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Attorney for Plaintiff
PROLUME, LTD.

UNITED STATES DISTRICT COURT

SOUTHERN DISTRICT OF CALIFORNIA

PROLUME, LTD., a Delaware Corporation,

Plaintiff,

v.

GENTARGET, INC., a California Corporation; BST SCIENTIFIC PTE LTD, a Singapore Corporation; FILGEN BIOSCIENCES, INC., a California Corporation; AMSBIO LLC, a California Limited Liability Company; GENTAUR MOLECULAR PRODUCTS BVBA, a Belgian Corporation, GENPRICE, INC., a California Corporation; DOES 1 through 10, inclusive,

Defendants.

Case No. '13CV0130 WQHBLM

COMPLAINT FOR PATENT INFRINGEMENT

DEMAND FOR JURY TRIAL

Plaintiff PROLUME, LTD. (hereinafter "PROLUME" or "Plaintiff") hereby alleges as follows:

PARTIES

1. On information and belief, Defendant GENTARGET, INC. (hereinafter "GENTARGET") is a California corporation with its principal place of business at 6640 Lusk Blvd., Suite A107, San Diego, California.

2. On information and belief, Defendant BST SCIENTIFIC PTE LTD is a foreign corporation existing under the laws of Singapore, with its principal place of business at 41 Science Park Road, #04-08 The Gemini, Singapore Science Park II, Singapore 117610.

3. On information and belief, Defendant FILGEN BIOSCIENCES, INC. is a California corporation with its principal place of business at 1313 Elm Street, El Cerrito, California.

4. On information and belief, Defendant AMSBIO LLC is a California limited liability company with its principal place of business at 23591 El Toro Road, #167, Lake Forest, California.

5. On information and belief, Defendant GENTAUR MOLECULAR PRODUCTS BVBA is a foreign corporation existing under the laws of Belgium, with its principal place of business at Voortstraat 49, 1910 Kampenhout, Belgium.

6. On information and belief, Defendant GENPRICE, INC. is a subsidiary of GENTAUR MOLECULAR PRODUCTS BVBA and is a California corporation with its principal place of business at 547 Yurok Circle, San Jose, California.

7. Plaintiff is not aware of the true names and capacities of the defendants sued herein as DOES 1 through 10, inclusive, and therefore sues said defendants by such fictitious names. When Plaintiff has ascertained the true names and capacities of said defendants, Plaintiff will seek leave of this court to amend this complaint accordingly. On information and belief, each of the fictitiously

named defendants is responsible in some manner for the occurrences alleged in this complaint, and Plaintiff alleges that his damages were proximately and legally caused by defendants' conduct.

8. At all material times, Plaintiff alleges, each defendant was the agent, servant and employee of each of the remaining defendants, and was acting within the purpose, scope and course of said agency, service and employment, with the express and/or implied knowledge, permission and consent of the remaining defendants, and each of them, and each of said defendants ratified, approved of, and/or accepted the benefits of such acts.

JURISDICTION/VENUE

9. This is an action for patent infringement arising under the patent laws of the United States, Title 35, United States Code.

10. This Court has subject matter jurisdiction pursuant to 28 U.S.C. §§ 1331 and 1338(a).

11. On information and belief, Defendants are subject to personal jurisdiction in the Southern District of California (the "District"), consistent with the principles of due process and the California Long Arm Statute, because Defendants maintain offices and facilities in this District, offer their products for sale in this District, have transacted business in this District, have committed and/or induced acts of patent infringement in this District, and/or have placed infringing products into the stream of commerce through established distribution channels with the expectation that such products will be purchased by residents of this District.

12. Venue is proper in this District pursuant to 28 U.S.C. § 1391(b), 139(c), 1391(d), and 1400(b). Defendant GENTARGET resides in this judicial district and, on information and belief, has committed acts of infringement in this judicial district.

