luciferase Assay System according to manufacturer's recommendations (Promega, Madison WI USA). In brief, HEK-293 cells were lysed in Cell Culture Lysis Reagent (CCLR) lysis buffer, and then centrifuged at 12,000 × g for 2 minutes at 4°C. Luciferase Assay Reagent (Promega; 100μl/well) was added to supernatant (20μl/well). Luminescence readout was taken on a luminometer reader (Fluostar; BMG Labtech, Offenburg Germany) according to the manufacturer's instructions.

The expression of pORF-eSEAP plasmid was used to normalize for transfection efficiency. This plasmid encodes for the secreted placental alkaline phosphatase (SEAP), which was assayed for enzymatic activity in cell culture medium using the Quanti-Blue colorimetric enzyme assay kit (InvivoGen, San Diego, CA) at selected time points. Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU; relative light units), then divided by SEAP absorbance to normalize for transfection efficacy (OD, optic density) ratio.

IV. Statistical analysis

All statistical analyses were performed using Student's t two-tailed test adjusted by Holm–Bonferroni correction for multiple testing, if appropriate. A p-value of less than 0.05 was regarded as being significant.

V. Gene reporter expression assay

A firefly luciferase reporter luminescence assay was used to assess the translatability of mRNA generated by the chimeric enzyme according to the invention or T7RNAP enzyme. The co-transfection of the pT7RNAP and pT7p-Luciferase plasmids triggered low but detectable luciferase expression signal in comparison to cells co-transfected with the pT7RNAP/BamHI-digested version of the pT7p-Luciferase plasmid (which therefore demonstrate that luciferase gene reporter expression is driven by the phage T7 promoter;Figures 4A and 4B). This is in agreement with previously published reports, which have shown that the T7RNAP expressed in eukaryotic cells can synthesize RNA molecules which are poorly translated because of their absence of 5'-capping (Fuerst, Niles et al. 1986; Chen, Li et al. 1994). A drastic reduction of the firefly luciferase gene expression signal was also observed when the transfection was performed without the pT7RNAP plasmid (which confirm that luciferase expression require the presence of T7RNAP) or the pT7p-Luciferase plasmid (which confirm the specificity of the luminescence signal), or both.

The pNP868R-T7RNAP plasmid was cotransfected with the pT7p-Luciferase plasmid and tested under same conditions as above. At peak, approximately 23-fold higher luciferase expression signal was observed with pNP868R-T7RNAP/pT7p-Luciferase than with the pT7RNAP/pT7p-Luciferase plasmids (Figure 4A). The specificity of the above findings was confirmed by the co-transfection of BamHI-digested version of the pT7p-Luciferase plasmid, as well as the transfection by pNP868R-T7RNAP or pT7p-Luciferase plasmids digested or not alone, which gave drastically reduced luciferase expression signal (Figure 4C). At peak, co-transfection of pNP868R-T7RNAP/pT7p-Luciferase plasmids gave 72% of the luciferase expression signal to that of pCMV-T7RNAP plasmid (Figure 4A).

In summary, the activity of the chimeric NP868R-T7RNAP enzyme according to the invention encoded by the pNP868R-T7RNAP plasmid has been demonstrated using a firefly luciferase reporter luminescence assay. The specificity of the present findings is supported by a series of controls, which suggest that both the mRNA capping and DNA-dependent RNA polymerase enzymatic activities of the NP868R-T7RNAP enzyme are retained when expressed in HEK-293 cells.

VI. Gene reporter expression assay in alpha-amanitin treated cells

To further demonstrate that the transcription by pNP868R-T7RNAP is dependent of its phage DNA-dependent T7 RNA polymerase moiety, gene transfection assays were also performed in α-amanitin treated cells. Alpha-amanitin is a specific inhibitor of the nuclear RNA polymerase II (Jacob, Sajdel et al. 1970; Kedinger, Gniazdowski et al. 1970; Lindell, Weinberg et al. 1970), which binds its Rpb1 subunit (Bushnell, Cramer et al. 2002). In contrast, alpha-amanitin has no effect on transcription by the phage T7 RNA polymerase which was used to engineer the NP868R-T7RNAP chimeric enzyme according to the invention (Kupper, McAllister et al. 1973; Engleka, Lewis et al. 1998).

