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(71) Applicant (for all designated States except US): BAYER HEALTHCARE AG [DE/DE]; 51368 Leverkusen (DE).

(72) Inventors; and

(75) Inventors/Applicants (US only): GOLZ, Stefan [DE/DE]; Bückmannsmühle 46, 45326 Essen (DE). MARKOVA, Svetlana [RU/RU]; Akademgorodok, 12a-68, Krasnoyarsk, 660036 (RU). BURAKOVA, Ludmila [RU/RU]; Solnechnaya St., 9-32, Sosnovoborsk, 662500 (RU). FRANK, Ludmila [RU/RU]; Akademgorodok, 19-82, Krasnoyarsk, 660036 (RU). VYSOTSKI, Eugene [RU/RU]; Lado Kechoveli St., 35-108, Krasnoyarsk, 660100 (RU).

(74) Joint Representative: BAYER HEALTHCARE AG; Law & Patents, Patents & Licensing, 51368 Leverkusen (DE).

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(54) Title: ISOLATED FLUORESCENT PROTEIN FROM *CLYTIA GREGARIA* CGFP AND USE THEREOF

(54) Bezeichnung: ISOLIERTES FLUORESZIERENDES PROTEIN AUS *CLYTIA GREGARIA* CGFP, SOWIE DESSEN VERWENDUNG

(57) Abstract: The invention relates to the nucleotide and amino acid sequence, in addition to the activity and use of the fluorescent protein CGFP (fluorescent protein from *clytia gregaria*).

(57) Zusammenfassung: Die Erfindung betrifft die Nukleotid- und Aminosäuresequenz, sowie die Aktivität und Verwendung des fluoreszierenden Proteins CGFP (fluoreszierendes Protein aus *clytiagregaria*).

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**ISOLATED FLUORESCENT PROTEIN FROM CLYTIA GREGARIA
(CGFP) AND USE THEREOF**

5 The invention relates to the nucleotide and amino acid sequences and to the activity and use of the fluorescent protein CGFP (fluorescence protein of clytia gregaria).

Fluorescent proteins

10 A multiplicity of coelenterates are bioluminescent (Morin et al., 1974) and emit blue or green light. Aequoria victoria aequorin which was identified as the first light-producing protein in 1962 (Shimomura et al., 1962) emitted, as an isolated protein, a blue light, rather than the phenotypically observed green light of Aequoria victoria. Later, the green fluorescent protein (GFP) was isolated from Aequoria victoria, which, owing to the excitation by aequorin, makes the medusa appear green
15 phenotypically (Johnson et al, 1962; Hastings et al., 1969; Inouye et al, 1994).

Green fluorescent proteins have been isolated from different organisms. These include the Hydozoa (aequoria, halistaura obelia) and anthropods (acanthotilum, sea cactus, cavernularia, renila, ptilosarcus, stylatula) (Morin et al., 1971; Morin et al.,
20 1971 II, Wampler et al., 1971, Wampler et al., 1973, Cormier et al., 1973, Cormier et al., 1974, Levine et al., 1982).

A compilation of some fluorescent proteins can be found in Table 1:

Table 1:

Overview over some fluorescent proteins. Indicated are the name, the organism from which the protein has been isolated and the identification number (Acc. No.) of the database entry.

Name	Organism	Identification no.
Green fluorescent protein	Aequorea macrodactyla	AF435433
Green fluorescent protein	Aequoria	L29345
Green fluorescent protein-like protein	Agaricia agaricites	AY037775
Green fluorescent protein-like protein	Agaricia fragilis	AY037765
Green fluorescent protein	Dendronephthya	AF420591
Red fluorescent protein	Entacmaea quadricolor	AY130757
Green fluorescent protein-like protein	Caribbean anemone	AY037777
Green fluorescent protein	Heteractis crispa	AF420592
Green fluorescent protein-like protein	Montastraea annularis	AY037766
Green fluorescent protein-like protein	Montastraea cavernosa	AY037768
Cyan fluorescent protein	Montastraea cavernosa	AY056460
Green fluorescent protein	Renilla muelleri	AY015996
Green fluorescent protein	Renilla reniformis	AF372525
Green fluorescent protein-like protein	Ricordea florida	AY037774

The fluorescent proteins differ not only due to their nucleotide and amino acid sequences but also due to their biochemical and physical properties. The spectral characteristics of the fluorescent proteins may differ both on the side of excitation and on the side of emission. An overview of the fluorescence spectra and the excitation wavelength can be found in Table 2.

Table 2:

Overview over some fluorescent proteins. Indicated are the organism from which the protein has been isolated, the excitation wavelength and emission wavelength determined in spectral analyses.

Organism	Excitation	Fluorescence
Aequoria	465-498 nm	509 nm
Halistaura	465 nm	497 nm
Phialidium	485 nm	498 nm
Renilla	498 nm	508 nm

The use of fluorescent proteins has already been described previously. An overview can be found in Table 3:

Table 3:

Overview over some fluorescent proteins. Indicated are the organism from which the protein has been isolated, the name of the fluorescent protein and a selection of patents or applications.

Organism	Fluorescent protein	Patent / application
Renilla mulleri	GFP	US patent no. 6,436,682 WO200168824 WO200257451 WO200134824 WO9949019 US patent no. 6,232,107

Organism	Fluorescent protein	Patent / application
Aequoria	GFP	WO200071565 WO9711094 WO9623898 US patent no. 5,958,713 US patent no. 6,172,188

5 It was shown that it is possible to alter the physical and biochemical properties of fluorescent proteins by altering the amino acid sequence thereof. Examples of mutagenized fluorescent proteins have been described in the literature (Delagrave et al., 1995; Ehrig et al., 1995; Heim et al., 1996).

10 Fluorescent proteins are already used in a wide variety of areas. The use of fluorescent proteins in 'Fluorescence Resonance Energy Transfer' (FRET), 'Bioluminescence Resonance Energy Transfer (BRET) and other energy transfer methods has already been described in the literature (Mitra et al., 1996; Ward et al., 1978; Cardullo et al, 1988; US patent no. 4,777,128; US patent no. 5,126,508; US patent no. 4,927,923; US patent no. 5,279,943). Further nonradioactive methods of energy transfer by means of GFP have likewise been described previously (PCT appl. WO 98/02571 and WO 97/28261)

15

Reporter systems

20 Reporter gene or indicator gene generally refers to genes whose gene products can be readily detected with the aid of simple biochemical or histochemical methods. At least 2 types of reporter genes are distinguished.

