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(54) Title: **METHODS AND MEANS FOR REGULATION OF GENE EXPRESSION**

(57) Abstract: A transcriptional activator which comprises a fusion protein, the fusion protein comprising at least two polypeptide components, a first polypeptide component which binds in a sequence specific manner to an operator sequence in DNA, and a second polypeptide component, comprising a Lux-R transcription factor regulatory domain which binds cognate Acy1HSL or an analogue thereof, whereby upon binding of the Acy1HSL or analogue the DNA binding function of the first polypeptide component is activated; the transcriptional activator additionally comprising a third polypeptide component which activates transcription in eukaryotic cells.

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METHODS AND MEANS FOR REGULATION OF GENE EXPRESSION

The present invention relates to regulation of gene expression, in particular regulation of expression in eukaryotic cells and expression systems, relevant for gene therapy applications.

Nucleic acid molecules and proteins useful for regulating the expression of genes in eukaryotic cells and organisms in a highly controlled manner are disclosed. In a regulatory system in accordance with the invention, transcription of a nucleotide sequence is activated by a transcriptional activator fusion protein composed of at least two and optionally at least three polypeptide components: (i) a first polypeptide component which binds DNA with selectivity for a particular operator sequence; (ii) a second polypeptide component comprising a regulatory domain which binds AcylHSL (or an analogue thereof) whereby upon AcylHSL binding, the DNA binding function of the first polypeptide is activated; and (iii) an optional third polypeptide which directly or indirectly activates transcription in eukaryotic cells. As an alternative to inclusion in a fusion protein the third polypeptide component can be provided separately, whereby the fusion protein and polypeptide component interact in order to provide a transcription factor.

With gene therapy, one problem is regulation of gene expression in humans. Several regulatory systems have been developed. In general these systems comprise three elements: (1) a target gene i.e. the gene whose expression needs to be regulated; (2) a gene coding for a regulatory protein, i.e. a protein that regulates the activity of the target gene via complexing with the third element; (3) a regulatory molecule, preferably of small molecular weight, that can be added to the system from outside. For example, the relevant regulatory molecule can be added to the culture media or introduced in the body of the animal.

To date the two systems most commonly used to regulate gene expression, in the context of gene therapy, are the one based on the use of steroid hormone receptor and the drug RU486 (Mifepristone or Mifegyne, a progesterone antagonist), and the Bujard system based on the use of the tet-operon and the administration of tetracyclines. For example, the Tet repressor (TetR), which binds to tet operator sequences in the absence of tetracycline and represses gene transcription, has been expressed in plant cells at sufficiently high concentrations to repress transcription from a promoter containing tet operator sequences (Gatz, C. et al. (1992) *Plants* 2:397-404). In other studies, TetR has been fused to the activation domain of VP16 to create a tetracycline-controlled transcriptional activator (tTA) (Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89:5547-5551). The tTA fusion protein is regulated by tetracycline in the same manner as TetR, i.e., tTA binds to tet operator sequences in the absence of tetracycline but not in the presence of tetracycline. Thus, in this system, in the continuous presence of Tc, gene expression is kept off, and to induce transcription, Tc is removed.

Both these systems suffer from a number of shortcomings and development of alternative gene-regulation systems is therefore an objective of great importance.

The present invention pertains to a regulatory system which utilizes components of the prokaryotic quorum sensing system in order to regulate gene expression in eukaryotic cells. Many prokaryotic organisms are endowed with a control mechanism called quorum sensing (Fuque, et al (1994), *J. Bacteriol.*, **176**: 269-275). In the well characterized marine microorganism *Vibrio fischeri*, the quorum sensing mechanism consists primarily of (1) the product of the gene LuxR; (2) a small effector molecule called the autoinducer, chemically a N-acyl-homoserine-lactone (AcylHSL), freely diffusible across bacterial membranes and that binds to the LuxR gene product;

(3) a DNA operator sequence to which the LuxR/AcylHSL complex binds thus activating transcription, termed the lux box B a 20 nucleotide inverted repeat; (4) the LuxI gene product, which is the AcylHSL synthase and whose expression is under the control of a lux box. Several bacteria genera contain regulatory system homologous to the quorum sensing system of *V. fischeri*. Proteins homologous to LuxR have often been identified. Examples of these are listed in Table I, and discussed in Fuqua, et al (1994,) *J. Bacteriol.*, **176**:269-275 and in Fuqua, C., et al (1996), *Annu. Rev. Microbiol.*, **50**:727-751. There are certainly highly conserved regions, although the overall level of sequence similarity between proteins of this group is rather low, often no higher than 18-25% identity. A number of bacteria with proteins homologous to LuxR also produce autoinducers similar to that produced by *V. fischeri*. All of the inducer signal compounds have identical homoserine lactone moieties but differ in the length and the structure of their acyl groups (Table II). *Vibrio fischeri* LuxR and its cognate autoinducer - N(3-oxohexanoyl)-L homoserine lactone - is employed in preferred embodiments of the present invention.

In other preferred embodiments, especially where a TraR regulatory domain is employed as described below, the AcylHSL inducer employed may be N(3-oxooctanoyl)-L homoserine lactone.

The system of the invention utilizes components of the prokaryotic quorum sensing/autoinducer pathway in order to regulate gene expression in eukaryotic cells. In particular, this invention provides AcylHSL-regulated transcriptional activators which are useful for activating gene expression, in a controlled manner, of a gene linked to one or more selected operator sequences.

The present invention relates to nucleic acid molecules and proteins which can be used to regulate the expression of genes in eukaryotic cells or animals. Regulation of gene expression

by the system of the invention involves at least two components: A gene which is operably or operatively linked to a regulatory sequence and a protein which, in presence of an inducible agent, binds to the regulatory sequence and activates transcription of the gene. The system of the invention utilizes components of the prokaryotic quorum sensing pathway in order to regulate gene expression in eukaryotic cells. More specifically, the present invention is based on the prokaryotic LuxR-type transcription factors. The term "LuxR-type transcription factor" is intended to include, homologues of the *Vibrio fischeri* LuxR protein. According to the general teaching of Henikoff, S., et al (Henikoff, S. Wallace, JC. Brown, JP., 1990; *Methods Enzimol.* **183**: 111-132) and more specifically of Fuqua, et al (Fuqua, et al.; 1994; *J. Bacteriol.*, **176**:269-275; Fuqua, C., et al; 1996; *Annu. Rev. Microbiol.*, **50**:727-751), members of a LuxR superfamily of LuxR-type transcription factor are defined by the following characteristics:

(1) are DNA-binding proteins that are a component of an N-acyl homoserine lactone based gene regulatory system;

(2) comprise a first cluster of sequence similarity in a region that aligns with the putative AcylHSL-binding region of LuxR;

(3) their carboxyl terminal thirds comprise a second cluster of sequence similarity in a region defined as a helix-turn-helix motif contained within the DNA binding domain. This helix-turn-helix motif is identified as containing a motif defined as a probe helix putatively involved in protein-DNA major groove interaction in a number of transcription factors (Suzuki, M. 1993; *EMBO J.*, **8**: 3221-3226). Sequence similarity is generally recognised using the ExpASY public server of the Swiss Institute of Bioinformatics. A signature pattern defining LuxR family membership, defined by PROSITE (Protein Family and Domain Database of the Swiss Institute of Bioinformatics, 1, Rue Michel-Servet, 1211, Genève, 4 Switzerland) is the following:

[GDC] -x(2) - [NSTAVY] -x(2) - [IV] - [GSTA] -x(2) - [LIVMFYWCT] -x-
[LIVMFYWCR] -x(3) - [NST] - [LIVM] -x(5) - [NRHSA] - [LIVMSTA] -x(2) -
[KR] .

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Addition LuxR signature patterns may be defined with reference to the ABLOCKS@ database at the Fred Hutchinson Cancer Research Center in Seattle, Washington, USA or at the Weizmann Institute of Science in Israel.

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A list of LuxR-type transcription factors that may be employed in embodiments of the present invention is shown in Table I. One preferred embodiment employs LuxR DNA binding-domain and/or regulatory domain. Another preferred embodiment
15 employs TraR DNA binding domain and/or regulatory domain.

The invention is widely applicable to a variety of situations where it is desirable to be able to turn gene expression on and off, or regulate the level of gene expression.

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The invention may be employed for gene therapy purposes, e.g. in treatments for genetic or acquired diseases.

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To use the system of the invention for gene therapy purposes, in one embodiment, cells of a subject in need of gene therapy are modified to contain 1) nucleic acid encoding a transactivator of the invention in a form suitable for expression of the transactivator in the host cells and 2) a gene of interest (e.g., for therapeutic purposes) operatively
30 linked to an AcylHSL-regulated transcription unit. Expression of the gene of interest in the cells of the subject is then stimulated by administering AcylHSL or a AcylHSL analogue to the patient. The level of gene expression can be modulated by adjusting the dose of the AcylHSL or analogue administered to
35 the patient.

To stop expression of the gene of interest in cells of the

subject, administration of the inducing agent is stopped.
Thus, the regulatory system of the invention offers the
advantage over constitutive regulatory systems of allowing for
modulation of the level of gene expression depending upon the
requirements of the therapeutic situation.

Additionally, the system of the invention can be used to
conditionally express a suicide gene in cells, thereby
allowing for elimination of the cells after they have served
an intended function. For example, cells used for vaccination
can be eliminated in a subject after an immune response has
been generated the subject by inducing expression of a suicide
gene in the cells by administering AcylHSL or a AcylHSL
analogue to the subject.

Large scale production of a protein of interest can be
accomplished using cultured cells *in vitro* which have
been modified to contain a nucleic acid encoding a
transactivator fusion protein of the invention in a form
suitable for expression of the transactivator in the cells and
a gene encoding the protein of interest operatively linked to
an AcylHSL-regulated transcription unit.

The invention also provides for large scale production of a
protein of interest in transgenic animals. Transgenic
livestock carrying in their genome the components of the
inducible regulatory system of the invention can be
constructed, wherein a gene encoding a protein of interest
is operatively linked to an AcylHSL-regulated transcription
unit. Gene expression, and thus protein production, is induced
by administering AcylHSL or analogue thereof to the transgenic
animal.

The transcriptional activator proteins of the invention can be
used alone or in combination to stimulate or inhibit
expression of specific genes in animals to mimic the
pathophysiology of human disease to thereby create

animal models of human disease. Ability to regulate the expression of the specific gene may be advantageous over gene "knock out" by homologous recombination to create animal models of disease, since the AcylHSL-regulated system described herein allows for control over both the levels of expression of the gene of interest and the timing of when gene expression is regulated.

Brief Description Of The Figures

Figure 1 is a schematic diagram of the construction of LuxR/VP16 fusion constructs by in-frame fusion of nucleic acid encoding LuxR and the activation domain of the VP16 protein.

Figure 2 is a schematic diagram of the construction of HNF-1/LuxR/VP16 fusion constructs by in-frame fusion of nucleic acid encoding the DNA binding domain, of HNF-1, the regulatory domain of LuxR and the activation domain of the VP16 protein.

Figure 3 is a schematic diagram of a promoter construct for regulation of a genes of interest operatively linked to the lux-box containing promoter for regulation by a AcylHSL-regulated transcriptional activator.

Figure 4 is a schematic diagram of a promoter construct for regulation of a genes of interest operatively linked to an hHNF1-box containing promoter for regulation by a AcylHSL-regulated transcriptional activator.

Figure 5 illustrates individual embodiments that are preferred in accordance with different aspects of the present invention. Shown are constructs providing a eukaryotic chimeric trans-activator of transcription that is regulated by N-(3-oxo-octanoyl)-L-homoserine lactone.