To initiate the expression of the NP868R-T7RNAP enzyme, which is driven by the RNA polymerase II-dependent CMV promoter, cells were transfected with the pNP868R-T7RNAP 24 hours before addition of α-amanitin to cell medium (at concentrations ranging from 0 to 20µg/ml) and a second transfection with the pT7p-Luciferase plasmid (Figure 5A). For the pCMV-Luciferase plasmid, cells were simultaneously transfected and treated with α-amanitin (at 0 or 20µg/ml; Figure 5A). Gene reporter expression was expressed as the luciferase luminescence in studied cells subtracted for luminescence in cells treated with the transfection reagent only (RLU; relative light units), then divided by SEAP absorbance to normalize for transfection efficacy (OD, optic density) ratio.

As expected, α-amanitin nearly completely abolished firefly luciferase gene reporter expression of pCMV-Luciferase transfected cells (Figure 5B). In contrast, only a mild decrease of luciferase expression was triggered by α-amanitin at all concentrations in pNP868R-T7RNAP/pT7p-Luciferase transfected cells (Figure 5C).

The present findings, therefore confirms that the transcription by NP868R-T7RNAP enzyme depends of the enzymatic activity of its phage T7 DNA-dependent RNA polymerase moiety.

VII. Immunofluorescence

The subcellular distribution of the NP868R-T7RNAP enzyme was investigated by indirect immunofluorescence. HEK-293 cells were plated in 24 well plates at 8 x 10^4 cells/well, on poly-L-lysine coated coverslips (BD BioCoat; Bioscience, Mississauga, ON USA), then transfected as previously described. Six and 24-hours after transfection, cells were washed in phosphate buffered saline (PBS), and then fixed in 4% paraformaldehyde for 15 minutes. After fixation, cells were washed with PBS, and then permeabilised for 30 minutes in PBS containing 5% goat serum (Invitrogen), 0.1% Triton X-100 and 0.02% sodium azide. Cells were incubated overnight

at 4°C with the mouse monoclonal antibody raised against T7 RNA Polymerase (1:200, Novagen). After extensive washing with PBS, cells were incubated for 3 hours at room temperature with fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse IgG (Sigma-Aldrich). Cell nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI) for 5 minutes. Cells were then washed and mounted in the anti-fade medium Mowiol 4-88 (Calbiochem, Gibbstown, NJ USA). Cells were analyzed by using an epifluorescence microscope with appropriate filters.

As expected, a weak but detectable FITC signal was observed at both 6 and 24-hours in the cytoplasm of cells transfected with the pNP868R-T7RNAP plasmid, while their nuclei were stained by DAPI.

VIII. Cell viability, cytotoxicity and apoptosis assays

The ApoTox-Glo Triplex Assay (Promega, Madison WI) was used to investigate whether the expression of the NP868R-T7RNAP enzyme impair viability, or induce toxicity or apoptosis of transfected cells. Two protease activities were assayed by fluorescence: one is a marker of cell viability (i.e. the peptide substrate GF-AFC), and the other is a marker of cytotoxicity (i.e. the peptide substrate bis-AAF-R110). Apoptosis was assayed by the luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity.

Cell culture and transfections were performed as previously, except that HEK-293 cells were plated in 96-well plates at densities ranging of 1.2 x 10^4 cells per well. Cells were transfected with the pNP868R-T7RNAP plasmid, the pT7RNAP plasmid, or the transfection reagent only. ApoTox-Glo Triplex Assay was performed according to manufacturer's recommendations. In brief, at selected time points, the viability/cytotoxicity reagent containing both GF-AFC Substrate and bis-AAF-R110 substrates were added to the wells and incubated for 30 minutes at 37°C, before fluorescence assessment at two different wavelength sets for viability and cytotoxicity. The caspase reagent was then added to all wells, and luminescence was measured after 30 minutes incubation at room temperature. Statistical analysis was performed as above. Cell viability, cytotoxicity and apoptosis levels were expressed as the luminescence/fluorescence signal in studied cells subtracted for luminescence/fluorescence in untreated cells.