1. Resistance genes. Resistance genes refer to genes whose expression imparts to a cell the resistance to antibiotics or other substances whose presence in the growth medium leads to cell death when the resistance gene is absent.
- 5 2. Reporter gene. The products of reporter genes are used as fused or nonfused indicators in genetic engineering. The most commonly used reporter genes include beta-galactosidase (Alam et al., 1990), alkaline phosphatase (Yang et al., 1997; Cullen et al., 1992), luciferases and other photoproteins (Shinomura, 1985; Phillips GN, 1997; Snowdowne et al., 1984).

10

Luminescence refers to the emission of photons in the visible spectral range, said emission being effected by excited emitter molecules. In contrast to fluorescence, the energy is supplied here not from the outside in the form of radiation of shorter wavelength.

15

A distinction is made between chemiluminescence and bioluminescence. Chemiluminescence refers to a chemical reaction resulting in an excited molecule which luminesces itself when the excited electrons return to the ground state. If this reaction is catalyzed by an enzyme, then this process is called bioluminescence. The enzymes involved in the reaction are generally referred to as luciferases.

20

Classification of the species *Clytia gregaria*

Cnidaria→ Leptomedusae→ Campanulariidae→ *Clytia gregaria*

25

The species *Clytia gregaria* belongs to the cnidaria, especially to the medusae. The bioluminescent or fluorescent phenotype has already been described in 1998 (Ward et al., 1998).

Isolation of cDNA

In order to study the fluorescent activity of the species *Clytia gregaria*, specimens were caught in Friday Harbor in Washington State (USA) and stored in liquid nitrogen. The cDNA library was prepared using exclusively the bioluminescent ring of one medusa specimen. The *Clytia gregaria* cDNA libraries were generated by isolating the RNA by means of isothiocyanate according to the method of Krieg (Krieg et al., 1996).

The cDNA was prepared by carrying out an RT-PCR. To this end, 10 µg of RNA were incubated with reverse transcriptase (Superscript Gold II) according to the following plan:

PCR	1.	30	seconds	95°C
	2.	6	minutes	68°C
	3.	10	seconds	95°C
	4.	6	minutes	68°C

17 cycles of step 4 after step 3

In order to inactivate the polymerase, the reaction products were incubated at 37°C with proteinase K for 30 minutes and cDNA was precipitated with ethanol. The cDNA was dissolved in water and incubated with SfiI at 37°C for one hour. The reaction products were gel-filtrated in order to remove small fragments. The fractionated cDNA was then ligated into the SfiI-cut and dephosphorylated λTriplex2 vector. A λ-phage expression library was then prepared by packing the cloned cDNA fragments into λ phages by means of the in vitro packing system SMART cDNA Library Construction Kits (Clontech).

The recombinant phages containing a cDNA insertion with potential expression of fluorescent proteins were identified by carrying out a „library screening“.

5 To this end, bacterial lawns of transformed E. coli XL1-Blue were plated on 90 mm culture dishes and incubated at 31°C for 12-15 hours. Induction of protein expression was started by adding to the plates 60 µl of a 20 mM IPTG (isopropylthiogalactoside) solution. After 24 hours of incubation at room temperature, the plates were stored at 4 °C for 72 hours. This was followed by measuring the fluorescence.

10 To this end, the bacteria were irradiated using an argon laser (LGN502) with 100 mV at 488 nm or 366 nm (UVL21). The fluorescence was measured using a 510 nm ZSV filter.

15 To isolate the clones and for spectral analysis, the biomass of fluorescence-positive clones was removed from the culture plates and resuspended in PBS (phosphate buffered saline). The cells were disrupted by means of ultrasound. After clarifying the lysate by centrifugation, the fluorescence of the supernatant was determined in a fluorimeter.

20 A fluorescent protein was identified. The fluorescent protein was referred to as CGFP (fluorescence protein of clytia gregaria). The fluorescent protein CGFP is illustrated in detail below.

CGFP

25

The fluorescent protein CGFP shows the highest homology at the amino acid level to Aequoria GFP with an identity of 44% (depicted in Example 8; Figure 5). At the nucleic acid level, the identity is below 30% (depicted in Example 9; Figure 6). The BLAST method was used for sequence comparison (Altschul et al., 1997).

30

The invention also relates to functional equivalents of CGFP. Functional equivalents are those proteins which have comparable physicochemical properties and which are at least 70% homologous. Preference is given to a homology of 80% or 90%. Particular preference is given to a homology of 95%.

5

The fluorescent protein CGFP is suitable as a reporter gene for cellular systems, especially for receptors, for ion channels, for transporters, for transcription factors or for inducible systems.

10

The fluorescent protein CGFP is suitable as a reporter gene in bacterial and eukaryotic systems, especially in mammalian cells, in bacteria, in yeasts, in baculo, in plants.

15

The fluorescent protein CGFP is suitable as a reporter gene for cellular systems in combination with bioluminescent or chemiluminescent systems, especially systems with luciferases, with oxygenases, with phosphatases.

20

The fluorescent protein CGFP is suitable as a marker protein, especially in FACS (fluorescence activated cell sorter) sorting.

25

The fluorescent protein CGFP is suitable as a fusion protein, especially for receptors, for ion channels, for transporters, for transcription factors, for proteinases, for kinases, for phosphodiesterases, for hydrolases, for peptidases, for transferases, for membrane proteins, for glycoproteins.

30

The fluorescent protein CGFP is suitable for immobilization, especially by antibodies, by biotin, by magnetic or magnetizable carriers.

The fluorescent protein CGFP is suitable as a protein for energy transfer systems, especially the FRET (Fluorescence Resonance Energy Transfer), BRET

(Bioluminescence Resonance Energy Transfer), FET (field effect transistors), FP (fluorescence polarization), HTRF (Homogeneous time-resolved fluorescence) systems.

- 5 The fluorescent protein CGFP is suitable as a label of substrates or ligands, especially for proteases, for kinases, for transferases.

10 The fluorescent protein CGFP is suitable for expression in bacterial systems, especially for titer determination, as substrates for biochemical systems, especially for proteinases and kinases.

15 The fluorescent protein CGFP is suitable as a marker, especially coupled to antibodies, coupled to enzymes, coupled to receptors, coupled to ion channels and other proteins.

20 The fluorescent protein CGFP is suitable as a reporter gene in pharmacological drug screening, especially in HTS (High Throughput Screening).

25 The fluorescent protein CGFP is suitable as a component of detection systems, especially for ELISA (enzyme-linked immunosorbent assay), for immunohistochemistry, for Western blotting, for confocal microscopy.