Figure 5A illustrates a transcriptional activator according to individual embodiments of the present invention in which a

eukaryotic trans-activation domain (e.g. VP16, F3, p65, LFB 1/HNF-1 or other) is fused in frame with the TraR protein or another member of the LuxR protein family.

5 In one embodiment the activation domain is placed in the fusion protein N-terminal to TraR (or other DNA binding/regulatory protein). In another embodiment the activation is placed C-terminal. TraR (or other) may be separated from the activation domain by a linker amino acid
10 sequence (linker). A nuclear localization sequence (NLS) is an optional component of embodiments of the invention. An NLS where used may, as illustrated, be provided at the N-terminal or at the C-terminal of the fusion protein. In a further embodiment, the NLS is introduced between TraR and the
15 activation domain or as part of the linker sequence.

Figure 5B illustrates how in other embodiments of the present invention TraR or a portion of it can be used to replace the dimerization domain in a eukaryotic dimeric transcription
20 factor. TraR* indicates full-length TraR protein or a portion of it containing the TraR dimerization or the N-(3-oxo-octanoyl)-L-homoserine lactone binding domain or both. "DNA binding domain" indicates the DNA binding domain of a eukaryotic transcription factor whereby the original
25 dimerization domain has been deleted or inactivated. The activation domain module may be placed at either the N-terminal of the fusion protein, or at the C-terminus. If required, a nuclear localization sequence maybe added to the fusion protein.

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According to one aspect of the present invention there is provided a transcriptional activator which comprises a fusion protein comprising at least two polypeptide components: (i) a first polypeptide component which binds in a sequence specific
35 manner to an operator sequence in DNA; and (ii) a second polypeptide component, comprising a Lux-R transcription factor regulatory domain which binds cognate AcylHSL or an analogue

thereof whereby upon binding of the AcylHSL or analogue the DNA binding function of the first polypeptide component is activated. The transcriptional activator additionally comprises a third polypeptide which activates transcription in eukaryotic cells, and this may be a third polypeptide component of the fusion protein. Where it is not a component of the fusion protein the third polypeptide is a transcriptional activator that interacts with the fusion protein comprising the first and second polypeptide components in order to activate transcription when the fusion protein binds to a cognate operator sequence.

A transcriptional activator according to the invention activates transcription when bound to an operator sequence, and binds to the operator sequence in the presence of AcylHSL, or an analogue thereof. Thus, in a host cell, transcription of a gene operatively linked to an operator may be controlled by altering the concentration of AcylHSL (or analogue) in contact with the host cell (e.g. adding AcylHSL from a culture medium, or administering AcylHSL to a host organism, etc.). The invention further pertains to transcription units for regulation by the transcriptional activators of the invention. Methods for stimulating transcription of a gene using AcylHSL (or analogues thereof) are also encompassed by the invention.

The term "AcylHSL analogue" encompasses compounds which need not be structurally related to AcylHSL but which bind to at least one of the LuxR-type transcription factors and induce the requisite change to induce DNA binding in an operably linked DNA binding component. Preferably an AcylHSL analogue binds a LuxR transcription factor with a K_a of at least about 10^6 M^{-1} , and more preferably K_a of about 10^9 M^{-1} or greater.

Transcription may be induced in a cell *in vitro* by culturing the cell in a medium containing AcylHSL or analogue thereof. In such a culture, the concentration of AcylHSL or analogue thereof is preferably between about 10 and about 1000 ng/ml.

To induce transcription *in vivo* AcylHSL or analogue thereof may be administered to the body, or a tissue of interested (e.g. by injection). The body to be treated may be that of an animal, particularly a mammal, which may be human or non-
5 human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken, or a plant. For administration to a human or animal, the dosage may preferably be designed to achieve a serum concentration of between about 0.05 and 1.0
10 µg/ml. Suitable routes of administration include oral (e.g. in drinking water) and, in the case of a plant, water administered to the plant.

The first, second, and third polypeptide components of a
15 fusion protein may be arranged in any order or sequence in a fusion transactivator protein of the invention.

The first and second polypeptide components may be derived from a bacterial LuxR transcription factor.

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The first and/or second polypeptide components may be derived from TraR of *Agrobacterium tumefaciens* (see below, including Table I, for references).

25 In one embodiment of the invention, both the first (the DNA binding) and the second (the regulatory) polypeptides of the trans-activator fusion protein are derived from the DNA binding domain and the regulatory domain, respectively, of LuxR or a LuxR-type transcription factor. In a preferred
30 version of the fusion transactivator of the invention, the DNA binding and the regulatory polypeptide components are both derived from the *Vibrio fischeri* LuxR protein. For instance the DNA binding domain may consist of or comprise residues 160-250 of *Vibrio fischeri* LuxR (containing a 20-amino acid
35 region (amino acids 190-210) believed to fold in a helix-turn-helix structure that is predicted to be involved in DNA

recognition), and/or the regulatory polypeptide component may consist of or comprise residues 20-159 of that protein. This part of the LuxR protein encompasses the AcylHSL-binding region (amino acids 79-120).

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In another embodiment of the invention, the first polypeptide component (the DNA binding domain) of the trans-activator fusion protein is derived from the DNA binding domain of a protein other than a LuxR transcription factor. For example,

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the first polypeptide may be derived from the DNA-binding domain of a eukaryotic transcription factor. In a preferred embodiment of the present invention, a HNF-1 DNA-binding domain may be used, which may for example consist of or comprise residues 1-281 of hHNF-1 (Bach, et al (1990),

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Genomics, 8(1):155-164 (Sequence accession number P20823)).

For example, the DNA-binding domain of hHNF-1 may be fused to the regulatory domain of a LuxR transcription factor in order to achieve AcylHSL-inducibility of DNA-binding.

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The third polypeptide component transcriptional activator domain may be any available to those skilled in the art.

Polypeptides which activate transcription in eukaryotic cells are well known in the art. In particular, transcriptional activation domains of many DNA binding proteins have been

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described and have been shown to retain their activation function when the domain is transferred to a heterologous protein. A preferred polypeptide for use in the fusion protein of the invention is the herpes simplex virus virion protein 16 (referred to herein as VP16, the amino acid sequence of which

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is disclosed in Triezenberg, S. J. et al. (1988) *Genes Dev.* 2:718-729). In one embodiment, about 127 of the C-terminal amino acids of VP16 are used. For example, a polypeptide

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having the amino acid sequence of the 127 C-terminal amino acids of VP 16 may be used as the second polypeptide component in a fusion protein according to the present invention. In another embodiment, at least one copy of about 11 amino acids from the C-terminal region of VP16 (amino acids 437-447) which

retain transcriptional activation ability is used as the second polypeptide component. Preferably, multimers (two to four monomers) of this region are used. Preferably, a dimer of this region (i.e., about 22 amino acids) is used. Suitable C-terminal peptide portions of VP16 are described in Seipel, K. et al. (EMBO J. (1992) 13:4961-4968). For example, a dimer of a peptide having an amino acid sequence DALDDFDLDM can be used as the second polypeptide in the fusion protein. Other polypeptides with transcriptional activation ability in eukaryotic cells can be used in a transcriptional activator in accordance with the invention. Transcriptional activation domains found within various proteins have been grouped into categories based upon similar structural features. Types of transcriptional activation domains include acidic transcription activation domains, proline-rich transcription activation domains, serine/threonine-rich transcription activation domains and glutamine-rich transcription activation domains. Examples of acidic transcriptional activation domains include the VP16 regions already described and amino acid residues 753-881 of GAL4. Examples of proline-rich activation domains include amino acid residues 399-499 of CTF/NF1 and amino acid residues 31-76 of AP2. Examples of serine/threonine-rich transcription activation domains include amino acid residues 1-427 of ITF1 and amino acid residues 2-451 of ITF2. Examples of glutamine-rich activation domains include amino acid residues 175-269 of Oct1 and amino acid residues 132-243 of Sp1. The amino acid sequences of each of the above described regions, and of other useful transcriptional activation domains, are disclosed in Seipel, K. et al. (EMBO J. (1992) 12:4961-4968). The transcriptional activation ability of a polypeptide can be assayed by linking the polypeptide to another polypeptide having DNA binding activity and determining the amount of transcription of a target sequence that is stimulated by the fusion protein. For example, a standard assay used in the art utilizes a fusion protein of a putative transcriptional activation domain and a GAL4 DNA binding domain (e.g., amino acid residues 1-93). This

fusion protein is then used to stimulate expression of a reporter gene linked to GAL4 binding sites (see e.g., Seipel, K et al. (1992) EMBO J. 11:4961-4968 and references cited therein).

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In a preferred embodiment, the fusion protein of the transactivator itself possesses transcriptional activation activity (i.e., the third polypeptide component directly activates transcription). In another embodiment, the transcription is activated by an indirect mechanism, through recruitment of a transcriptional activation protein to interact with the fusion protein. This may, for example, be via a polypeptide domain (e.g., a dimerization domain) which mediates a protein-protein interaction with a transcriptional activator protein, such as an endogenous activator present in a host cell. Examples of suitable interaction (or dimerization) domains include leucine zippers (Landschulz et al. (1989) Science 243:1681-1688), helix-loop-helix domains (Murre, C. et al. (1989) Cell 58:537-544) and zinc finger domains (Frankel, A. D. et al. (1988) Science 240:70-73). A fusion protein of the invention may further comprise one or more additional polypeptide components, such as a fourth polypeptide component which promotes transport of the fusion protein into a cell nucleus, a nuclear localization signal (NLS). Nuclear localization signals typically are composed of a stretch of basic amino acids. When attached to a heterologous protein (e.g., a fusion protein of the invention), the nuclear localization signal promotes transport of the protein to a cell nucleus. The nuclear localization signal is attached to a heterologous protein such that it is exposed on the protein surface and does not interfere with the function of the protein. Preferably, the NLS is attached to one end of the protein, e.g. the N-terminus. The amino acid sequence of a non-limiting example of an NLS that can be included in a fusion protein of the invention is Met-Pro-Lys-Arg-Pro-Arg-Pro. Preferably, a nucleic acid encoding the nuclear localization signal is spliced by standard recombinant

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DNA techniques in-frame to the nucleic acid encoding the fusion protein (e.g., at the 5' end).

Examples of preferred embodiments according to the present invention are described in detail in the Examples below. Preferred embodiments include transcriptional activators comprising a luxR+VP16 fusion, a TraR+VP16, TraR+LFB1/HNF1, TraR+ NF- κ B p65. The transcriptional activator component may be comprised in a fusion protein upstream or N-terminal to the regulatory and/or DNA binding domain. The transcriptional activator component may be comprised in a fusion protein downstream or C-terminal to the regulatory and/or DNA binding domain. One or more spacers may be used, e.g. polylinker of GS (poly-gly-ser), whether the activator component is N-terminal or C-terminal to the regulatory and/or DNA binding domain (i.e. towards the N-terminus or C-terminus, whether at the respective end of the protein or not). Preferred combinations of components and spacers are illustrated by the Examples below and shown in Figure 5A and Figure 5B. A transcriptional activator including a fusion protein according to the present invention may comprise of be composed of portions of naturally occurring proteins, of which examples have been mentioned. Furthermore, one or more of the polypeptide components may comprise an amino acid sequence which differs by one or more amino acid residues from a natural amino acid sequence, whether a mutant, allele, isoform, variant or derivative of a specific sequence. Instead of using a wild-type protein component, a transcriptional activator according to the present invention may include an amino acid sequence which differs by one or more amino acid residues from the wild-type amino acid sequence, by one or more of addition, insertion, deletion and substitution of one or more amino acids.