As previously reported (Patil, Rhodes et al. 2004), the cell viability, cytotoxicity and apoptosis were significantly impaired in cells treated with the transfection reagent (i.e. Lipofectamine 2000) as compared to untreated cells (Figures 6A, 6B and 6C). As also expected, cell viability, cytotoxicity and apoptosis were generally more impaired when plasmid DNA were

added to transfection mixture (Figures 6A, 6B and 6C). However, at all studied time points, the cell viability, cytotoxicity or apoptosis markers of cells transfected with the pNP868R-T7RNAP plasmid were not statistically different to that of pT7RNAP plasmid, except 24 hours after transfection for cell viability only, which is possibly due to hazard only (Figures 6A, 6B and 6C; two-tailed Student's t test for individual time points, P-value<0.05).

In conclusion, no obvious difference in cytotoxicity, cell viability, and apoptosis of the NP868R-T7RNAP enzyme can be demonstrated in comparison to T7RNAP, which has no recognized capping enzymatic activity.

EXAMPLE 2 – EXAMPLES OF ACTIVE MONOMERIC CHIMERIC ENZYMES NP868R-T3RNAP and NP868R-SP6RNAP

Two other types of monomeric chimeric enzymes according to the invention have been generated, which consist of NP868R, the monomeric mRNA capping enzyme of the African Swine Fever Virus, fused to the amino-terminal end of the wild type T3 or SP6 monomeric bacteriophage DNA-dependent RNA polymerases, via the flexible linker $(Gly_3Ser)_4$.

I. Methods

The sequences used to generate said monomeric chimeric enzymes were assembled from synthetic oligonucleotides using a PCR-based method, cloned and fully sequence verified. These sequences were subcloned in the pCMV-Script plasmid containing the subcloning cassette previously described. Finally, all the plasmids used for expression had the similar design: CMV IE1 promoter/enhancer promoter, Kozak sequence followed by the ORFs, poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal (Fiure 7(A-D)).

As a consequence of the subcloning strategy, amino-acids were added immediately downstream to the ATG of the Kozak sequence encoded by the plasmids (Glu-Phe-Leu-Glu for pT3RNAP and pSP6RNAP; Glu-Phe for pNP868R-T3RNAP and pNP868R-SP6RNAP). In addition, two amino-acids were added immediately upstream (Gly-Pro for pNP868R-T3RNAP and pNP868R-SP6RNAP) or downstream to the $(Gly_3Ser)_4$ linker (Leu-Glu for pNP868R-T3RNAP and pNP868R-SP6RNAP).

HEK-293 cells were grown as previously described in 24-wells plates and transfected using the Lipofectamine 2000 reagent, and the appropriate plasmids (0.4µg DNA/well, plus 1µL/well lipofectamine 2000, per transfected plasmid). The firefly luciferase luminescence was assayed as previously described using the pT7p-Luciferase (which also contains both the T3 and SP6 promoters) and the Luciferase Assay System. The expression of pORF-eSEAP plasmid was used

to normalize for transfection efficacy as previously described. Statistical analyses were performed using Student's t two-tailed test adjusted by Holm–Bonferroni correction for multiple testing, if appropriate. A p-value of less than 0.05 was regarded as being statistically significant.

II. Results

As shown in Figure 8, when co-transfected with the reporter pT7p-luciferase plasmid, both pNP868R-T3RNAP and NP868R-SP6RNAP show strong luciferase gene reporter signal, which was 14- and 56-folds higher than pT3RNAP or pSP6RNAP, respectively ($p<0.001$ for each comparisons, Student's t-test). The NP868R-T3RNAP enzyme has 36% activity to that of pCMV-T7RNAP plasmid ($p<0.001$, Student's t-test), whereas or NP868R-SP6RNAP heterodimeric enzyme shows 1.1-fold luciferase reporter gene expression to that of pCMV-T7RNAP plasmid (non-statistically significant difference, Student's t-test).

These results demonstrate the activity of different types of monomeric chimeric enzymes according to the invention.