30 The fluorescent protein CGFP is suitable as a marker for the analysis of interactions, especially for protein-protein interactions, for DNA-protein interactions, for DNA-RNA interactions, for RNA-RNA interactions, for RNA-protein interactions (DNA : deoxyribonucleic acid; RNA : ribonucleic acid).

35 The fluorescent protein CGFP is suitable as a marker or fusion protein for expression in transgenic organisms, especially in mice, in rats, in hamsters and other mammals, in primates, in fish, in worms, in plants.

The fluorescent protein CGFP is suitable as a marker or fusion protein for analyzing embryonic development.

- 5 The fluorescent protein CGFP is suitable as a marker via a coupling mediator, especially via biotin, via NHS (N-hydroxysulfosuccimides), via CN-Br.

The fluorescent protein CGFP is suitable as a reporter coupled to nucleic acids, especially to DNA, to RNA.

10

The fluorescent protein CGFP is suitable as a reporter coupled to proteins or peptides.

- 15 The fluorescent protein CGFP coupled to nucleic acids or peptides is suitable as a probe, especially for Northern blots, for Southern blots, for Western blots, for ELISA, for nucleic acid sequencings, for protein analyses, chip analyses.

The fluorescent protein CGFP is suitable as a label of pharmacological formulations, especially of infectious agents, of antibodies, of small molecules.

20

The fluorescent protein CGFP is suitable for geological studies, especially for sea currents, groundwater currents and river currents.

- 25 The fluorescent protein CGFP is suitable for expression in expression systems, especially in in-vitro translation systems, in bacterial systems, in yeast systems, in baculo systems, in viral systems, in eukaryotic systems.

The fluorescent protein CGFP is suitable for visualizing tissues or cells in surgical procedures, especially in invasive, in noninvasive, in minimally invasive procedures.

30

The fluorescent protein CGFP is also suitable for labeling tumor tissues and other phenotypically altered tissues, especially in histological examination, in surgical interventions.

- 5 The invention also relates to purifying the fluorescent protein CGFP, especially as wild-type protein, as fusion protein, as mutagenized protein.

10 The invention also relates to the use of the fluorescent protein CGFP in the field of cosmetics, especially of bath additives, of lotions, of soaps, of body paints, of toothpaste, of body powders.

The invention also relates to the use of the fluorescent protein CGFP for coloring, especially of food, of bath additives, of ink, of textiles, of plastics.

- 15 The invention also relates to the use of the fluorescent protein CGFP for the coloring of paper, especially of greeting cards, of paper products, of wallpapers, of handicraft articles.

20 The invention also relates to the use of the fluorescent protein CGFP for the coloring of liquids, especially for water pistols, for fountains, for beverages, for ice cream.

The invention also relates to the use of the fluorescent protein CGFP for producing toys, especially finger paints, face paints.

- 25 The invention relates furthermore to nucleic acid molecules, selected from the group consisting of

- a) nucleic acid molecules encoding the polypeptide disclosed by SEQ ID NO: 2;
- 30 b) nucleic acid molecules containing the sequence depicted by SEQ ID NO: 1;

- 5
- c) nucleic acid molecules whose complementary strand hybridizes under stringent conditions with a nucleic acid molecule of a) or b) and which have the biological function of a fluorescent protein;
- d) nucleic acid molecules which differ from those mentioned under c) due to the degeneracy of the genetic code;
- 10 e) nucleic acid molecules whose sequences are at least 95% homologous to SEQ ID NO: 1 and which have the biological function of a fluorescent protein; and
- 15 f) nucleic acid molecules whose sequences are at least 65% homologous to SEQ ID NO: 1 and which have the biological function of a fluorescent protein.

The invention relates to the abovementioned nucleic acid molecules whose sequence contains a functional promoter 5' of the sequence.

- 20 The invention also relates to nucleic acid molecules as described above which are a part of recombinant DNA or RNA vectors.

The invention relates to organisms which contain such a vector.

- 25 The invention refers to oligonucleotides, having more than 10 contiguous nucleotides which are identical or complementary to the DNA or RNA sequence of the CGFP molecules.

- 30 The invention relates to fluorescent proteins which are encoded by the above-described nucleotide sequences.

The invention refers to methods of expressing the fluorescent polypeptides of the invention in bacteria, eukaryotic cells or in *in vitro* expression systems.

- 5 The invention also relates to methods of purifying/isolating a fluorescent polypeptide of the invention.

10 The invention relates to peptides, having more than 5 contiguous amino acids which are recognized immunologically by antibodies to the fluorescent proteins of the invention.

The invention relates to the use of the fluorescent proteins of the invention as marker genes and reporter genes, in particular for pharmacological drug screening and diagnostics.

15

Expression of the fluorescent proteins of the invention

20 Expression refers to the production of a molecule which, after introduction of the gene into a suitable host cell, allows the foreign gene cloned into an expression vector to be transcribed and translated. Expression vectors contain the control signals required for expression of genes in cells of prokaryotes or eukaryotes.

25 Expression vectors may be constructed in principle in two different ways. In the case of „transcription fusions“, the protein encoded by the cloned-in foreign gene is synthesized as an authentic, biologically active protein. To this end, the expression vector carries all 5' and 3' control signals required for expression.

30 In the case of „translation fusions“, the protein encoded by the cloned-in foreign gene is expressed as a hybrid protein together with another protein which can be readily detected. The 5' and 3' control signals required for expression, including the start

codon and possibly part of the sequences coding for the N-terminal regions of the hybrid protein to be formed, are derived from the vector. The additional protein part introduced not only stabilizes, in many cases, the protein encoded by the cloned-in foreign gene against degradation by cellular proteases but can also be used for
5 detecting and isolating the hybrid protein formed. Expression may be either transient or stable. Suitable host organisms are bacteria, yeasts, viruses and eukaryotic systems.

Purification of the fluorescent proteins of the invention

10

The isolation of proteins (also after overexpression) is frequently referred to as protein purification. A multiplicity of established methods and processes are available for protein purification.

15

Solid-liquid separation is a basic operation in protein isolation procedures. This process step is required both in the removal of cells from the culture medium and in clarifying the crude extract, after cell disruption and removal of the cell debris, in removing precipitates after precipitations, etc. It is carried out by way of centrifugation and filtration.

20

By obtaining intracellular proteins requires the cell wall to be destroyed or made permeable. Depending on the scale and organism, high-pressure homogenizers or stirred ball mills or glass bead mills are used for this purpose. On the laboratory scale, mechanical cell integration and ultrasound treatment are used inter alia.