Preferably, the amino acid sequence shares homology with a fragment of the relevant protein, preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85%,

90% or 95% homology. Thus, a protein component may include 1, 2, 3, 4, 5, greater than 5, or greater than 10 amino acid alterations such as substitutions with respect to the wild-type sequence.

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As is well-understood, homology at the amino acid level is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine.

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Similarity may be as defined and determined by the TBLASTN or other BLAST program, of Altschul et al., (1990) *J. Mol. Biol.* 215, 403-10, which is in standard use in the art, or, and this may be preferred, either of the standard programs BestFit and GAP, which are part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (Advances in Applied Mathematics (1981) 2, pp. 482-489). GAP uses the Needleman and Wunsch algorithm to align two complete sequences that maximizes the number of matches and minimizes the number of gaps. Generally, the default parameters are used, with a gap creation penalty = 12 and gap extension penalty = 4. Homology is generally over the full-length of the relevant sequence compared with the relevant wild-type amino acid sequence.

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A further way of defining similarity or identity between sequences is to consider ability of nucleic acid to hybridize under stringent conditions. As noted further below, a fusion protein and polypeptide components thereof according to the present invention are generally provide by expression from

encoding nucleic acid. Such encoding nucleic acid may be employed in hybridisation experiments.

Preliminary experiments may be performed by hybridising under low stringency conditions. Preferred conditions are those which are stringent enough for there to be a simple pattern with a small number of hybridisations identified as positive which can be investigated further.

For example, hybridisations may be performed, according to the method of Sambrook et al. (below) using a hybridisation solution comprising: 5X SSC (wherein $5\times\text{SSC} = 0.15\text{ M sodium chloride; } 0.15\text{ M sodium citrate; pH } 7$), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 $\mu\text{g/ml}$ denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridisation is carried out at 37-42°C for at least six hours. Following hybridisation, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

One common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified sequence homology is (Sambrook et al., 1989): $T_m = 81.5^\circ\text{C} + 16.6\text{Log} [\text{Na}^+] + 0.41 (\% \text{ G+C}) - 0.63 (\% \text{ formamide}) - 600/\#\text{bp in duplex}$.

As an illustration of the above formula, using $[\text{Na}^+] = [0.368]$ and 50-% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1% decrease in homology. Thus, targets with greater than about 75% sequence identity would be observed using a hybridisation temperature of 42°C.

It is well known in the art to increase stringency of hybridisation gradually until only a few positive clones remain. Other suitable conditions include, e.g. for detection
5 of sequences that are about 80-90% identical, hybridisation overnight at 42°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 55°C in 0.1X SSC, 0.1% SDS. For detection of sequences that are greater than about
10 90% identical, suitable conditions include hybridisation overnight at 65°C in 0.25M Na₂HPO₄, pH 7.2, 6.5% SDS, 10% dextran sulfate and a final wash at 60°C in 0.1X SSC, 0.1% SDS.

One convenient way of producing a polypeptide or fusion
15 protein according to the present invention is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Accordingly the present invention also provides in various
20 aspects nucleic acid encoding the transcriptional activator of the invention, which may be used for production of the encoded protein.

Generally whether encoding for a protein or component in
25 accordance with the present invention, nucleic acid is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except
30 possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as encompassing reference
35 to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding a polypeptide or fusion

protein in accordance with the present invention can be readily prepared by the skilled person using the information and references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, A
5 Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), and Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, (1994)), given the nucleic acid sequence and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to
10 amplify samples of such nucleic acid, e.g. from genomic sources, (ii) chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding portions of full-length coding sequences (e.g. a DNA binding domain, or regulatory domain as the case may be) may be generated and used in any suitable way
15 known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard
20 commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA with suitable PCR primers. Modifications to the relevant sequence may be made, e.g. using site directed mutagenesis, to lead to the expression of modified peptide or to take account of codon
25 preference in the host cells used to express the nucleic acid.

In order to obtain expression of the nucleic acid sequences, the sequences may be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to
30 control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that
35 the polypeptide produced in the host cell is secreted from the cell. Polypeptide can then be obtained by transforming the vectors into host cells in which the vector is functional,

culturing the host cells so that the polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of *E. coli*,
5 yeast, and eukaryotic cells such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or fusion protein as disclosed, the method including expression from nucleic acid encoding the
10 product (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide.

Polypeptides may also be expressed in *in vitro* systems, such
15 as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and
20 yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is *E. coli*.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as
30 appropriate. Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook et al., 1989, Cold Spring Harbour Laboratory Press.

Many known techniques and protocols for manipulation of
35 nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into

cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

5 For use in mammalian cells, a recombinant expression vector's control functions may be provided by viral genetic material. Exemplary promoters include those derived from polyoma, Adenovirus 2, cytomegalovirus and SV40. For example, an expression vector similar to that described in Example 1 can
10 be used.

A regulatory sequences of a recombinant expression vector used in the present invention may direct expression of a polypeptide or fusion protein preferentially in a particular
15 cell type, i.e., tissue-specific regulatory elements can be used. In one embodiment, the recombinant expression vector of the invention is a plasmid, such as that described in Example 1. Alternatively, a recombinant expression vector of the invention can be a virus, or portion thereof, which allows
20 for expression of a nucleic acid introduced into the viral nucleic acid. For example, replication defective retroviruses, adenoviruses and adeno-associated viruses can be used. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can
25 be found in Ausubel, et al. (*supra*). The genome of a virus such as adenovirus can be manipulated such that it encodes and expresses a transactivator protein but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle.

30

Thus, a further aspect of the present invention provides a host cell containing heterologous nucleic acid as disclosed herein.

35 The host cell can be, for example, a mammalian cell (e.g., a human cell), a yeast cell, a fungal cell or an insect cell. Moreover, the host cell can be a fertilized non-human oocyte,

in which case the host cell can be used to create a transgenic organism having cells that express the transcriptional inhibitor fusion protein. Still further, the recombinant expression vector can be designed to allow homologous recombination between the nucleic acid encoding the transactivator and a target gene in a host cell. Such homologous recombination vectors can be used to create homologous recombinant animals that express a fusion protein of the invention.

The nucleic acid of the invention may be integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

Examples of mammalian cell lines which may be used include CHO dhfr- cells (Urlaub and Chasin (1980) Proc. Natl. Acad Sci. USA 77:4216-4220), 293 cells (Graham et al. (1977) J. Gen. Virol. 36: pp 59) and myeloma cells like SP2 or NS0 (Galfre and Milstein (1981) Meth. Enzymol. 73(B):3-46). In addition to cell lines, the invention is applicable to normal cells, such as cells to be modified for gene therapy purposes or embryonic cells modified to create a transgenic or homologous recombinant animal. Examples of cell types of particular interest for gene therapy purposes include hematopoietic stem cells, myoblasts, hepatocytes, lymphocytes, muscle cells, neuronal cells and skin epithelium and airway epithelium. Additionally, for transgenic or homologous recombinant animals, embryonic stem cells and fertilized oocytes can be modified to contain nucleic acid encoding a transactivator fusion protein.

Nucleic acid a transactivator fusion protein can transferred into a fertilized oocyte of a non-human animal to create

a transgenic animal which expresses the fusion protein of the invention in one or more cell types.

In addition to transgenic animals, the present invention is
5 useful in other transgenic organisms, such as transgenic
plants. Transgenic plants can be made by conventional
techniques known in the art. Accordingly, aspects of the
invention further provide non-human transgenic organisms,
including animals and plants, that contains cells which
10 express transcriptional activator protein of the invention
(i.e., a nucleic acid encoding the transactivator is
incorporated into one or more chromosomes in cells of the
transgenic organism).

15 A still further aspect provides a method which includes
introducing the nucleic acid into a host cell. The
introduction, which may (particularly for *in vitro*
introduction) be generally referred to without limitation as
Atransformation@, may employ any available technique. For
20 eukaryotic cells, suitable techniques may include calcium
phosphate transfection, DEAE-Dextran, electroporation,
liposome-mediated transfection and transduction using
retrovirus or other virus, e.g. vaccinia or, for insect cells,
baculovirus. For bacterial cells, suitable techniques may
25 include calcium chloride transformation, electroporation and
transfection using bacteriophage. As an alternative, direct
injection of the nucleic acid could be employed.

Marker genes such as antibiotic resistance or sensitivity
30 genes may be used in identifying clones containing nucleic
acid of interest, as is well known in the art.

The introduction may be followed by causing or allowing
expression from the nucleic acid, e.g. by culturing host cells
35 (which may include cells actually transformed although more
likely the cells will be descendants of the transformed cells)
under conditions for expression of the gene, so that the

encoded product is produced. If the polypeptide is expressed coupled to an appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired, e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

Introduction of nucleic acid encoding a polypeptide according to the present invention may take place *in vivo* by way of gene therapy. One option is to introduce nucleic acid into cells *ex vivo*, which cells may then be implanted or otherwise administered to an individual. Such cells may have been taken from the individual and may be returned after treatment with nucleic acid of the invention.

Thus, a host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence endogenous to the cell or ancestor (which introduction or alteration may take place *in vivo* or *ex vivo*), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken, or a plant. Genetically modified or transgenic animals, birds and plants comprising such a cell are also provided as further aspects of the present invention.

A host cell containing a transcriptional activator of the invention (e.g. a fusion protein provided by transformation of the host cell with encoding nucleic acid) may additionally contain (e.g. as a result of transformation) one or more nucleic acids which serve as a target for the transcriptional

activator. A target nucleic acid comprises a nucleotide sequence to be transcribed operatively linked to at least one operator sequence.

5 A transcriptional activator in accordance with the present invention may be used to regulate transcription of a target nucleotide sequence which is operatively or operably linked to a regulatory sequence to which the transcriptional activator binds. The nucleotide sequence to be transcribed typically
10 includes a minimal promoter sequence which is not itself transcribed but which serves (at least in part) to position the transcriptional machinery for transcription. The minimal promoter sequence is located upstream of the transcribed sequence to form a contiguous nucleotide sequence. The
15 activity of such a minimal promoter is dependent upon the binding of a transcriptional activator to an operatively linked regulatory operator sequence. The minimal promoter may be from the human cytomegalovirus (as described in Boshart et al. (1985) Cell 41:521-530), and other suitable minimal
20 promoters are available to those skilled in the art.

The target nucleotide sequence is operatively linked to at least one oligonucleotide sequence to which a transcriptional activator of the invention binds, an operator sequence. The
25 operator is usually 5' to the sequence to be transcribed and, where appropriate, minimal promoter. An operator sequence may be operatively linked downstream (i.e., 3') of the nucleotide sequence to be transcribed.

30 The operator sequence may correspond to that of a lux-box operator sequence. The term "lux-box operator sequence" is intended to encompass all DNA sequences that are binding sites for LuxR-type transactivators (Fuque, et al (1994), *J. Bacteriol.*, **176**:269-275). A nucleotide sequence to be
35 transcribed can be operatively linked to a single lux box operator sequence, or to multiple lux-box operator sequences (e.g., two, three, four, five, six, seven, eight, nine, ten or

more operator sequences).

A transcription factor in accordance with the present invention binds to the appropriate DNA operator sequences in the presence but not the absence of a cognate acylhomoserine lactone (AcylHSL) or an analogue thereof. In the presence of a cognate acylhomoserine lactone (AcylHSL), a fusion protein of the invention binds to its cognate operator sequence. Where the operator sequence is operatively linked to a further sequence of interest, transcription of the further sequence of interest is thereby activated.