EXAMPLE 3 – EXAMPLES OF ACTIVE DIMERIC AND TRIMERIC CHIMERIC ENZYMES

Different types of active oligomeric chimeric enzymes according to the invention have been generated as shown in Figure 9:

- one heterodimeric chimeric enzyme, resulting of the non-covalent linkage between the monomeric African Swine Fever Virus mRNA capping enzyme NP868R and the monomeric T7 RNA polymerase, via the $EE_{1234}L$ and $RR_{1234}L$ leucine-zippers, which form the heterodimeric $RR_{1234}L$-pNP868R/$EE_{1234}L$-T7RNAP chimeric enzyme,
- two heterodimeric chimeric enzymes obtained by fusion between each of the two subunits of the vaccinia virus mRNA capping enzyme (i.e. D1R or D12L) with the monomeric T7 RNA polymerase, via the flexible $(Gly_3Ser)_4$ linker. Each fusion proteins are coexpressed with the other subunit of the vaccinia virus mRNA capping enzyme, in order to form the heterodimeric D12L/D1R-T7RNAP and D1R/D12L-T7RNAP chimeric enzymes,
- two heterotrimeric chimeric enzymes, which are generated by fusion of the $EE_{1234}L$ and $RR_{1234}L$ leucine-zippers to the amino-terminal ends of one of the subunits of the vaccinia virus mRNA capping enzyme and the T7 RNA polymerase, respectively. Co-expression of $RR_{1234}L$-D1R or p$RR_{1234}L$-D12L, with the other subunit of the vaccinia virus mRNA capping enzyme, plus $EE_{1234}L$-T7RNAP, form the heterotrimeric D1R/$RR_{1234}L$-D12L/$EE_{1234}L$-T7RNAP and D12L/$RR_{1234}L$-D1R/$EE_{1234}L$-T7RNAP chimeric enzymes.

I. Methods

The sequences used to generate the chimeric enzymes were assembled from synthetic oligonucleotides using a PCR-based method, cloned and fully sequence verified. These sequences were subcloned in the pCMV-Script plasmid with the subcloning cassette previously described. Finally, all the plasmids used for expression had the similar design: CMV IE1 promoter/enhancer promoter, Kozak sequence followed by the open-reading frames (ORFs), poly[A]-track, TΦ terminator for phage RNA polymerase transcription, and SV40 polyadenylation signal (Figure 10 (A-H)).

As a consequence of the subcloning strategy, two amino-acids were added immediately downstream to the ATG of the Kozak sequence of some plasmids (Leu-Glu for pT7RNAP; Glu-Phe for pNP868R, pD1R, pD12L, pD1R-T7RNAP, and pD12L-T7RNAP), immediately downstream to the leucine-zipper sequences (Leu-Glu for pEE1234L-T7RNAP; Glu-Phe for pRR1234L-NP868R, pRR1234L-D1R and pRR1234L-D12L), and at the carboxyl-terminal end of some encoded proteins (Gly-Pro for pNP868R, pRR1234L-NP868R, pD1R, pD12L, pRR1234L-D1R and pRR1234L-D12L). In addition, two amino-acids were added immediately upstream (Gly-Pro for pD1R-T7RNAP and pD12L-T7RNAP) or downstream to the $(Gly_3Ser)_4$ linker (Leu-Glu for pD1R-T7RNAP and pD12L-T7RNAP).

As previously described, the Human Embryonic Kidney 293 cells (HEK-293) were grown in 24-wells plates. HEK-293 cells were transfected using the lipofectamine 2000 reagent, and the appropriate plasmids (0.4μg DNA/well, plus 1μL/well lipofectamine 2000, per transfected plasmid) as previously described. The firefly luciferase luminescence was assayed as previously described using the pET-22b(+)T7RNAPp-Luciferase reporter plasmid (designated pT7p-Luciferase thereafter) and the Luciferase Assay System. The expression of pORF-eSEAP plasmid was used to normalize the transfection efficacy as previously described.

Gene reporter expression was expressed as the luciferase luminescence in studied condition subtracted by the luminescence in cells treated with the transfection reagent only (RLU, relative light units), then divided by SEAP absorbance (OD, optic density) ratio. Statistical analyses were performed using Student's t two-tailed test adjusted by Holm–Bonferroni correction for multiple testing, if appropriate. A p-value of less than 0.05 was regarded as being statistically significant.