25

Various precipitation methods involving salts (in particular ammonium sulfate) or organic solvents (alcohols, acetone) are a rapid and deficient method of concentrating proteins, both for extracellular and intracellular proteins (after cell disruption). When purifying intracellular proteins, removal of the soluble nucleic acids is desirable
30 (precipitation with streptomycin or protamine sulfate, for example). The recovery of

extracellular proteins frequently involves the addition of carriers (e.g. starch, kieselguhr), prior to addition of the precipitants, in order to obtain better manageable precipitates.

- 5 Numerous chromatographic and partition methods are available for fine purification (absorption chromatography and ion exchange chromatography, gel filtration, affinity chromatography, electrophoreses). Column chromatography is also applied on the industrial scale. Affinity chromatography which makes purification factors of up to several 100 per step possible is especially important on the laboratory scale.

10

Extracellular proteins are obtained in relatively diluted solutions. Like extracellular proteins, they must be concentrated prior to further use. Ultrafiltration is a proven method, also on the industrial scale, in addition to the methods already mentioned.

- 15 Inorganic salts are frequently undesired accompanying substances of proteins in specific applications. They may be removed, inter alia, by gel filtration, dialysis and diafiltration.

- 20 Numerous proteins are used as dry preparations. Important drying processes are vacuum drying, freeze drying and spray drying.

Nucleotide and amino acid sequences

- 25 The fluorescent protein CGFP is encoded by the following nucleotide sequence (SEQ ID NO: 1):

5' -

- atgactgcacttaccgaaggagcaaaactgttcgagaaagaaattccctacattaca
gagttggaaggagacgttgaaggaatgaaattcatcatcaaaggtgaagggtactggc
gacgctactactggcaccatcaaagcgaaatatatttgcacaactggtgaccttcct
30 gtaccatgggctaccatcttgagtagtttgctcgatggtgttttctgtttcgctaag
tatccacgccacattgccgactttttcaagagcacacaaccagatggttattcacia

gacagaatcattagttttgacaatgatggacaatacgaatgtcaaagccaagggttact
tatgaaaacggaacactttataatagagtcacagtcacaaaggtactggcttcaaataca
aacggcaacatccttggtatgagagttctctaccattcaccaccacacgctgtctac
atccttcctgaccgtaaaaatggtggcatgaaaattgaatacaataaggctttcgac
5 gttatggcggtggtcaccaaatggcgcgctcacgccaattcaataaaccactagga
gcctgggaagaagattatccgttgatcatcatcttaccgtatggacttctttcgga
aaagatccggatgatgatgaaactgaccatttgaccatcgtcgaagtcacaaagct
gttgatttggaacataccggtga-3`.

10 This results in an amino acid sequence of (SEQ ID NO: 2):
MTALTEGAKLFEKEIPYITELEGDVEGMKFIIKGEGTGDATTGTIKAKYICTTGDLP
VPWATILSSLSYGVFCFAKYPRHIADFFKSTQPDGYSQDRIISFDNDGQYDVKAKVT
YENGTLYNRVTVKGTGFKSNGNILGMRVLYHSPPHAVYILPDRKNGGMKIEYNKAFD
VMGGGHQMARHAQFNKPLGAWEEDYPLYHHLTVWTSFGKDPDDDETDLTIVEVIKA
15 VDLETYR

These sequences can be found in the sequence listing.

Description of the figures

20

Fig. 1 depicts the plasmid map of the pTriplEX2-CGFP vector.

Fig. 2 depicts the plasmid map of the pcDNA3-CGFP vector.

25

Fig. 3 depicts the transient expression of CGFP in CHO cells in the pcDNA3-CGFP expression vector. The figure depicts the microscopic image of the transiently transfected cells, at an excitation of 480 nm and an emission of 520 nm.

Fig. 4 depicts the excitation of CGFP and of the control lysate.

30

Fig. 5 depicts the emission of CGFP and of the control lysate.

Fig. 6 depicts the alignment of CFGP, GFP (Aequoria) and GFP (Renilla) at the amino acid level.

5 CGFP_Cly : CGFP from Clytia gregaria
 GFP_Ren : GFP from Renilla
 GFP_Aeq . GFP from Aequoria

Fig. 7 depicts the alignment of CFGP, GFP (Aequoria) and GFP (Renilla) at the nucleic acid level.

10 CGFP_Cly : CGFP from Clytia gregaria
 GFP_Ren : GFP from Renilla
 GFP_Aeq . GFP from Aequoria

Examples

Example 1

5 The vector used for preparing the construct illustrated below was the plasmid pTriplEx2 from Clontech. The derivative of said vector was referred to as pTriplEx2-CGFP. The pTriplEx2-CGFP vector was used for expressing CGFP in bacterial systems.

10 Fig. 1 depicts the plasmid map of the pTriplEX2-CGFP vector.

Example 2

15 The vector used for preparing the construct illustrated below was the plasmid pcDNA3.1(+) from Clontech. The derivative of said vector was referred to as pcDNA3-CGFP. The pcDNA3-CGFP vector was used for expressing CGFP in eukaryotic systems.

Fig. 2 depicts the plasmid map of the pcDNA3-CGFP vector.

20

Example 3

Bacterial expression

25 Bacterial expression was carried out in the E. coli strain BL21(DE3) by transforming the bacteria with the expression plasmids pTriplEX2-CGFP and pTriplEX2. The transformed bacteria were incubated in LB medium at 37°C for 3 hours and expression was induced for 4 hours by adding IPTG up to a final concentration of 1 mM. The induced bacteria were harvested by centrifugation, resuspended in PBS
30 and sonicated. The fluorescence was determined with the aid of a fluorimeter.

Example 4

Eukaryotic expression

5

Constitutive eukaryotic expression was carried out in CHO cells by transfecting said cells with the expression plasmids pcDNA3-CGFP and pcDNA3.1(+) in transient experiments. To this end, 10000 cells per well were plated in DMEM-F12 medium on 96-well microtiter plates and incubated at 37°C overnight. Transfection was carried out with the aid of the Fugene 6 kit (Roche) according to the manufacturer's information. The transfected cells were incubated in DMEM-F12 medium at 37°C overnight. The fluorescence was measured in a fluorimeter at room temperature.

10

Figure 3 depicts expression of CGFP in CHO cells.

15

Example 5

Spectrum of the fluorescent protein CGFP

20

To measure the emission spectrum, E. coli BL21(DE3) were transformed with the plasmids pTriplex2-CGFP and pTriplex2. Induction was carried out by adding 1 mM IPTG and incubating at 37 °C for 4 hours. The bacteria were subsequently harvested and resuspended in PBS. The lysis was carried out using ultrasound. Subsequently, fluorescence was measured in a fluorimeter.

25

Figure 4 depicts the excitation of CGFP and of the control lysate.