Where a LuxR DNA binding domain is employed the operator sequence may be a Lux box (Fuque, et al (1994)). Other DNA binding domains that may be employed include DNA binding domain of other prokaryotic or eukaryotic DNA-binding proteins with their cognate operator sequence. A preferred embodiment of the invention utilises DNA-binding domains of eukaryotic origin, more preferably from mammals, more preferably from human.

Examples of DNA binding sites are GAL4 DNA, virus DNA binding sites and insect DNA binding sites. A preferred embodiment of the invention utilises a DNA-binding domain of a human transcription factor. In one embodiment of the invention, the DNA binding domain of human transcription factor HNF-1 is utilised (amino acids 1-281, Sequence accession number P20823). In this embodiment, the operator sequence may correspond to that of an HNF-1 cognate binding site. A nucleotide sequence to be transcribed can be operatively linked to a single or multiple HNF-1 binding sites.

The further sequence operably linked to the promoter and operator sequences may be a coding sequence for a polypeptide or peptide, an antisense sequence or a ribozyme.

A polypeptide of which expression may be controlled using the

present invention may be selected according to the desires and aims of the person performing the invention, and may be a therapeutic protein or a cytotoxic protein.

5 Polypeptide expression may be inhibited by using appropriate nucleic acid to influence expression by antisense regulation, and an antisense sequence may be placed under transcriptional control in accordance with the present invention. The use of anti-sense genes or partial gene sequences to down-regulate
10 gene expression is now well-established. Double-stranded DNA is placed under the control of a promoter in a "reverse orientation" such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene.
15 The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of action is still uncertain. However, it is established fact that the technique works.

20

Another possibility is that nucleic acid is used which on transcription produces a ribozyme, able to cut nucleic acid at a specific site - thus also useful in influencing gene expression. Background references for ribozymes include
25 Kashani-Sabet and Scanlon (1995). *Cancer Gene Therapy*, 2, (3) 213-223, and Mercola and Cohen (1995). *Cancer Gene Therapy* 2, (1) 47-59.

30 An AcylHSL-regulated transcription unit of the invention may be incorporated into a recombinant vector (e.g., a plasmid or viral vector), and may be introduced into a host cell or animal, optionally along with a transcriptional activator as disclosed or encoding nucleic acid therefor.

35 A further aspect of the present invention provides a composition comprising:

(i) a transcriptional activator as disclosed, or a first nucleic acid encoding a transcriptional activator as disclosed (which nucleic acid may encode a fusion protein comprising first, second and third polypeptide components as disclosed or may comprise separate sequences encoding a fusion which comprises first and second polypeptide components and a third polypeptide to interact with the fusion to provide a transcription factor as discussed above); and

(ii) a second nucleic acid comprising a nucleotide sequence to be transcribed operatively linked to a AcylHSL-regulated transcription unit.

In one embodiment, where both a first and a second nucleic acid are included, the first and second nucleic acids are separate molecules (e.g., two different vectors). In this case, a host cell may be cotransfected with the two nucleic acid molecules or successively transfected first with one nucleic acid molecule and then the other nucleic acid molecule. Furthermore, the components of a transcriptional activator comprising a fusion protein which comprises first and second components and another polypeptide providing transcriptional activation which interacts with the fusion to provide a transactivator may be provided as separate molecules. In another embodiment, the nucleic acids are linked (i.e., colinear) in the same molecule (e.g., a single vector). In this case, a host cell may be transfected with the single nucleic acid molecule.

The invention further provides a method of treatment which includes administering to a patient an agent which comprises (i) a transcriptional activator according to the invention, or nucleic acid encoding such a fusion protein, and/or (ii) an AcylHSL-regulated transcription unit as disclosed. The invention further provides for use of such components (i) and (ii) in the manufacture of a medicament for administration to an individual.

A transcriptional activator according to the present invention may be used to regulate transcription of a sequence by means of an operator sequence operably linked to the sequence to be transcribed. As discussed, this operator/transcribed sequence construct may be introduced into host cells. In an alternative, a sequence to be transcribed may be endogenous to a host cell. An endogenous sequence may be operatively linked to a AcylHSL-regulated transcription unit by means of homologous recombination. For example, a homologous recombination vector can be prepared which includes at least one Lux-box operator sequence and a minimal promoter sequence flanked at its 3' end by sequences representing the coding region of the endogenous gene and flanked at its 5' end by sequences from the upstream region of the endogenous gene by excluding the actual promoter region of the endogenous gene. The flanking sequences are of sufficient length for successful homologous recombination of the vector DNA with the endogenous gene. Preferably, several kilobases of flanking DNA are included in the homologous recombination vector. Upon homologous recombination between the vector DNA and the endogenous gene in a host cell, a region of the endogenous promoter is replaced by the vector DNA containing one or more Lux-box operator sequences operably linked to a minimal promoter. Thus, expression of the endogenous gene is no longer under the control of its endogenous promoter but rather is placed under the control of the AcylHSL-regulated transcription unit.

In another embodiment, an operator sequence may be inserted elsewhere within an endogenous gene, preferably within a 5' or 3' regulatory region, via homologous recombination to create an endogenous gene whose expression can be regulated by a AcylHSL-regulated fusion protein described herein. For example, one or more Lux-box sequences can be inserted into a promoter or enhancer region of an endogenous gene such that promoter or enhancer function is maintained. Application or provision of cognate Acyl-HSL, e.g. by

administration, may be used to regulate transcription from an AcylHSL-regulated transcription unit by means of a transcriptional activator according to the present invention.

5 A composition according to the present invention that is to be given to an individual, administration is preferably in a prophylactically effective amount or a "therapeutically effective amount" as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit
10 to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical
15 doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

20

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer,
25 stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by
30 injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid
35 pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose

or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

5 For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art
10 are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

15 Liposomes, particularly cationic liposomes, may be used in carrier formulations.

Examples of techniques and protocols mentioned above can be
20 found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

The agent may be administered in a localised manner to a tumour site or other desired site or may be delivered in a
25 manner in which it targets tumour or other cells.

Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands.
30 Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

35 Instead of administering these agents directly, they may be produced in the target cells by expression from an encoding gene introduced into the cells, e.g. in a viral vector. The

vector may be targeted to the specific cells to be treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

5 A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, such as cancer, virus infection or any other condition in which an effect mediated by activity of the fusion protein is desirable.

10 Nucleic acid according to the present invention, encoding a transcriptional activator may be used in methods of gene therapy, for instance in treatment of individuals, e.g. with the aim of preventing or curing (wholly or partially) a
15 disorder or for another purpose as discussed elsewhere herein.

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target
20 cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells,
25 providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO
30 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

35

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes

electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

5 Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells, is an example of a technique for specifically
10 targeting nucleic acid to particular cells.

Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. Certain aspects and embodiments of
15 the invention will now be illustrated by way of example and with reference to the sequences discussed already above.

All documents mentioned anywhere in this text are incorporated by reference.

20

EXAMPLE 1

Construction of a N-(3-oxohexanoyl)-homoserine lactone-regulated transcriptional activator fusion protein comprising
25 the LuxR protein from *Vibrio fischeri* and VP16 activator domain from *Herpes simplex virus*

In order to construct an eukaryotic expression vector encoding the luxR protein, a nucleic acid fragment was amplified by the
30 polymerase chain reaction (PCR) using the plasmid pHK724 (G. B. Kaplan and E. P. Greenberg. 1987. Proc. Natl. Acad. Sci. USA 84: 6639-6643) as template. The 5'-oligonucleotide used for PCR (5'-CGCGG ATCCA TATGA AAGAC ATAAA TGCCG ACGAC-3') contains a *Bam*HI restriction site which is used for cloning
35 into the eukaryotic expression vector pcDNA3 (Invitrogen), a *Nde*I restriction site which contains the ATG start codon and

encodes the first seven N-terminal amino acids of luxR
(Accession number M19039, J. H. Devine, C. Countryman, and T.
O. Baldwin. 1988. Biochemistry 27, p 837-842, ATCC 7744).

5 The 3'-oligonucleotide (5'-CGTGC TCGAG TCGAC TTAGA ATTCA CTTTT
AAAGT ATGGG CAATC-3') was designed such that the VP16
activation domain can be cloned in frame to the C-terminal end
of the luxR protein. To this end, the 3'-oligonucleotide
encodes the last seven C-terminal amino acids of luxR protein
10 (amino acids 244B250), a *EcoRI* restriction site, which can be
used for in-frame cloning of VP16, the stop codon UAA and a
XhoI restriction site suitable for cloning into pcDNA3.

A DNA fragment was amplified using 25 cycles of 1= 94°C, 1=
15 55°C and 1= 72°C. The resulting DNA fragment was digested
with *BamHI* and *XhoI* and ligated into the *BamHI* and *XhoI* sites
of the pcDNA3 vector. The resultant vector is named pcD-luxR.
Standard DNA sequencing techniques were used to isolate and
confirm its identity.

20

In order to construct a fusion protein between luxR and the
transcriptional activator domain VP16 a nucleic acid fragment
encoding amino acids 363B490 of herpes simplex virus VP16 was
amplified by PCR using the plasmid pUHD 15-1 (M. Grossen and
25 H. Bujard. 1992. Proc. Natl. Acad. Sci. USA 89: 5547-5551) as
template. The 5'-oligonucleotide primer contains an *EcoRI*
restriction site which is used for in-frame cloning with the
luxR protein and encodes aa 363B369 of VP16. The 3'-
oligonucleotide encodes amino acids 484B490 of VP16, a UAG-
30 stop codon and a *XhoI* restriction site suitable for cloning
into pcD-luxR. After a standard PCR reaction, the amplified
PCR fragment and pcD-luxR were digested with *EcoRI* and *XhoI*.
The amplified fragment was then ligated directionally into
pcD-luxR to create the expression vector pcD-luxR/VP16. The
35 resultant expression vector contains nucleotide sequences
encoding the fusion protein comprising amino acids 1B250 of

wild type luxR protein linked in-frame to amino acids 363B490 of VP16. The nucleotide and amino acid sequence across the junction of the fusion is as follows: TTT AAA AGT GAA TTC GCG TAC AGC and Phe-Lys-Ser-Glu-Phe-Ala-Tyr-Ser, respectively.

Phe-Lys-Ser corresponds to amino acids 248-250 of the luxR protein. Glu-Phe are encoded by the *EcoRI* restriction site and Ala-Tyr-Ser correspond to the amino acids 363B365 of VP16.

EXAMPLE 2

Construction of a plasmid for N-(3-oxohexanoyl)-homoserine lactone dependent expression of mouse erythropoietin (mEPO) in eukaryotic cells

Mouse-erythropoietin under the control of a minimal CMV promoter (Gossen and Bujard 1992) is used as reporter gene to measure N-(3-oxohexanoyl)-homoserine lactone dependent transcription.

The test operators present in the plasmid pOR/EPO (described in Rizzuto et al. (1999) Proc. Natl. Acad. Sci. USA 96, pp. 6417-6422) is removed by restriction digest using the restriction enzymes *NdeI* and *KpnI* and protruding ends are removed by incubation with Klenow polymerase and T4 DNA polymerase.