II. Results

II.1 Heterodimeric RR1234L-NP868R/EE1234L-T7RNAP chimeric enzyme

The activity of the heterodimeric enzyme RR1234L-NP868R/EE1234L-T7RNA chimeric enzyme (encoded by pRR1234L-pNP868R and pEE1234L-T7RNAP plasmids, respectively) has

been demonstrated. This heterodimeric enzyme is generated by non-covalent linkage between the monomeric African Swine Fever Virus mRNA capping enzyme pNP868R and the monomeric T7 RNA polymerase, via the EE1234L and RR1234L leucine-zippers (Figure 9). The EE1234L (acidic leucine-zipper; LEIEAAFLEQENTALETEVAELEQEVQRLENIVSQYETRYGPLGGGK, one letter amino-acid code) and RR1234L leucine-zippers (basic leucine-zipper with slight modification of the GGGK orientation, which is not involved in leucine-zipper dimerization (Moll, Ruvinov et al. 2001); LEIRAAFLRRRNTALRTRVAELRQRVQRLRNIVSQYETRYGPLGGGK, one letter amino-acid code), which were respectively added to the amino-terminal end of NP868R and T7RNAP, respectively. The RR1234L and EE1234L leucine zippers are dimeric coiled-coil peptide structures consisting of two amphipathic α-helices that preferably melt as heterodimer in antiparallel orientation (Moll, Ruvinov et al. 2001).

As expected, the transfection of the plasmid encoding for the African Swine Fever Virus mRNA capping enzyme alone with or without leucine-zipper sequences (i.e. pRR1234L-NP868R and pNP868R, respectively) do not induce any detectable luciferase reporter gene expression (Figure 11). As also expected, cells transfected with the plasmid encoding for the T7 RNA polymerase with the EE1234L leucine zipper (encoded by pEE1234L-T7RNA) show very similar activity to the T7 RNA polymerase without leucine-zipper (encoded by pT7RNA), which provide further evidence that the native amino-terminal end of the T7 RNA polymerase can be modified without major impairment of its enzymatic processivity.

The HEK293 cells co-transfected with the pRR1234L-NP868R and pEE1234L-T7RNA plasmids (encoding for NP868R and T7RNAP with leucine-zippers), together with the reporter pT7p-luciferase plasmid, show strong luciferase reporter gene expression signal, which is 87% to that of pCMV-T7RNAP plasmid (non-statistically significant difference, Student's t-test; (Figure 11). Furthermore, cells co-transfected in presence of the reporter pT7p-luciferase plasmid with the pRR1234L-NP868R and pEE1234L-T7RNA plasmids, displays 3.7-fold higher luciferase reporter gene expression than cells cotransfected with pNP868R and pT7RNAP (encoding for NP868R and T7RNAP without leucine-zippers; $p<0.05$, Student's t-test).

These results demonstrate the activity of heterodimeric chimeric enzymes according to the invention and that the non-covalent linkage between NP868R and T7RNAP by leucine-zippers increases significantly the expression of the gene reporter driven by said chimeric enzymes.

II.2 Heterodimeric D1R/D12L-T7RNAP and D12L/D1R-T7RNAP chimeric enzymes

The activity of other types of heterodimeric chimeric enzyme has also been demonstrated, using the vaccinia mRNA capping enzyme.

By itself, the vaccinia mRNA capping enzyme is a heterodimer consisting of: (*i*) a 95 kDa subunit encoded by the vaccinia virus D1R gene (genomic sequence ID# NC_006998.1; GeneID# 3707562; UniProtKB/Swiss-Prot ID# YP_232988.1), designated hereafter as D1R, which has RNA-triphosphatase, RNA guanylyltransferase and RNA N7-guanine methyltransferase enzymatic activities (Cong and Shuman 1993; Niles and Christen 1993; Higman and Niles 1994; Mao and Shuman 1994; Gong and Shuman 2003), (*ii*) and a 31-kDa subunit encoded by the vaccinia virus D12L gene (genomic sequence ID# NC_006998.1; GeneID#3707515; UniProtKB/Swiss-Prot ID#YP_232999.1), designated hereafter as D12L, which has no intrinsic enzymatic activity, but enhances drastically the RNA N7-guanine methyltransferase activity of the D1R subunit (Higman, Bourgeois et al. 1992; Higman, Christen et al. 1994; Mao and Shuman 1994).