Figure 5 depicts the emission of CGFP and of the control lysate.

Example 6

BLAST

5 Result of a BLAST analysis of CFGP at the amino acid level.

10 >AA2002:ABB06186 Abb06186 Green fluorescent protein GFPxml9 SEQ ID, NO:15.
5/2002, Length = 271, Score = 219 bits (558), Expect = 3e-56, Identities =
102/228 (44%), Positives = 151/228 (65%), Gaps = 3/228 (1%)

15 >gb|AAK02065.1| mutant green fluorescent protein [synthetic construct],
Length = 238, Score = 219 bits (557), Expect = 4e-56, Identities = 102/227
(44%), Positives = 150/227 (65%), Gaps = 3/227 (1%)

20 >gb|AAL33915.1|AF435430_1 green fluorescent protein [Aequorea macrodactyla],
Length = 238, Score = 218 bits (556), Expect = 5e-56, Identities = 102/227
(44%), Positives = 150/227 (65%), Gaps = 3/227 (1%)

25 >gb|AAL33918.1|AF435433_1 green fluorescent protein [Aequorea macrodactyla],
Length = 238, Score = 218 bits (555), Expect = 7e-56, Identities = 101/227
(44%), Positives = 149/227 (65%), Gaps = 3/227 (1%)

30 >gb|AAL33916.1|AF435431_1 green fluorescent protein [Aequorea macrodactyla],
Length = 238, Score = 218 bits (554), Expect = 9e-56
Identities = 102/227 (44%), Positives = 150/227 (65%), Gaps = 3/227 (1%)

35 >gb|AAL33917.1|AF435432_1 orange fluorescent protein [Aequorea
macrodactyla], Length = 238, Score = 218 bits (554), Expect = 9e-56,
Identities = 101/227 (44%), Positives = 149/227 (65%), Gaps = 3/227 (1%)

40 >AA2002:ABB06185 Abb06185 Green fluorescent protein GFPxml8 SEQ ID, NO:13.
5/2002, Length = 271, Score = 217 bits (552), Expect = 1e-55, Identities =
101/228 (44%), Positives = 151/228 (65%), Gaps = 3/228 (1%)

45 >AA2002:ABB06184 Abb06184 Green fluorescent protein GFPxml6 SEQ ID, NO:11.
5/2002, Length = 271, Score = 216 bits (551), Expect = 2e-55, Identities =
101/228 (44%), Positives = 150/228 (65%), Gaps = 3/228 (1%)

50 >AA2002:ABB06181 Abb06181 Green fluorescent protein GFPxm SEQ ID, NO:5.
5/2002, Length = 271, Score = 216 bits (551), Expect = 2e-55, Identities =
101/228 (44%), Positives = 150/228 (65%), Gaps = 3/228 (1%)

>gb|AAL33912.1|AF435427_1 green fluorescent protein [Aequorea macrodactyla],
Length = 238, Score = 216 bits (551), Expect = 2e-55, Identities = 101/227
(44%), Positives = 150/227 (65%), Gaps = 3/227 (1%)

>gb|AAK02064.1| mutant green fluorescent protein [synthetic construct],
Length = 238, Score = 216 bits (551), Expect = 2e-55, Identities = 101/227
(44%), Positives = 150/227 (65%), Gaps = 3/227 (1%)

Example 7

BLAST

- 5 Result of a BLAST analysis of CFGP at the nucleic acid level.

>gb|AF468563.1| Crassostrea gigas clone c077 microsatellite sequence, Length = 415, Score = 41.1 bits (21), Expect = 1.4, Identities = 25/27 (92%)

10 >gb|AC079685.2| Oryza sativa chromosome 10 clone OSJNBb0012A20, complete sequence, Length = 131599, Score = 41.1 bits (21), Expect = 1.4, Identities = 27/30 (90%)

15 >gb|AF427906.1|AF427906 Solenopsis globularia littoralis putative odorant binding protein, precursor (Gp-9) gene, complete cds, Length = 1767, Score = 41.1 bits (21), Expect = 1.4, Identities = 23/24 (95%)

20 >gb|AF297617.1|AF297617 Echinococcus granulosus genotype 1 mitochondrion, complete genome, Length = 13588, Score = 41.1 bits (21), Expect = 1.4, Identities = 23/24 (95%)

Example 8

- 25 Figure 6 depicts the alignment of CFGP, GFP (Aquoria) and GFP (Renilla) at the nucleic acid level.

Example 9

- 30 Figure 7 depicts the alignment of CFGP, GFP (Aquoria) and GFP (Renilla) at the amino acid level.

Literature / patents

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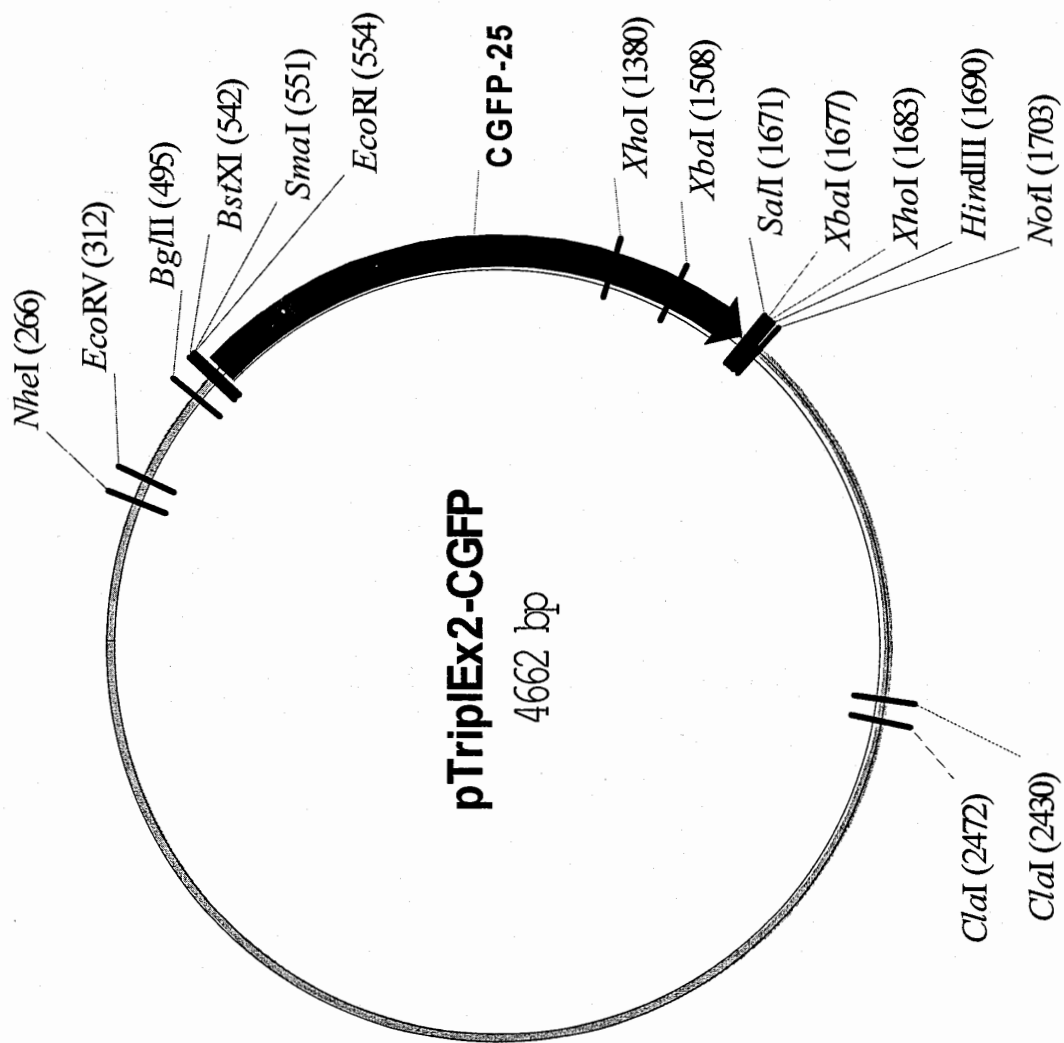
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Claims