In order to combine the minimal CMV promoter with the luxR binding site (lux box, bases 918 B 937 of sequence accession number M19039) the 20 bp inverted repeat sequence was synthesized as part of a 42-bp DNA fragment (upper strand: 5'-GATCC CATAA GCACC TGTAG GATCG TACAG GTTTA GCGAAB3'; lower strand: 5'-GATCT TCGCT AAACC TGTAC GATCC TACAG GTGCT TATGGB3'). Upon annealing, the two complementary strands exposed the compatible protruding ends of a *BamHI* and a *BglII* cleavage site at the 5' and 3' ends. Annealed

oligonucleotides are ligated, digested with *Bam*HI and *Bgl*II in order to select only head-to-tail ligation products, filled-in with Klenow polymerase and cloned blunt into the reporter construct. Plasmids containing one to seven head-to-tail repeats of the luxR binding sites are isolated, verified by DNA sequencing. The resulting constructs are named pVIJ-nXlux-bdg mEpo, where n indicates the number of luxR-binding sites present. The plasmids are then examined for their ability to be activated by the LuxR/VP16 chimeric transcription factor utilizing transient transfection of DNA plasmids in cultured cells.

EXAMPLE 3

N-(3-oxohexanoyl)-homoserine lactone Induced stimulation of transcription by pcD-luxR/VP16 transient transfection

1.5 x 10⁵ HeLa cells grown in DMEM are transiently transfected by standard calcium phosphate method (F. L. Graham and A. J. van der Eb. 1973. Virology 52: 456-461) with 10 ug of the pcD-luxR/VP16 expression vector and 10 ug of the reporter plasmid pVIJ 6Xlux-bdg mEpo, in which hexameric luxR-binding sites are fused upstream of a minimal CMV-promoter and murine EPO reporter gene. After 16 hours of transfection, cells are incubated for additional 24 hours in the presence or absence of increasing amounts of N-(3-oxohexanoyl)-homoserine lactone (or an analogue thereof). Secreted erythropoietin present in the cell medium is assayed following the instructions of the Quantikine IVD kit supplied by R&D systems.

EXAMPLE 4

Construction of a N-(3-oxo-octanoyl)-L-homoserine lactone-regulated transcriptional activator fusion protein comprising the TraR protein from *Agrobacterium tumefaciens* and either the

full-length VP16 activator domain from *Herpes simplex* virus or a minimal activator domain derived from VP16 (F3)

In order to construct an eukaryotic expression vector encoding the TraR protein (Accession numbers AF065244; L08596), the plasmid pJZ358 (J. Zhu and S. C. Winans. 1999. Proc. Natl. Acad. Sci. USA 96: 4832-4837) was digested with *NdeI/EcoRI*.

The nucleic acid fragment encoding the TraR protein was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen)

digested with *BamHI/EcoRI*. 5'-protruding ends of *NdeI* and *BamHI* were filled with Klenow polymerase. The resulting

plasmid was called **pcD-TraR**. In order to add the VP16 activation domain in-frame to the C-terminal end of TraR an *EcoRI* site was introduced at the C-terminal end of TraR by

PCR. The 3'-oligonucleotide (5'-ATACGATCGCTCGAGTTAGAATTCGATGAGTTCCGCCGATGGCGAG-3') encodes the last eight C-terminal amino acids of TraR protein (amino acids 227-234), a *EcoRI* restriction site, which can be used for C-terminal in-frame cloning of the desired activation domains, the stop codon UAA and a *XhoI* restriction site suitable for cloning into pcDNA3. The resulting plasmid was called **pcD-TraR/EcoRI**.

In order to construct a fusion protein between TraR and the transcriptional activator domain VP16 the plasmid pcD-LuxR/VP16 was digested with *EcoRI/XhoI* and the resulting DNA fragment coding for VP16 was cloned into pcD-TraR/EcoRI. The resultant expression vector was named **pcD-TraR/VP16**. The

nucleotide and amino acid sequence across the junction of the fusion is as follows: AAA CTC ATC GAA TTC GCG TAC AGC and Lys-Leu-Ile-Glu-Phe-Ala-Tyr-Ser, respectively. Lys-Leu-Ile correspond to amino acids 232-234 of the TraR protein. Glu-Phe are encoded by the *EcoRI* restriction site and Ala-Tyr-Ser correspond to the amino acids 363-365 of VP16.

In addition to the full-length VP16 activator domain in other embodiments we fused three minimal activation modules derived from VP16 (F3) either to the N-terminus or to the C-terminus of TraR. These minimal activation modules have been demonstrated to be equally active as the full-length VP16 activation domain (U. Baron, M. Gossen and H. Bujard. 1997. Nucl. Acids Res.: 2723-2729). The amino acid sequence of one minimal activation module is Pro-Ala-Asp-Ala-Leu-Asp-Asp-Phe-Asp-Leu-Asp-Met-Leu and comprises the amino acids 435-447 of VP16. F3 was fused in frame to the C-terminus of TraR using the *EcoRI* restriction site of pCD-TraR/*EcoRI*. The N-terminal *EcoRI* site of F3 and the stop codon were artificially introduced by PCR. The resulting plasmid was called **pcD-TraR/F3**. The nucleotide and amino acid sequence across the junction of the fusion is as follows: AAA CTC ATC GAA TTC CCG GCC GAC and Lys-Leu-Ile-Glu-Phe-Pro-Ala-Asp, respectively. Lys-Leu-Ile correspond to amino acids 232-234 of the TraR protein. Glu-Phe are encoded by the *EcoRI* restriction site and Pro-Ala-Asp correspond to the last three amino acids of F3. For the fusion of F3 to the N-terminus of TraR, a *BamHI* site was introduced at the N-terminus of TraR (**pcD-Bam/TraR**) and at the C-terminus of F3 by PCR. The resulting plasmid was called **pcD-F3/TraR**. The nucleotide and amino acid sequence across the junction of the fusion is as follows: GAC ATG CTC GGA TCC ATG CAG CAC and Asp-Met-Leu-Gly-Ser-Met-Gln-His, respectively. Asp-Met-Leu correspond to the last three amino acids of F3. Gly-Ser are encoded by the *BamHI* restriction site and Met-Gln-His correspond to the first three amino acids of TraR.

EXAMPLE 5

Construction of a N-(3-oxo-octanoyl)-L-homoserine lactone-regulated transcriptional activator fusion protein comprising the TraR protein from *Agrobacterium tumefaciens* and the minimal activation domain F3 derived from VP16 separated from

TraR in different embodiments by different peptide-linkers

Oligonucleotides were designed which introduce different peptides as linkers between the minimal activation domain F3 and the TraR protein either at the C-terminus or at the N-terminus of TraR. A GS-linker is fused to the C-terminus of TraR by PCR using a 5'-oligonucleotide annealing to the internal coding region of TraR and the 3'-oligonucleotide (5'-GACTCTAGATTAACCTCGAGCCTGAACCGCTCCCGGATCCGATGAGTTCCGCCGGATGGC-3') which adds the GS-linker and suitable restriction sites to TraR. The PCR fragment coding for TraR fused to the GS linker is cloned into pcD-TraR by *SacII/XbaI* restriction digest. The resulting plasmid is called **pcD-TraR/GS**. The nucleotide and amino acid sequence at the C-terminus of TraR is as follows:

AAA CTC ATC GGA TCC GGG AGC GGT TCA GGC TCG AGT TAA TCT AGA and Lys-Leu-Ile-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Ser-*, respectively. Lys-Leu-Ile correspond to the last three amino acids of TraR. All other amino acids are encoded by the GS-linker. A second linker is cloned to the C-terminus of TraR by ligation of the two suitable, annealed oligonucleotides into pcD-TraR/GS digested with *BamHI/XhoI* restriction enzymes. This plasmid is called **pcD-TraR/Poly**. The nucleotide sequence of the oligonucleotides are:

oligonucleotide sense:

5'-GATCAGCTCTAGATGGCATGCTGCTAGCTGAGGATCCCCGGGGAGAATTC-3';

oligonucleotide antisense:

5'-TCGAGAATTCTCCCCGGGGATCCTCAGCTAGCAGCATGCCATCTAGAGCT-3'.

The amino acid sequence at the C-terminus of TraR is as follows: Lys-Leu-Ile-Gly-Ser-Ala-Leu-Asp-Gly-Met-Leu-Leu-Ala-Glu-Asp-Pro-Arg-Gly-Glu-Phe-Ser-Ser. Lys-Leu-Ile correspond to the last three amino acids of TraR. All other amino acids are encoded by the Poly-linker.

The C-terminal fusion protein of TraR and F3, separated by the GS linker is obtained by restriction digest of pcD-Tra/F3 with

EcoRI/XbaI and the DNA fragment coding for F3 is ligated into pcD-TraR/GS digested *XhoI/XbaI*. 5'-protruding ends of *EcoRI* and *XhoI* were filled with Klenow polymerase. The resulting plasmid is called **pcD-TraR/GS/F3**.

- 5 The C-terminal fusion protein of TraR and F3, separated by the Poly-linker is obtained by restriction digest of pcD-Tra/F3 with *EcoRI/XbaI* and the DNA fragment coding for F3 is ligated into pcD-TraR/Poly digested *EcoRI/XbaI*. The resulting plasmid is called **pcD-TraR/poly/F3**.

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A similar strategy is used for the N-terminal fusion of F3 to TraR, separated by two different linkers. The N-terminal linkers are similar, but not identical to the C-terminal linkers. Two suitable oligonucleotides are designed such that they are annealed and directly cloned into the *HindIII/BamHI* restriction sites of pcD-F3/TraR. The resulting plasmid is called **pcD-GS2/Tra**. The sequence of the oligonucleotides are:

oligonucleotide sense:

5'-AGCTTTCTAGAATGGGATCCGGGAGCGGTTCTAGCTAGCA-3';

- 20 oligonucleotide antisense:

5'-GATCTGCTAGCTGAACCGCTCCCGGATCCCATTCTAGAA-3'.

The N-terminal amino acid sequence is as follows: Met-Gly-Ser-Gly-Ser-Gly-Ser-Ala-Ser-Arg-Ser-Met-Gln-His. Met-Gly-Ser-Gly-Ser-Gly-Ser-Ala-Ser-Arg-Ser are encoded by the GS2 linker.

- 25 Met-Gln-His represent the first three amino acids of TraR. For the second linker two suitable oligonucleotides are designed such that they are annealed and directly cloned into the *BamHI/NheI* restriction sites of pcD-GS2/TraR. The resulting plasmid is called **pcD-Poly2/TraR**. The sequence of the

oligonucleotides are:

oligonucleotide sense:

5'-GATCCGCTCGAGATGGCAGAATTCAGGAAGGGATATCTGCAG-3';

oligonucleotide antisense:

- 35 5'-CTAGCTGCAGATATCCCTTCCTGAATTCTGCCATCTCGAGCG-3'.

The N-terminal amino acid sequence is as follows: Met-Gly-Ser-Ala-Arg-Asp-Gly-Arg-Ile-Gln-Glu-Gly-Ile-Ser-Ala-Ala-Ser-Arg-Ser-Met-Gln-His. Met-Gly-Ser-Ala-Arg-Asp-Gly-Arg-Ile-Gln-Glu-Gly-Ile-Ser-Ala-Ala-Ser-Arg-Ser are encoded by the Poly2 linker. Met-Gln-His represent the first three amino acids of TraR.

The N-terminal fusion protein of TraR and F3, separated by the GS2 linker is obtained by restriction digest of pcD-F3/TraR with *HindIII*/*BamHI* and the DNA fragment coding for F3 is

ligated into pCD-GS2/TraR digested *HindIII*/*BamHI*. The resulting plasmid is called **pcD-F3/GS2/TraR**. The same strategy is used to clone F3 in frame to the N-terminus of TraR separated by the Poly2 linker, except that the F3-containing DNA fragment is cloned into pcD-Poly2/TraR, digested with *HindIII*/*BamHI*. This plasmid is called **pcD-F3/Poly2/TraR**.