D1R or D12L were fused to the amino-terminal end of the T7 RNA polymerase, via the $(Gly_3Ser)_4$ linker (encoded by D1R-T7RNAP or D12L-T7RNAP, respectively). When co-expressed, each fusion proteins, together with the other vaccinia mRNA capping enzyme subunit (encoded by pD12L or D1R, respectively), generate two different heterodimeric chimeric enzymes designated as D12L/D1R-T7RNAP and D1R/D12L-T7RNAP, respectively (Figure 9). In presence of the reporter pT7p-luciferase plasmid, the heterodimeric chimeric enzymes generate a strong luciferase gene reporter signal in HEK293 cells (Figure 12). The heterodimeric D12L/D1R-T7RNAP chimeric enzyme has 32% activity to that of pCMV-T7RNAP plasmid ($p<0.01$, Student's t-test), whereas the D1R/D12L-T7RNAP heterodimeric enzyme has 1.5-fold higher luciferase reporter gene expression than the pCMV-T7RNAP plasmid (non-statistically significant difference, Student's t-test). Furthermore, the coexpression of the two subunits of vaccinia mRNA capping enzyme unbound to the T7 RNA polymerase (encoded by pD1R, pD12L and pT7RNAP) shows 9- and 42-fold lower luciferase reporter gene expression signal than the heterodimeric D12L/D1R-T7RNAP and D1R/D12L-T7RNAP chimeric enzymes, respectively ($P<0.05$ for both statistical comparison, Student's t-test).

These results demonstrate the activity of different types of heterodimeric chimeric enzymes according to the invention and that covalent linkage between the subunits of the vaccinia mRNA capping enzyme and the T7RNAP stimulates significantly the gene reporter expression. As also expected, in presence of the reporter pT7p-luciferase plasmid, the expression of D1R and/or D12L without T7 RNA polymerase induces virtually no detectable luciferase expression.

II.3 Heterotrimeric D12L/RR1234L-D1R/EE1234L-T7RNAP and D1R/RR1234L-D12L/EE1234L-T7RNAP chimeric enzymes

The activity of heterotrimeric chimeric enzyme has also been demonstrated.

The basic RR1234L leucine zipper was fused to the amino-terminal ends of either the D1R or D12L subunits of the vaccinia virus mRNA capping enzyme (encoded by pRR1234L-D1R and RR1234L-D12L, respectively), while the complementary acidic EE1234L leucine-zipper was added to the amino-terminal end of T7 RNA polymerase (encoded by the pEE1234L-T7RNA plasmid). The co-expression of pEE1234L-T7RNAP, together with either pRR1234L-D1R or pRR1234L-D12L, plus the other vaccinia mRNA capping enzyme subunit (pD12L and pD1R plasmids, respectively), therefore generate two different heterotrimeric CCPP enzymes, designated as D12L/RR1234L-D1R/EE1234L-T7RNAP and D1R/RR1234L-D12L/EE1234L-T7RNAP, respectively (Figure 9).

The T7 RNA polymerase displayed 7-fold higher luciferase gene reporter signal when coexpressed with the D1R/D12L subunits of the vaccinia virus mRNA capping enzyme than in their absence. These results are therefore in line with those obtained by the vaccinia virus/bacteriophage RNAP hybrid expression system, in which the translatability of uncapped T7 transcripts is increased by the expression of the vaccinia mRNA capping enzyme provided by a recombinant virus (Fuerst, Niles et al. 1986; Fuerst, Earl et al. 1987; Elroy-Stein, Fuerst et al. 1989; Fuerst, Fernandez et al. 1989; Fuerst and Moss 1989; Elroy-Stein and Moss 1990).

A strong luciferase gene reporter signal was shown in HEK-293 cells expressing either the D1R/RR1234L-D12L/EE1234L-T7RNAP or the D12L/RR1234L-D1R/EE1234L-T7RNAP CCPP enzymes, in presence of the reporter pT7p-luciferase plasmid (Figure 13). The heterodimeric D12L/RR1234L-D1R/EE1234L-T7RNAP and D1R/RR1234L-D12L/EE1234L-T7RNAP chimeric enzymes have respectively 57% and 33% activity to that of pCMV-T7RNAP plasmid (non-statistically significant difference, Student's t-test). The heterodimeric D12L/RR1234L-D1R/EE1234L-T7RNAP and D1R/RR1234L-D12L/EE1234L-T7RNAP chimeric enzymes show respectively 11-and 6.7-fold stronger luciferase gene reporter expression signal than cells expressing D1, D12L and T7RNAP without leucine-zippers ($p=0.05$ and non-statistically significant difference, respectively; Student's t-test).