1. A nucleic acid molecule, selected from the group consisting of
 - 5 a) nucleic acid molecules encoding the polypeptide disclosed by SEQ ID NO: 2;
 - b) nucleic acid molecules containing the sequence depicted by SEQ ID NO: 1;
 - 10 c) nucleic acid molecules whose complementary strand hybridizes under stringent conditions with a nucleic acid molecule of a) or b) and which have the biological function of a fluorescent protein;
 - 15 d) nucleic acid molecules which differ from those mentioned under c) due to the degeneracy of the genetic code;
 - e) nucleic acid molecules whose sequences are at least 95% homologous to SEQ ID NO: 1 and which have the biological function of a
20 fluorescent protein; and
 - f) nucleic acid molecules whose sequences are at least 65% homologous to SEQ ID NO: 1 and which have the biological function of a
25 fluorescent protein.
2. The molecule as claimed in claim 1, whose sequence contains a functional promoter 5' of the sequence.
3. The molecule as claimed in claim 2, which is a part of recombinant DNA or
30 of RNA vectors.

4. An organism, which contains a vector described as claimed in claim 3.
5. An oligonucleotide, having more than 10 contiguous nucleotides which are identical or complementary to DNA or RNA sequences as claimed in claim 1.
- 5 6. A peptide, which is encoded by the nucleotide sequence as claimed in claim 1.
7. A method of expressing the CGFP polypeptide as claimed in claim 6 in bacteria, eukaryotic cells or in *in vitro* expression systems.
- 10 8. A method of purifying/isolating a CGFP polypeptide as claimed in claim 6.
9. A peptide, having more than 5 contiguous amino acids which are recognized immunologically by antibodies to the fluorescent protein CGFP.
- 15 10. The use of the fluorescent protein CGFP as claimed in claims 1 to 7 as a marker gene and reporter gene.

Figures**Fig. 1**

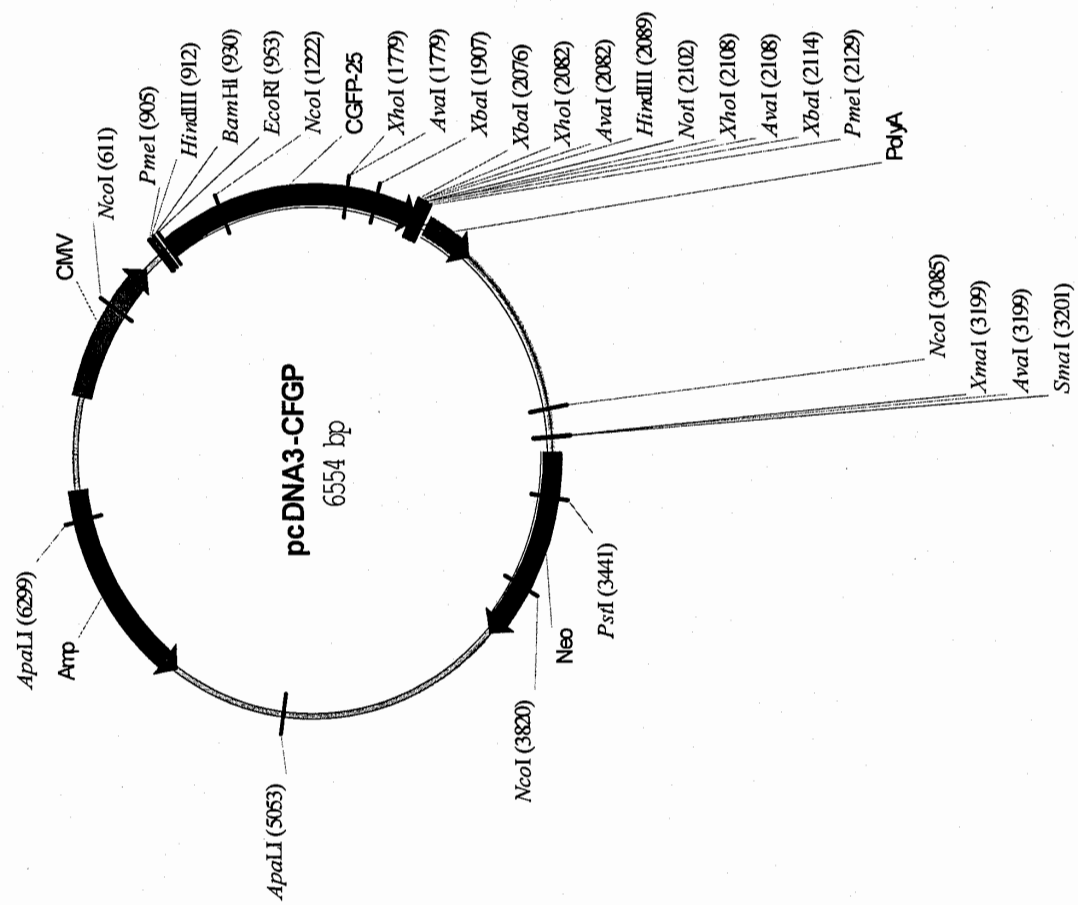
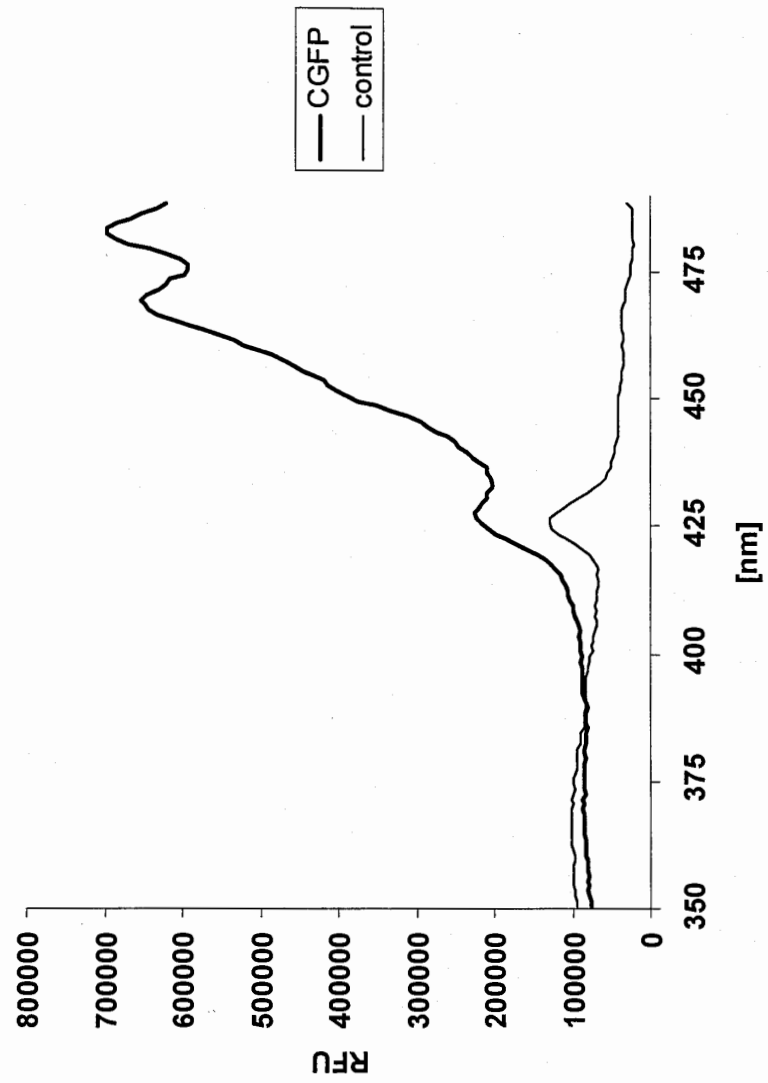