EXAMPLE 6

Construction of a N-(3-oxo-octanoyl)-L-homoserine lactone-regulated transcriptional activator fusion protein comprising the TraR protein from *Agrobacterium tumefaciens* and the human NF-KB p65 activation domain

As an alternative to the *Herpes simplex* virus activation domain VP16 or its derivative F3, the human NF-KB p65 (Accession No. M62399) (M. L. Schmitz and P. A. Baeuerle. 1991. EMBO J. 10: 3805-3817) activation domain is fused in frame either C-terminally or N-terminally to TraR. To this end the coding region of p65 is amplified by PCR using oligonucleotides which introduce restriction sites suitable for cloning and either an *EcoRI* restriction site at the N-terminus of p65 or a *BamHI* restriction site at the C-terminus of p65. These sites can be used for cloning p65 either to the C-terminus of TraR or to the N-terminus, respectively. For the fusion of p65 to the C-terminus of TraR, the plasmids pcD-

TraR/GS and pcD-TraR/Poly are used, which introduce the two different linker-regions described above between TraR and p65. The PCR fragment encoding p65 which contains an *EcoRI* restriction site at its N-terminus is digested with *EcoRI* cloned into pcD-TraR/GS digested *XhoI*. 5'-protruding ends of *EcoRI* and *XhoI* are filled in by Klenow polymerase. The plasmid is called **pcD-TraR/GS/p65**. The nucleotide and amino acid sequence across the junction of the fusion is as follows: AAA CTC ATC GGA TCC GGG AGC GGT TCA GGC TCG AAA TTC CAG TAC CTG and Lys-Leu-Ile-Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Lys-Phe-Gln-Tyr-Leu. Lys-Leu-Ile correspond to the last three amino acids of TraR. Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser represent the GS linker region. Lys-Phe correspond to the filled *XhoI/EcoRI* restriction sites and Gln-Tyr-Leu correspond to the first three amino acids of p65. For cloning p65 to the C-terminus of pcD-TraR/Poly the PCR fragment encoding p65 is digested with *EcoRI* and cloned into pcD-TraR/Poly, digested *EcoRI/XbaI*. The plasmid is called **pcD-TraR/Poly/p65**. 5'-protruding ends of *EcoRI* and *XbaI* are filled in by Klenow polymerase. The nucleotide and amino acid sequence across the junction of the fusion is as follows: AAA CTC ATC GGA TCA GCT CTA GAT GGC ATG CTG CTA GCT GAG GAT CCC CGG GGA GAA TTC CAG TAC CTG and Lys-Leu-Ile-Gly-Ser-Ala-Leu-Asp-Gly-Met-Leu-Leu-Ala-Glu-Asp-Pro-Arg-Gly-Glu-Phe-Gln-Tyr-Leu. Lys-Leu-Ile correspond to the last three amino acids of TraR. Gly-Ser-Ala-Leu-Asp-Gly-Met-Leu-Leu-Ala-Glu-Asp-Pro-Arg-Gly represent the Poly linker region. Glu-Phe correspond to the *EcoRI* restriction site and Gln-Tyr-Leu correspond to the first three amino acids of p65. A similar strategy for cloning p65 to the N-terminus of TraR is used. In this case an ATG codon as the first amino acid of p65 is introduced by PCR. The PCR fragment containing a *BamHI* restriction site at the C-terminus of p65 is digested with *BamHI* and inserted into the vectors pcD-GS2/TraR and pcD-Poly2/TraR, respectively, which have been digested with *HindIII/BamHI*. 5'-protruding ends of *HindIII* are filled in by

Klenow polymerase. The nucleotide and amino acid sequence across the junction of the fusion of **pcD-p65/GS2/TraR** is: ATC AGC TCC GGA TCC GGG AGC GGT TCA GCT AGC AGA TCC ATG CAG CAC and Ile-Ser-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Ala-Ser-Arg-Ser-Met-Gln-His. Ile-Ser-Ser correspond to the last three amino acids of p65. Gly-Ser correspond to the BamHI restriction site, Gly-Ser-Gly-Ser-Ala-Ser-Arg-Ser represent the GS2 linker region and Met-Gln-His are the first three amino acids of TraR. The nucleotide and amino acid sequence across the junction of the fusion of **pcD-p65/Poly2/TraR** is: ATC AGC TCC GGA TCC GCT CGA GAT GGC AGA ATT CAG GAA GGG ATA TCT GCA GCT AGC AGA TCC ATG CAG CAC and Ile-Ser-Ser-Gly-Ser-Ala-Arg-Asp-Gly-Arg-Ile-Gln-Glu-Gly-Ile-Ser-Ala-Ala-Ser-Arg-Ser-Met-Gln-His. Ile-Ser-Ser are the last three amino acids of p65. Gly-Ser correspond to the BamHI restriction site, Ala-Arg-Asp-Gly-Arg-Ile-Gln-Glu-Gly-Ile-Ser-Ala-Ala-Ser-Arg-Ser are encoded by the Poly2 linker and Met-Gln-His represent the first three amino acids of TraR.

EXAMPLE 7

Construction of a N-(3-oxo-octanoyl)-L-homoserine lactone-regulated transcriptional activator fusion protein comprising the TraR protein from *Agrobacterium tumefaciens* and the rat LFB1/HNF1 activation domain

The activation domain of rat LFB1/HNF1 (Accession No. J02170) (Frain et al. 1989. Cell 59: 145-157) is fused in frame to the C-terminus of TraR, leaving two different peptide linkers between the TraR protein and the LFB1/HNF1 activation domain.

pcD-TraR/link4/B1AD - The plasmid contains the coding sequence of the full-length TraR protein from residue M1 to I234 fused to the rat LFB1/HNF1 activation domain (residues A282 to Q628). The cloning introduces four amino acids (Gly-Ser-Ala-Leu) between the C-terminus of TraR and the N-terminus of the

activation domain of LFB1/HNF1. To obtain this construct, a *MscI/BglIII* fragment is excized from the plasmid pB1.2 (rat LFB1/HNF1 cDNA fragment from nucleotide 1 to 3311 inserted in Bluescript KS+; described in Nicosia et al. 1990. Cell 61: 1225-1236) and cloned into the *XbaI/BamHI* sites of the plasmid pcD-TraR/Poly. After cleavage, the *XbaI* site of the pcD-TraR/Poly vector is filled-in in a reaction catalyzed by the Klenow enzyme.

- 10 **PcD-TraR/GS/B1AD** - The plasmid contains the coding sequence of the full-length TraR protein from residue M1 to I234 fused to the N-terminus of rat LFB1/HNF1 activation domain from residue M283 to Q628. The cloning introduces nine amino acids (Gly-Ser-Gly-Ser-Gly-Ser-Gly-Ser-Thr) between the C-terminus of TraR and the N-terminus of LFB1/HNF1. To obtain this construct, a *MscI/BglIII* fragment is excized from the plasmid pB1.2 and cloned into the *XhoI* site of the plasmid pcD-TraR/GS. Both the *BglIII* site of the inserting fragment and the *XhoI* site of the receiving vector are filled-in a reaction catalyzed by the Klenow enzyme.

EXAMPLE 8

- 25 Construction of a fusion protein between TraR and LFB1/HNF1, in which TraR functions as a N-(3-oxo-octanoyl)-L-homoserine lactone regulated dimerization domain in place of the LFB1/HNF1 dimerization domain

- LFB1/HNF1 binds to DNA only as a dimer thereby activating transcription (Nicosia et al. 1990). The dimerization domain is contained within the N-terminal 28 amino acids. Here we replace the LFB1/HNF1 dimerization domain by the full-length TraR protein or different C-terminal deletion mutants of TraR.
- 30 **PcD-TraRf1/B1delDD** - The plasmid contains the coding sequence of the full-length TraR protein from residue M1 to I234 fused

to the N-terminus of rat LFB1/HNF1 from residue P33 to Q628, in place of the LFB1/HNF1 dimerization domain. To obtain this construct, an *SphI/BglIII* fragment is excized from the plasmid LFB1/HNF1 Δ 1-FL and cloned into the *SphI/BamHI* sites of the

5 plasmid pcD-TraR/Poly. The plasmid LFB1/HNF1 Δ 1-FL derives from the construct LFB1mutant Δ 1 described by Nicosia et al. 1990 (Cell 61: 1225-1236), however, contains the sequence coding for the entire C-terminal activation domain of LFB1/HNF1

10 protein followed by 220 nt of the 3'-non coding sequence of the LFB1/HNF1 cDNA. The cloning introduces a 7 aminoacids long spacer (Gly-Ser-Ala-Leu-Asp-Gly-Met) between the TraR and LFB1 sequences.

PcD-TraR182/B1delDD - The plasmid contains the coding sequence

15 of the TraR protein from residue M1 to L182 fused to the N-terminus of rat LFB1/HNF1 from residue P33 to Q628, in place of the LFB1/HNF1 dimerization domain. An oligonucleotide comprising the sequence from nt 495 to nt 546 of the TraR open reading frame (oligo TRA166-182sense) is annealed to an

20 oligonucleotide with the complementary sequence (oligo TRA166-182rev). The resulting double-strand oligonucleotide is digested with the *SacII* restriction enzyme and cloned into the *SacII/SphI* sites of the plasmid pcD-TraRfl-B1delDD. The *SphI* of the receiving vector is filled-in in a reaction catalyzed

25 by the Klenow enzyme. The sequences of the oligonucleotide TRA166-182sense and the oligo TRA166-182rev are as follows:

oligo TRA166-182sense:

5'- CCTACCGCGGAAGATGCCGCATGGCTCGATCCGAAGGAGGCCAGCCTATCTG - 3'

oligo TRA166-182rev:

30 5'- CAGATAGGTGGCCTCCTTCGGATCGAGCCATGCGGCATCTTCCGCGGTAGG - 3'

pcD-TraR167/B1delDD - The plasmid contains the coding sequence

of the TraR protein from residue M1 to T167 fused to the N-terminus of rat LFB1/HNF1 from residue P33 to Q628, in place

35 of the LFB1/HNF1 dimerization domain. To obtain this

construct, an *SphI*/*BglIII* fragment is excized from the plasmid LFB1/HNF1 Δ 1-FL and cloned into the *SacII*/*BamHI* sites of the plasmid pcD-TraR. Both the *SphI* and *SacII* sites are flushed in a reaction catalyzed by the Klenow enzyme.

EXAMPLE 9

Insertion of a nuclear localization signal (NLS) into the fusion protein of TraR and an eukaryotic activation domain

The nuclear localization signal (NLS) Met-Pro-Lys-Arg-Pro-Arg-Pro is added to the TraR fusion protein in order to promote the transport of the protein to the cell nucleus.

Oligonucleotides encoding the above mentioned peptide sequence and restriction sites suitable for cloning into the expression vectors are designed.

For the addition of the NLS to the N-terminus of TraR the two following oligonucleotides are annealed and directly cloned into the *HindIII*/*BamHI* restriction sites of pcD-Bam/TraR:

oligonucleotide-sense: 5'-AGCTTATGCCCAAAGACCACGCCCTG-3';

oligonucleotide-antisense: 5'-GATCCAGGGCGTGGTCTTTTGGGCATA-3'.