PATENT INFRINGEMENT COUNTS

13. Plaintiff PROLUME is the owner of all right, title, and interest in U.S. Patent No. 6,232,107 (the "107" patent) and U.S. Patent No. 7,109,315 (the '315) (collectively, the "PROLUME Patents"), which Defendants are infringing and/or inducing others to infringe by making, using, offering to sell, and/or selling in the United States, and/or importing into the United States, products or processes that practice one or more inventions claimed in the PROLUME Patents.

14. Defendants have profited through infringement of the PROLUME Patents. As a result of Defendants' unlawful infringement of the PROLUME Patents, Plaintiff has suffered and will continue to suffer damage. Plaintiff is entitled to recover from Defendants, and from each of them, the damages suffered by Plaintiff as a result of Defendants' unlawful acts.

15. Commencing on or about January 11, 2012, Plaintiff gave notice to Defendant GENTARGET of the PROLUME Patents. However, GENTARGET refused, and continues to refuse, to cease its infringement of the PROLUME Patents. Further, on information and belief, Defendant GENTARGET, in addition to its own infringement, has continued its inducement of third-party infringement of the PROLUME Patents, despite Plaintiff's above notice.

16. On information and belief, Defendants' infringement, including without limitation the infringement of Defendant GENTARGET was, is, and continues to be willful and deliberate, entitling Plaintiff to enhanced damages and attorneys' fees and costs.

17. On information and belief, Defendants continue their unlawful infringing activities, and Plaintiff continues to and will continue to suffer irreparable harm – for which there is no adequate remedy at law – from such unlawful activity unless Defendants are enjoined by this Court.

COUNT I

INFRINGEMENT OF U.S. PATENT NO. 6,232,107

18. Plaintiff hereby adopts, incorporates, and reiterates all of the preceding allegations of this Complaint.

19. Plaintiff is the owner of all right, title, and interest in the '107 patent, entitled "Luciferases, Fluorescent Proteins, Nucleic Acids Encoding The Luciferases And Fluorescent Proteins And The Use Thereof In Diagnostics, High Throughput Screening And Novelty Items", duly and properly issued by the U.S. Patent and Trademark Office on May 15, 2001. An excerpt of the '107 patent is attached as **Exhibit "A"** and made a part hereof.

20. The '107 patent claims, among other things, proprietary biological materials ("Gaussia Luciferase" and "Green Fluorescent Proteins") that are used to directly and easily discover new drug compounds, as well as identifying potential proteins of therapeutic interest in cells and live animals.

21. Defendants have been and/or are directly infringing and/or inducing infringement of and/or contributorily infringing the '107 patent by, among other things, making, using, offering to sell or selling in the United States, or importing and/or exporting products and/or services that are covered by at least claims 1 through 5 of the 63 claims of the '107 patent, including, by way of example and not limitation, GenTarget Product # LVP366 & LVP366-PBS is described using Gaussia Luciferase RNA (infringing at least patent claim nos. 1-22), since a Gaussia nucleotide sequence is combined with a retrovirus which is a vector, which incorporates the Gaussia Luciferase into a cell, cell line, organ, or animal (so that the RNA virus inserts itself into a host cell is converted into DNA), this DNA is integrated into the cell's DNA just like the well known HIV virus, and if combined with it an off/on switch which causes the Gaussia DNA to make and secrete the Gaussia Luciferase (active light emitting protein) from the infected cell, cell line, animal tissue, or animal organ can be visualized optically. Gaussia

Luciferase generates light which is clearly detectable, or may stop the generation of light if a drug is applied to kill that cell or block the Gaussia Luciferase from being produced. Gentarget product LVP 362 is a combination product composed of Gaussia Luciferase and a Green Fluorescent Protein (GFP) infringing at least claims 1-22 and additionally claims 43-56 and 58-63. A search of the word "gaussia" at www.gentarget.com reveals 14 products that incorporate Gaussia Luciferase in various combinations including stable cell lines HEK 293 containing Gaussia Luciferase with product sales prices from $295-650.00/each. Another search of the Gentarget website for the search term "GFP" produced 118 products ranging in price from $295-800.00 which infringe multiple '107 patent claims.