Fig. 2



Fig. 3

**Fig. 4**

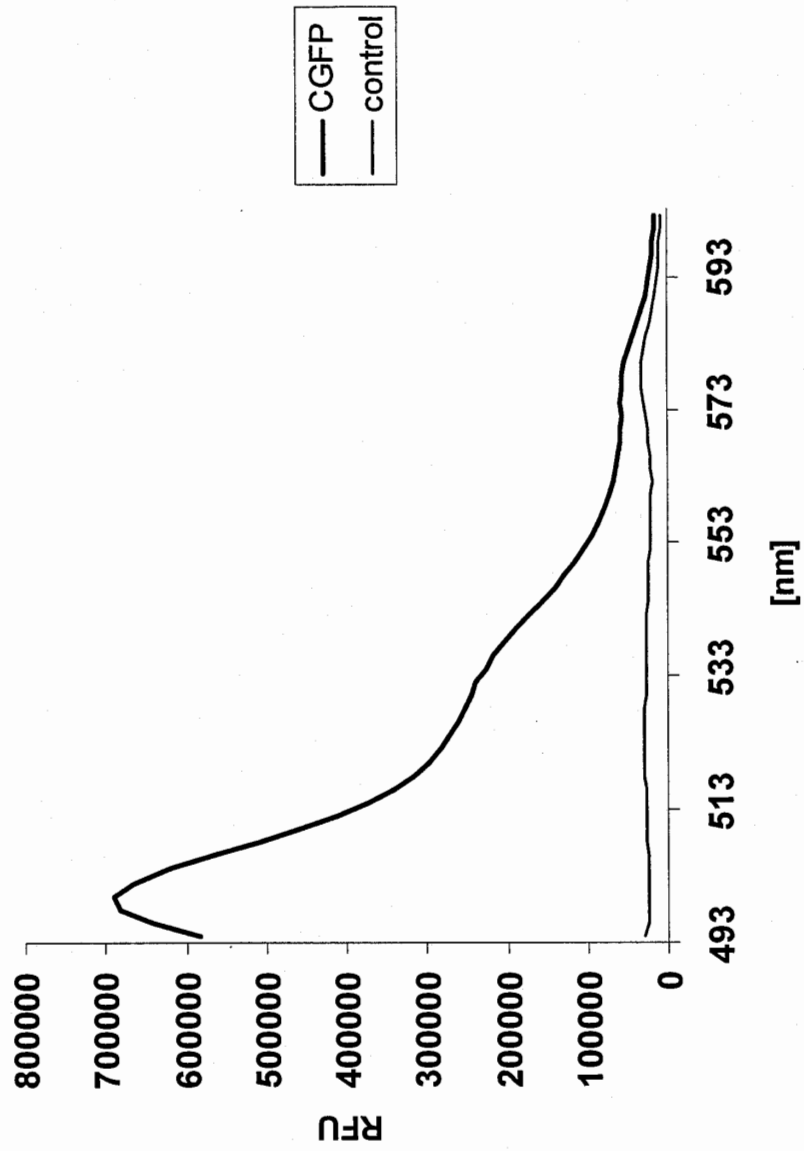


Fig. 5

Fig. 6

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GFP_Ren 000:MSKQILKNTCLQEVMSYKVNLEGI VNNHVFTMEGCGKGNILFGNQLVQIRVTKGAPLPFA

CGFP_cly 060:WATILSSLSYGVFCFAKYPRHIA--DEFFKSTQPDGYSQDRIIISFDNDGGQYDVKAKVTYEN
GFP_Aeq 060:WPTLVTTFSYGVQCFSRYPDHMKQHDEFFKSAMPEGYVQERTIFYKDDDGNYKTRAEVKFEG
GFP_Ren 060:FDIVSPAFAQYGNRTFTKYPNDIS--DYFIQSFPAQFMYERTLLRYEDGGLVEIRSDINLIE