The resulting plasmid is called **pcD-NLS/TraR**. The DNA fragment coding for TraR containing the NLS at its N-terminus is cloned into any of the above mentioned vectors expressing the fusion proteins between TraR and C-terminal activation domains by *HindIII*/*SacII* restriction digest.

For the addition of the NLS to the C-terminus of TraR the two following oligonucleotides are annealed and directly cloned into the *EcoRI*/*XbaI* restriction sites of pcD-TraR/*EcoRI*:

oligonucleotide-sense: 5'-AATTCCTCCCAAAGACCACGCCCTTAAT-3';

oligonucleotide-antisense: 5'-CTAGATTAAGGGCGTGGTCTTTTGGGG-3'.

The resulting plasmid is called **pcD- TraR/NLS**. The DNA

fragment coding for TraR containing the NLS at its C-terminus

is cloned into any of the above mentioned vectors expressing the fusion proteins between TraR and N-terminal activation domains by *XbaI/SacII* restriction digest. The NLS sequence can also be inserted into the linker regions between TraR and the activation domain. The two following oligonucleotides are annealed and directly cloned into the *EcoRI* restriction site of pcD-p65/Poly2/TraR:

oligonucleotide-sense: 5'-AATTCAGCCCAAAGACCACGCCCTGG-3';

oligonucleotide-antisense: 5'-AATTCCAGGGCGTGGTCTTTTGGGCTG-3'.

The resulting plasmid is called **pcD-p65/NLS/TraR**.

EXAMPLE 10

Construction of a plasmid for N-(3-oxo-octanoyl)-L-homoserine lactone-dependent expression of secreted alkaline phosphatase (SEAP) in eukaryotic cells

The heptamerized tet-operators present in the plasmid pOr/EPO (described in Rizzuto et al. 1999. Proc. Natl. Acad. Sci. U.S.A. 96: pp6417-6422) are removed by restriction digest with *NdeI/KpnI*. In place of the tet-operators we clone a DNA fragment containing the 18 bp inverted repeat sequence defined as DNA binding site of TraR (C. Fuqua and S.C. Winans. 1996. J. Bacteriol. 178: 435-440). This was obtained by annealing the two complementary oligonucleotides (upper strand: 5'-TATGCTGAAAGGGAATGTGCAGATCTGCACATCGGCAACGCGGTAC-3'; lower strand: 5'-CGCGTTGCCGATGTGCAGATCTGCACATTCCTTTTCAGCA-3') and cloning directly into the digested vector pOr/EPO. Upon annealing the two complementary strand expose the compatible protruding ends of *NdeI* and *KpnI*. This vector is called **p-trabox/EPO**. A DNA fragment containing the CMV minimal promoter and the trabox is excised from the plasmid p-trabox/EPO by restriction digest using the enzymes *AatII/EcoRI*. 3'-protruding ends produced by *AatII* are removed by T4-DNA polymerase and the DNA fragment is cloned into the eukaryotic

expression vector pSEAP2-Basic (Clontech), digested with *NheI/EcoRI*. The 5'-protruding ends of the *NheI* restriction site are filled in by Klenow polymerase. The resulting plasmid is called **p-tra-box/SEAP2**.

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EXAMPLE 11

The DNA-binding activity of TraR expressed *in vitro* in rabbit reticulocyte lysate strictly depends on the presence of N-(3-oxo-octanoyl)-L-homoserine lactone

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PcD-TraR, linearized with the restriction enzyme *XbaI*, was taken as a DNA template for *in vitro* transcription using T7 RNA polymerase. The RNA transcript was subsequently used for *in vitro* translation with nuclease-treated rabbit reticulocyte lysate in the presence of ³⁵S-labelled methionine. *In vitro* translation was performed either in the presence or in the absence of 10 μM N-(3-oxo-octanoyl)-L-homoserine lactone.

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TraR was translated equally well under both conditions.

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TraR translated *in vitro* was then tested for its DNA-binding activity on oligonucleotides containing the DNA sequence of the tra-box. For this experiment two complementary oligonucleotides (described in the section above) were annealed and labelled at the 5'-end of the oligonucleotides with T4-polynucleotide kinase using [γ-³²P]ATP. 40 fmoles of the resulting duplex were added to a reaction mixture containing 25 mM Hepes pH 7.5, 1 mM DTT, 2 % glycerol, 5 mM EDTA, 100 mM NaCl, 100 ng poly[d(I.C)-d(I.C)] with (+) or without (-) 10 μM N-(3-oxo-octanoyl)-L-homoserine lactone in a total volume of 20 μl. 8 μl of TraR translated *in vitro* were then added to the mixture and allowed to stand at room temperature for 30 min. 5 μl of the mixture were loaded onto a

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30

6 % nondenaturing polyacrylamid gel containing 0.25 x TBE (Maniatis). After electrophoresis at 10 V/cm the gel was dried and autoradiographed.

5 The results show that only TraR translated *in vitro* in the presence of N-(3-oxo-octanoyl)-L-homoserine lactone is capable of binding to the tra-box. Binding to the DNA cannot be further stimulated by the addition of N-(3-oxo-octanoyl)-L-homoserine lactone to the DNA binding reaction mixture. TraR
10 translated without N-(3-oxo-octanoyl)-L-homoserine lactone is unable to bind DNA, even if N-(3-oxo-octanoyl)-L-homoserine lactone is added to the DNA-binding reaction.

EXAMPLE 12

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The DNA-binding activity of TraR expressed in Hep3B cells using recombinant vaccinia virus vTF7-3 depends on the presence of N-(3-oxo-octanoyl)-L-homoserine lactone

20 Hep3B cells (5.5×10^5 in 60-mm-diameter dishes) were infected with vaccinia virus vTF7-3 for 1 hour at 37°C and then transfected with 20 µg of pcD-TraR or 20 µg of control vector (pcDNA3) by the calcium precipitation technique. Transfection was performed in the presence (+) or absence (-) of 10 µM N-
25 (3-oxo-octanoyl)-L-homoserine lactone. After 6 hours of transfection, cells were harvested and cell extract was prepared in 150 µl of lysis buffer (25 mM Tris pH 8.0, 300 mM NaCl, 20 % glycerol, 2 mM DTT, 2 mM PMSF, 1 % Triton-X100). 80 µg of total protein were loaded onto a 12 % SDS-PAGE, proteins
30 were transferred onto nitrocellulose and Western Blotting was performed using the SuperSignal West Pico system (Pierce). The specific antibody reacting with the TraR protein was prepared by ourselves.

35 TraR protein was expressed at comparable amounts when

transfected in the presence or absence of N-(3-oxo-octanoyl)-L-homoserine lactone. No signal was present in the control-transfection. Purified TraR was employed as a positive control. A non-specific cellular protein reacted with the α -TraR antibody.

Cellular extracts were then tested for DNA binding activity. Binding reaction was performed exactly as described above, except that 1 μ g of poly[d(I.C)-d(I.C)] and 10 μ l of cellular extract were used. No NaCl was added to the reaction mix. As already demonstrated above for TraR translated *in vitro*, TraR expressed in Hep3B cells only bound to DNA, when N-(3-oxo-octanoyl)-L-homoserine lactone was present during transfection. No DNA-binding was detectable when TraR was expressed without ligand, even if N-(3-oxo-octanoyl)-L-homoserine lactone was added during the DNA-binding reaction.

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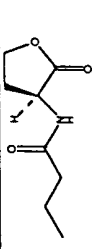
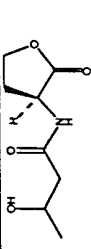
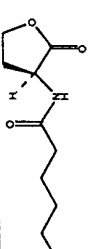
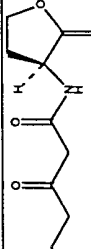
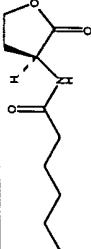
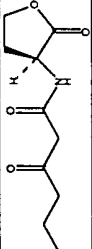
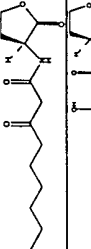
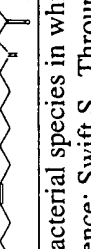
SdiA

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24. Blatner et al., Direct Submission to EMBL/GenBank/DDBJ
data banks
25. Itoh et al., DNA Res. 3(6), 379-392 (1996)
26. Wang et al., EMBO J. 10(11), 3363-3372 (1991)

Table I

Bacterial species	Signal molecules	Regulatory Protein	References	SwissProt/ NCBI Accession #
<i>Vibrio fischeri</i>	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	LuxR	1-5	P12746 P35327
<i>Pseudomonas aeruginosa</i>	N-3-(oxododecanoyl)-L-homoserine lactone (PAI-1)	LasR	6,7	P25084
<i>Pseudomonas aeruginosa</i>	N-(butyryl)-L-homoserine lactone (PAI-2)	RhlR	8-12	P54292
<i>Pseudomonas aurcofaciens</i>	(PRAI, not characterized)	PhzR	13	P54303
<i>Agrobacterium tumefaciens</i>	N-3-(oxooctanoyl)-L-homoserine lactone (AAI)	TraR	14, 15	P54294 P33905
<i>Erwinia carotovora</i> subsp. Carotovora SCRI193	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	ExpR	16	Q47189
<i>Erwinia carotovora</i> subsp. Carotovora SCC3193	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	CarR	17	Q46751
<i>Erwinia stewartii</i>	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	EsaR	18	P54293
<i>Rhizobium leguminosarum</i>	N-(3R-hydroxy-7-cis-tetradecanoyl)-L-homoserine lactone (RLAI)	RhlR	19	Q03316
<i>Enterobacter agglomerans</i>	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	EagR	20	
<i>Yersenia enterocolifica</i>	N-3-(oxohexanoyl)-homoserine lactone (VAI-1)	YenR	21	P54295
<i>Aeromonas hydrophila</i>	(AHAI, not characterized)	AhyR	22	CAA61654
<i>Escherichia coli</i>		SdiA	23-26	P07026

TABLE II

Chemical Formula	Name	Abbreviation	Microorganism
	N-butanoyl-L-homoserine lactone	PAI-2 or BHL	<i>Pseudomonas aeruginosa</i> <i>Serratia liquefacens</i>
	N-(3-hydroxy)-butanoyl-L-homoserine lactone	HAI-1 or HBHL	<i>Vibrio harveyi</i>
	N-hexanoyl-L-homoserine lactone	HHL	<i>Chromobacterium violaceum</i> <i>Yersinia enterocolitica</i> <i>Pseudomonas aeruginosa</i>
	N-(3-oxo)-hexanoyl-L-homoserine lactone	VAI-1 or OHHL	<i>Vibrio fischeri</i> <i>Erwinia carotovora</i> <i>Erwinia stewartii</i> <i>Nitrosomas europea</i> <i>Enterobacter agglomerans</i> <i>Yersinia enterocolitica</i>
	N-octanoyl-L-homoserine lactone	VAI-2 or OHL	<i>Vibrio fischeri</i>
	N-(3-oxo)-octanoyl-L-homoserine lactone	AAI-1 or OOHHL	<i>Vibrio fischeri</i> <i>Agrobacter tumefaciens</i>
	N-(3-oxo)-dodecanoyl-L-homoserine lactone	PAI-1 or OdDHL	<i>Pseudomonas aeruginosa</i>
	N-(3-hydroxy-7 <i>cis</i>)-tetradecenoyl-L-homoserine lactone	RLAI or HtDeHI	<i>Rhizobium leguminosarum</i>

The bacterial species in which the production and activities of ASHL autoinducers have been demonstrated are indicated.

Reference: Swift S., Throup J.P., Williams P., Salmond G.P.C. and Stewart G.S.A.B (1996) Trends Biochem. Sci. 21:214-219.