These results demonstrate the activity of heterotrimeric chimeric enzymes according to the invention and that the non-covalent linkage between any of the subunits of the vaccinia mRNA capping enzyme and the T7 RNA polymerase increases significantly the gene reporter expression.

III. Conclusion

These present results show the activity of different types of heterodimeric and heterotrimeric chimeric enzymes according to the invention, generated by covalent or non-covalent linkage.

The present results also provide evidences that covalent or non-covalent linkage between the different catalytic domain of the chimeric enzyme and in particular between capping enzymes and RNA polymerases allows the optimization of the gene reporter expression by the chimeric enzymes.

EXAMPLE 4 – STIMULATION OF LUCIFERASE REPORTER GENE EXPRESSION BY SILENCING SEQUENCES AGAINST THE CELLULAR RNA POLYMERASE II AND CAPPING ENZYME

I. Methods

HEK-293 cells were grown as previously described in 24-wells plates and transfected using the Lipofectamine 2000 reagent, and the appropriate concentration of siRNA (Qiagen; Hilden, Germany) and plasmids (0.4µg DNA/well, plus 1µL/well lipofectamine 2000, per transfected plasmid). The NP868R-SP6 chimeric enzyme, which has strong demonstrated activity, was used in the present experiment. The firefly luciferase luminescence was assayed as previously described using the pT7p-Luciferase (which also contains both the T3 and SP6 promoters) and the Luciferase Assay System. The expression of pORF-eSEAP plasmid was used to normalize for transfection efficacy as previously described.

Four siRNA that target the human POLR2A (NCBI Gene ID# 5430; mRNA sequence ID# NM_000937.4; NCBI protein sequence ID# NP_000928.1) were used: SI04364381 (mRNA sequence 1255-1275: CAGCGGTTGAAGGGCAAGGAA), SI04369344 (mRNA sequence 830-850: ATGCGGAATGGAAGCACGTTA), SI04250162 (mRNA sequence 2539-2559: ATGGTCGTGTCCGGAGCTAAA), and SI04354420 (mRNA sequence 4896-4916: CAGCGGCTTCAGCCCAGGTTA).

In addition, four siRNA that target the human RNGTT (Gene ID# 8732; mRNA sequence ID# NM_003800.3; NCBI protein sequence ID# NP_003791.3) were used: SI00055986 (mRNA sequence 3187- 3207: ATGGATTTAAAGGGCGGCTAA), SI03021508 (mRNA sequence 430-450: TTCAAGGTTCTATGACCGAAA), SI00055972 (mRNA sequence 2530-2550: CAGGGTTGTTAAGTTGTACTA) and SI00055979 (mRNA sequence 4132-4152: TACCATCTGCAGTATTATAAA).

II. Results

In a first series of experiments, the effects of four POLR2A siRNA and four RNGTT siRNA were tested at 25nM final concentration (Figure 14). The siRNA were co-transfected with the pNP868R-SP6RNAP chimeric enzyme plasmid and the reporter pT7p-luciferase plasmid. Collectively, the POLR2A siRNA trend to increase the luciferase gene reporter expression by 127% on average, in comparison the same condition without siRNA. Similarly, the addition of RNGTT siRNA collectively increased the luciferase gene reporter expression to 147% on average in comparison the same condition without siRNA.

The POLR2A SI04369344 and the RNGTT SI00055972 siRNA, which have show the highest stimulation rate, were selected for a second series of experiments. Expression of the luciferase reporter gene driven by NP868R-SP6RNAP was assayed in presence of siRNA at concentrations ranging from 0 to 100 nM (Figure 15). Dose-response was observed with both siRNA. The strongest expression stimulation of 3.8-folds was observed at 100 nM with POLR2A SI04369344, and of 5.1-folds with the RNGTT SI00055972 siRNA at 100 nM.

III. Conclusion

The present findings demonstrate that the silencing of the cellular transcription and post-transcriptional machineries by siRNA stimulate the reporter gene expression driven by the NP868R-SP6RNAP chimeric enzyme.

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CLAIMS

1. A chimeric enzyme comprising:
- at least one catalytic domain of a RNA triphosphatase,
- at least one catalytic domain of a guanylyltransferase,
- at least one catalytic domain of a N^7-guanine methyltransferase, and
- at least one catalytic domain of a DNA-dependant RNA polymerase.