22. Plaintiff has been damaged as a result of Defendants' conduct. Defendants are, therefore, liable to Plaintiff in an amount that adequately compensates Plaintiff for Defendants' infringements, which, by law, cannot be less than a reasonable royalty, together with interest and costs as fixed by this Court under 35 U.S.C. § 284.

COUNT II

INFRINGEMENT OF U.S. PATENT NO. 7,109,315

23. Plaintiff hereby adopts, incorporates, and reiterates all of the preceding allegations of this Complaint.

24. Plaintiff is the owner of all right, title, and interest in the '315 patent, entitled "Renilla reniformis fluorescent proteins, nucleic acids encoding the fluorescent proteins and the use thereof in diagnostics, high throughput screening and novelty items", duly and properly issued by the U.S. Patent and Trademark Office on September 19, 2006. An excerpt of the '315 patent is attached as **Exhibit "B"** and made a part hereof.

25. The '315 patent claims, among other things, proprietary biological materials ("Renilla reneformis Green Fluorescent Proteins") that are used to

directly and easily discover new drug compounds, as well as identifying potential proteins of therapeutic interest in cells and live animals.

26. Defendants have been and/or are directly infringing and/or inducing infringement of and/or contributorily infringing the '315 patent by, among other things, making, using, offering to sell or selling in the United States, or importing and/or exporting products and/or services that are covered by claims under the '315 patent. A search of www.gentarget.com using search term "GFP" produced 118 products ranging from $295-800.00. From the Gentarget website it can not be determined which claims are being infringed without an analysis of the DNA sequence used in their products, which is not clearly published on the Gentarget website; however the color of the blue light (as listed in their product literature) that stimulates the Green Fluorescent Protein used in the Gentarget GFP product(s) is 450-490 nm and the color reflected back is Green (525 nm) which matches the spectra of protein covered in claims #1-26 of the '315 patent. Historically, the Renilla Green Fluorescent Protein was the second Green Fluorescent Protein discovered and any subsequent Green Fluorescent Proteins with the same characteristics would be covered by the doctrine of equivalents.

27. Plaintiff has been damaged as a result of Defendants' conduct. Defendants are, therefore, liable to Plaintiff in an amount that adequately compensates Plaintiff for Defendants' infringements, which, by law, cannot be less than a reasonable royalty, together with interest and costs as fixed by this Court under 35 U.S.C. § 284.

WHEREFORE, Plaintiff prays for judgment against Defendants, and each of them, as follows:

1. For a judicial determination and declaration that one or more claims of the '107 and '715 patents has been infringed, either literally and/or under the doctrine of equivalents, by Defendants and/or by others to whose infringement

Defendants have contributed and/or by others whose infringement has been induced by Defendants;

2. For an order that Defendants account for and pay to Plaintiff all damages to and costs incurred by Plaintiff because of Defendants' infringing activities and other conduct complained of herein;

3. For an order that Defendants, their officers, agents, servants, and employees, and those persons in active concert and participation with any of them, be preliminarily and permanently enjoined from infringement of the '107 and '715 patents. In the alternative, should the Court find that injunctive relief is not warranted, Plaintiff requests an award of post judgment royalty to compensate for future infringement;

4. For an order that Plaintiff be granted pre-judgment and post-judgment interest on the damages caused to it by reason of Defendants' infringing activities and other conduct complained of herein;

5. For a judicial determination and declaration that Defendants' infringement has been, and continues to be, willful and deliberate;

6. For a judicial determination and declaration that this is an exceptional case and award Plaintiff its reasonable attorney's fees and costs in accordance with 35 U.S.C. § 285;

7. For an order that Defendants notify all of their customers and users of infringing products of the Court's judgment, and that Defendants be ordered to encourage all customers to cease all such infringing actions; and

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8. For any such other and further relief as the Court may deem just and proper.