CLAIMS:

1. A transcriptional activator which comprises a fusion protein, the fusion protein comprising at least two polypeptide components:

(i) a first polypeptide component which binds in a sequence specific manner to an operator sequence in DNA; and

(ii) a second polypeptide component, comprising a luxR-type transcription factor regulatory domain which binds cognate AcylHSL or an analogue thereof, whereby upon binding of the AcylHSL or analogue the DNA binding function of the first polypeptide component is activated;

the transcriptional activator additionally comprising a third polypeptide component which activates transcription in eukaryotic cells.

2. A transcriptional activator according to claim 1 wherein said fusion protein comprises said third polypeptide component.

3. A transcriptional activator according to claim 1 or claim 2 wherein the first and/or second polypeptide components are derived from a bacterial LuxR-type transcription factor.

4. A transcriptional activator according to claim 3 wherein the first and second polypeptide components are derived from a LuxR-type transcription factor.

5. A transcriptional activator according to claim 4 wherein the first and second polypeptide components are both derived from the *Vibrio fischeri* LuxR protein.

6. A transcriptional activator according to claim 4 or claim 5 wherein the first component comprises residues 160-250 of *Vibrio fischeri* LuxR protein and/or the second component comprises residues 20-159 of *Vibrio fischeri* LuxR protein.

7. A transcriptional activator according to claim 1 or claim 2 wherein the first polypeptide component is derived from the DNA binding domain of a protein other than a LuxR transcription factor.

5

8. A transcriptional activator according to claim 7 wherein the first polypeptide component comprises a DNA-binding portion of a eukaryotic transcription factor.

10 9. A transcriptional activator according to claim 8 wherein the first polypeptide component comprises a DNA-binding portion of hHNF-1.

15 10. A transcriptional activator according to claim 9 wherein the first polypeptide component comprises residues 1-281 of hHNF-1.

11. A transcriptional activator according to claim 9 or claim 10 wherein the fusion protein comprises a DNA-binding portion of hHNF-1 fused to a regulatory domain of a LuxR-type transcription factor, providing AcylHSL-inducibility of DNA-binding.

20 12. A transcriptional activator according to claim 3 comprising a TraR DNA-binding domain.

13. A transcriptional activator according to claim 12 wherein the third polypeptide component comprises a transcriptional activation portion of LFB1/HNF1.

30

14. A transcriptional activator according to claim 12 wherein the third polypeptide component comprises a transcriptional activation portion NF- κ B p65.

35 15. A transcriptional activator according to any one of claims 1 to 12 wherein the third polypeptide component comprises a transcriptional activation portion of herpes

simplex virus virion protein 16 (VP16).

16. A transcriptional activator according to claim 15 comprising the 127 C-terminal amino acids of VP16.

5

17. A transcriptional activator according to claim 15 comprising amino acids 437-447 of VP16.

18. A transcriptional activator according to claim 17 comprising a multimer of amino acids 437-447 of VP16.

10

19. A transcriptional activator according to any one of claims 2 to 18 wherein said fusion protein comprises a fourth polypeptide component which comprises a nuclear localization signal (NLS).

15

20. A transcriptional activator according to any one of claims 1 to 19 wherein said fusion protein comprises one or more spacers between any of said polypeptide components.

20

21. A transcriptional activator according to claim 20 comprising a GS spacer.

22. Nucleic acid encoding the fusion protein of a transcriptional activator according to any one of claims 1 to 21.

25

23. A nucleic acid vector comprising nucleic acid according to claim 22.

30

24. A nucleic acid vector according to claim 23 wherein the nucleic acid encoding the fusion protein is under control of regulatory sequences for expression of the fusion protein.

25. A host cell transformed with a nucleic acid vector according to claim 24.

35

26. A method of making a transcriptional activator, the method comprising culturing a host cell according to claim 25 under conditions for production of the transcriptional activator.

5

27. A method of stimulating transcription, the method comprising binding a transcriptional activator according to any one of claims 1 to 21 to an operator sequence operatively linked to a target nucleotide sequence.

10

28. A method according to claim 27 wherein said binding occurs within a host cell, the method comprising treating the host cell with AcylHSL or an analogue thereof to activate binding of the transcriptional activator to the operator sequence.

15

29. A method according to claim 28 wherein said host cell is cultured *in vitro* in a medium containing AcylHSL or analogue thereof.

20

30. A method according to claim 28 or claim 29 wherein said AcylHSL is N-(3-oxohexanoyl)-L homoserine lactone or N-(3-oxooctanoyl)-L homoserine lactone.

25

31. A method according to any one of claims 27 to 30 wherein the target nucleotide sequence encodes a product polypeptide.

32. A method according to claim 31 wherein the polypeptide is produced by expression from the target nucleotide sequence, the method further comprising isolating and/or purifying the product polypeptide.

30

33. A method according to claim 32 wherein the product polypeptide is formulated into a composition comprising at least one additional component.

35

34. A method according to any one of claims 27 to 30 wherein

the target nucleotide sequence provides, on transcription, an antisense sequence.

35. A method according to any one of claims 27 to 30 wherein the target nucleotide sequence provides, on transcription, a ribozyme.

36. A composition comprising:

(i) a transcriptional activator according to any one of claims 1 to 21, or first nucleic acid encoding said transcriptional activator; and

(ii) a second nucleic acid which comprises a target nucleotide sequence to be transcribed operatively linked to a AcylHSL-regulated transcription unit.

37. A composition according to claim 36 comprising said first nucleic acid.

38. A composition according to claim 37 wherein said first nucleic acid encodes a fusion protein comprising said first, second and third polypeptide components.

39. A composition according to claim 37 wherein said first nucleic acid comprises separate sequences encoding (i) a fusion which comprises said first and second polypeptide components and (ii) a third polypeptide to interact with the fusion to provide the transcriptional activator.

40. A composition according to claim 39 wherein said separate sequences are within separate nucleic acid molecules.

41. A composition according to claim any one of claims 37 to 40 wherein said first and second nucleic acids are separate molecules.

42. A host cell comprising a composition according to any one of claims 36 to 41.

Figure 1

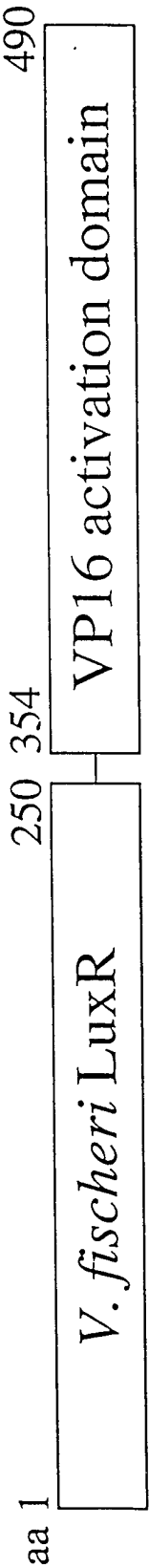


Figure 2

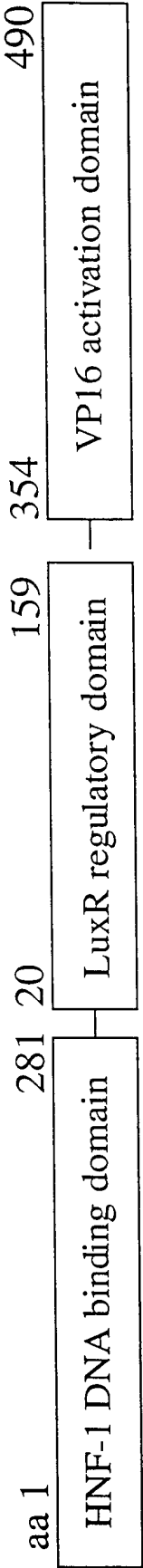


Figure 3

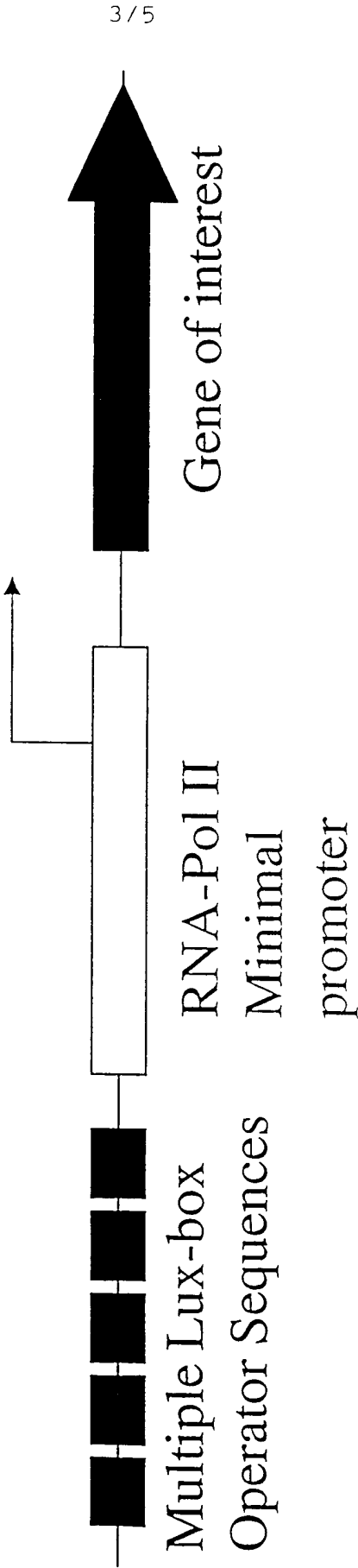


Figure 4

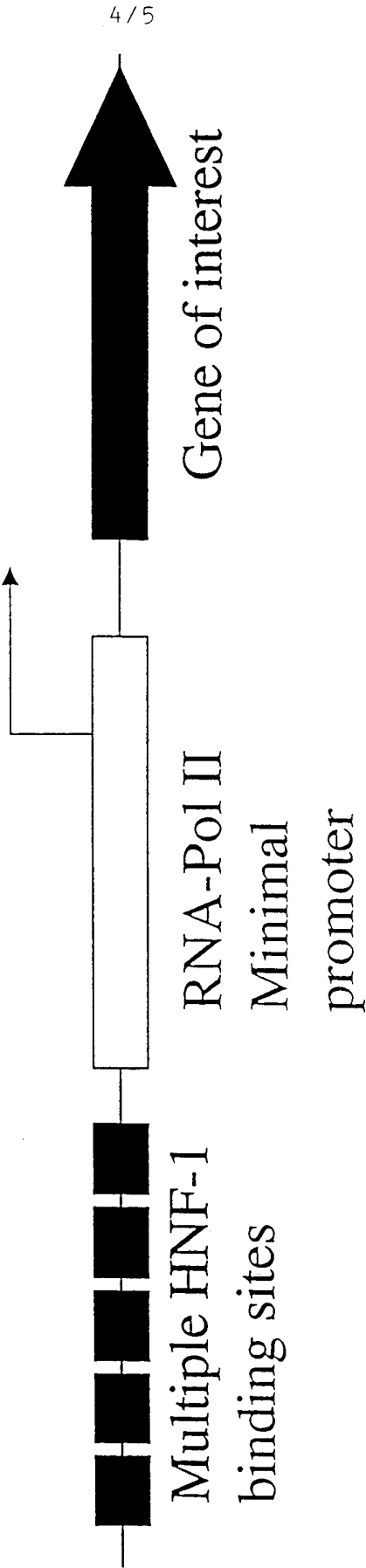


Figure 5