2. The chimeric enzyme according to Claim 1, characterized in that it is a cytoplasmic chimeric enzyme.

3. The chimeric enzyme according to Claim 1 or 2, wherein said catalytic domain of a RNA triphosphatase, said catalytic domain of a guanylyltransferase, said catalytic domain of a N^7-guanine methyltransferase, are included in a monomer.

4. The chimeric enzyme according to any one of Claims 1 to 3, wherein said catalytic domain of a DNA-dependant RNA polymerase is included in a monomer.

5. The chimeric enzyme according to any one of Claims 1 to 4, wherein said catalytic domain of a DNA-dependant RNA polymerase and at least one of the catalytic domain chosen in the group consisting of:
- said catalytic domain of a RNA triphosphatase;
- said catalytic domain of a guanylyltransferase; and
- said catalytic domain of a N^7-guanine methyltransferase;

are included in a monomer.

6. The chimeric enzyme according to any one of Claims 1 to 5, wherein said catalytic domain of a DNA-dependant RNA polymerase is a catalytic domain of a bacteriophage DNA-dependant RNA polymerase.

7. The chimeric enzyme according to any one of Claims 1 to 6, wherein at least one of said catalytic domain chosen in the group consisting of:
- said catalytic domain of a RNA triphosphatase;
- said catalytic domain of a guanylyltransferase; and

- said catalytic domain of a N^7-guanine methyltransferase;

is a catalytic domain of a virus capping enzyme.

8. The chimeric enzyme according to any one of Claims 1 to 7, characterized in that it is a monomeric enzyme.

9. The chimeric enzyme according to any one of Claims 1 to 8, wherein at least two of said catalytic domains chosen in the group consisting of:

- a catalytic domain of a RNA triphosphatase,
- a catalytic domain of a guanylyltransferase,
- a catalytic domain of a N^7-guanine methyltransferase, and
- a catalytic domain of a DNA-dependant RNA polymerase.

are bound by a linking peptide.

10. An isolated nucleic acid molecule or a group of isolated nucleic acid molecules, said nucleic acid molecule(s) encoding a chimeric enzyme according to any one of Claims 1 to 9.

11. The nucleic acid molecule according to Claim 10, which is operatively linked to at least one promoter chosen from the group consisting of:

- a promoter for RNA polymerase II; and
- a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

12. A vector comprising a nucleic acid molecule according to Claim 10 or 11.

13. A host cell comprising a nucleic acid molecule or a group of isolated nucleic acid molecules according to Claim 10 or 11 or a vector according to Claim 12.

14. A genetically engineered non-human eukaryotic organism, which expresses a chimeric enzyme according to any one of Claims 1 to 9.

15. *In vitro* or *ex vivo* use of a chimeric enzyme according to any one of Claims 1 to 9, for the production of RNA molecule with 5'-terminal m^7GpppN cap.

16. An *in vitro* or *ex vivo* method for producing a RNA molecule with 5'-terminal m^7GpppN cap encoded by a DNA sequence, in a host cell, said method comprising the step of

expressing in the host cell a nucleic acid molecule or a group of isolated nucleic acid molecules according to Claim 10 or 11, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.

17. The method according to claim 16, wherein said method further comprises the step of inhibiting the expression of at least one of the subunits of the endogenous DNA-dependent RNA polymerase and/or of the endogenous capping enzyme in said host cell.

18. A kit for the production of a RNA molecule with 5'-terminal m^7GpppN cap, comprising at least one chimeric enzyme according to any one of Claims 1 to 9, and/or an isolated nucleic acid molecule and/or a group of isolated nucleic acid molecules according to Claim 10 or 11, and/or a vector according to Claim 12.

19. A pharmaceutical composition comprising a chimeric enzyme according to any one of Claims 1 to 9, and/or an isolated nucleic acid molecule and/or a group of isolated nucleic acid molecules according to Claim 10 or 11, and/or a vector according to Claim 12.

20. The pharmaceutical composition according to Claim 19, which further comprise:

- at least one DNA sequence of interest, wherein said DNA sequence is operatively linked to a promoter for said catalytic domain of a DNA-dependant RNA polymerase.