CGFP_cly 120:GTLYNRVTVKGTGFKSNGNILGMRVLYHSPPHAVYILPDRKNGGGMKIEYNKAFDVMGGGH
GFP_Aeq 120:DTLVNRIELKGIDFKEDGNILGHKMEYNYNSHNVYIMADKPKNGIKVNFKIRHNIKDGSV
GFP_Ren 120:DKFVYRVEYKGSNFPDDGPMQKTIILGIEPSFEAMYM----NNGVLVGEVILVYKLN SGK

CGFP_cly 180:QMARHAQFNKPLGAWEEDYPLYHHLT--VWTSFGKDPDDDETDLTIVEVIKAVDL-----
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GFP_Ren 180:YYSCHMKTLMKSKGVVKEFPFHYFIQHRLEKTYVEDGGFVEQHEHETAIAQMTSIGKPLGSL

CGFP_cly 240:-ETYR
GFP_Aeq 240:DELYK
GFP_Ren 240:HEWV

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Fig. 7

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GFP_Aeq_180:gtcactactttctcttattgtgtgttcaatgcttttcaagatacccagatcatatgaaacag

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 03/13281

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/435 A01K67/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

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

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

EPO-Internal, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEVINE L D ET AL: "ISOLATION AND CHARACTERIZATION OF A PHOTO PROTEIN PHIALIDIN AND A SPECTRALLY UNIQUE GREEN FLUORESCENT PROTEIN FROM THE BIO LUMINESCENT JELLYFISH PHIALIDIUM-GREGARIUM" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY B, vol. 72, no. 1, 1982, pages 77-86, XP009028577 ISSN: 0305-0491 cited in the application	1,2,5,6, 8,9
Y	abstract page 79, right-hand column, paragraph 2 -page 80, right-hand column, paragraph 1; figure 3; table 3 --- -/--	3,4,7,10

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Date of the actual completion of the international search

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12/05/2004

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NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Weiland, S

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/13281

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHALFIE, M. AND KAIN, S.: "Green fluorescent protein: properties, applications, and protocols" August 1998 (1998-08), WILEY-LISS, INC. XP009028583 cited in the application page 49; figure 3.3 page 70	3,4,7,10
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Y	TSIEN R Y: "THE GREEN FLUORESCENT PROTEIN" ANNUAL REVIEW OF BIOCHEMISTRY, PALTO ALTO, CA, US, vol. 67, 1998, pages 509-544, XP000946725 ISSN: 0066-4154 page 511, paragraph 3 page 539, paragraph 3	3,4,7,10
A	INOUE SATOSHI ET AL: "Cloning and sequence analysis of cDNA for the calcium-activated photoprotein, clytin" FEBS (FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES) LETTERS, vol. 315, no. 3, 1993, pages 343-346, XP001180448 ISSN: 0014-5793 page 343, right-hand column, paragraph 1	1-10
A	PRASHER D C ET AL: "PRIMARY STRUCTURE OF THE AEQUOREA VICTORIA GREEN-FLUORESCENT PROTEIN" GENE, ELSEVIER, AMSTERDAM, NL, vol. 111, 1992, pages 229-233, XP001018985 ISSN: 0378-1119 figure 2	1-10
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INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen

PCT/EP 03/13281

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 7 C07K14/435 A01K67/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C07K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

EPO-Internal, SEQUENCE SEARCH

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	LEVINE L D ET AL: "ISOLATION AND CHARACTERIZATION OF A PHOTO PROTEIN PHIALIDIN AND A SPECTRALLY UNIQUE GREEN FLUORESCENT PROTEIN FROM THE BIO LUMINESCENT JELLYFISH PHIALIDIUM-GREGARIUM" COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY B, Bd. 72, Nr. 1, 1982, Seiten 77-86, XP009028577 ISSN: 0305-0491 in der Anmeldung erwähnt	1,2,5,6, 8,9
Y	Zusammenfassung Seite 79, rechte Spalte, Absatz 2 -Seite 80, rechte Spalte, Absatz 1; Abbildung 3; Tabelle 3 --- -/-	3,4,7,10

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen		☐ Siehe Anhang Patentfamilie	
* Besondere Kategorien von angegebenen Veröffentlichungen : *A* Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist *E* älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist *L* Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt) *O* Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht *P* Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist		*T* Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist *X* Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden *Y* Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist *Z* Veröffentlichung, die Mitglied derselben Patentfamilie ist	
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6. April 2004		12/05/2004	
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C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	CHALFIE, M. AND KAIN, S.: "Green fluorescent protein: properties, applications, and protocols" August 1998 (1998-08), WILEY-LISS, INC. XP009028583 in der Anmeldung erwähnt Seite 49; Abbildung 3.3 Seite 70	3,4,7,10
Y	PRASHER D C: "Using GFP to see the light" TRENDS IN GENETICS, ELSEVIER, AMSTERDAM, NL, Bd. 11, Nr. 8, 1995, Seiten 320-323, XP004207387 ISSN: 0168-9525 Seite 322, rechte Spalte, Absatz 5 -Seite 323, linke Spalte, Absatz 2; Abbildung 2; Tabelle 1	3,4,7,10
Y	TSIEN R Y: "THE GREEN FLUORESCENT PROTEIN" ANNUAL REVIEW OF BIOCHEMISTRY, PALTO ALTO, CA, US, Bd. 67, 1998, Seiten 509-544, XP000946725 ISSN: 0066-4154 Seite 511, Absatz 3 Seite 539, Absatz 3	3,4,7,10
A	INOUE SATOSHI ET AL: "Cloning and sequence analysis of cDNA for the calcium-activated photoprotein, clytin" FEBS (FEDERATION OF EUROPEAN BIOCHEMICAL SOCIETIES) LETTERS, Bd. 315, Nr. 3, 1993, Seiten 343-346, XP001180448 ISSN: 0014-5793 Seite 343, rechte Spalte, Absatz 1	1-10
A	PRASHER D C ET AL: "PRIMARY STRUCTURE OF THE AEQUOREA VICTORIA GREEN-FLUORESCENT PROTEIN" GENE, ELSEVIER, AMSTERDAM, NL, Bd. 111, 1992, Seiten 229-233, XP001018985 ISSN: 0378-1119 Abbildung 2	1-10
X	-& DATABASE GENBANK 'Online! 26. April 1993 (1993-04-26) PRASHER ET AL.: "Primary Structure of the Aequorea victoria green-fluorescent protein" Database accession no. M62653 XP002276253 das ganze Dokument	1-3,5