DATED: Jan. 15, 2013

LAW OFFICES OF PHILIP J. KAPLAN

By: _____
PHILIP J. KAPLAN
Attorney for Plaintiff
PROLUME, LTD.
E-mail: philipkaplanlaw@gmail.com

DEMAND FOR JURY TRIAL

Plaintiff hereby demands, pursuant to Rule 38 of the Federal Rules of Civil Procedure, a trial by jury in this matter.

DATED: Jan. 15, 2013

LAW OFFICES OF PHILIP J. KAPLAN

By: _____
PHILIP J. KAPLAN
Attorney for Plaintiff
PROLUME, LTD.
E-mail: philipkaplanlaw@gmail.com

EXHIBIT “A”

United States Patent **6,232,107**
Bryan , et al. **May 15, 2001**

Luciferases, fluorescent proteins, nucleic acids encoding the luciferases and fluorescent proteins and the use thereof in diagnostics, high throughput screening and novelty items

Abstract

Isolated and purified nucleic acid molecules that encode a luciferase from Renilla mulleri, Gaussia and Pleuromamma, and the proteins encoded thereby are provided. Isolated and purified nucleic acids encoding green fluorescent proteins from the genus Renilla and Ptilosarcus, and the green fluorescent proteins encoded thereby are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.

Inventors: **Bryan; Bruce J.** (Beverly Hills, CA), **Szent-Gyorgyi; Christopher** (Pittsburgh, PA)

Assignee: **Bryan; Bruce J.** (
Prolume, LTD (Pittsburgh, PA)

Appl. No.: **09/277,716**

Filed: **March 26, 1999**

Current U.S. Class: **435/189** ; 435/183; 435/252.2; 435/320.1; 435/6.13; 435/69.1; 435/8

Current International Class: A61K 49/00 (20060101); A61K 47/48 (20060101); C07K 14/435 (20060101); C12N 9/02 (20060101); A61K

38/00 (20060101); C12N 009/02 (); G01N 033/53 ();
C12Q 001/66 (); C12P 021/06 ()

Field of Search: 435/69.1,189,252.3,252.33,320.1,6,8,69.7,183 536/23.2

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Parent Case Text

RELATED APPLICATIONS

This application claims priority to U.S. provisional application Ser. No. 60/102,939, filed Oct. 1, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS". Priority is also claimed to U.S. provisional application Ser. No.60/089,367, filed Jun. 15, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "GAUSSIA LUCIFERASE, NUCLEIC ACIDS ENCODING THE LUCIFERASE AND METHODS USING THE LUCIFERASE", and to U.S. provisional

application Ser. No.60/079,624, filed Mar. 27, 1998, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "RENILLA GREEN FLUORESCENT PROTEIN COMPOSITIONS AND METHODS." For U.S. purposes, benefit of priority to each of these applications is claimed under 35 U.S.C. .sctn.119(e).

This application is also related to subject matter in U.S. application Ser. No. 08/757,046, filed Nov. 25, 1996, to Bruce Bryan entitled "BIOLUMINESCENT NOVELTY ITEMS", now U.S. Pat. No. 5,876,995, issued Mar. 2, 1999, and in U.S. application Ser. No. 08/597,274, filed Feb. 6, 1996, to Bruce Bryan, entitled "BIOLUMINESCENT NOVELTY ITEMS". This application is also related to U.S. application Ser. No. 08/908,909, filed Aug. 8, 1997, to Bruce Bryan entitled "DETECTION AND VISUALIZATION OF NEOPLASTIC TISSUE AND OTHER TISSUES". The application is also related to U.S. application Ser. No. 08/990,103, filed Dec. 12, 1997, to Bruce Bryan entitled "APPARATUS AND METHODS FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS".

The subject matter of each of the above noted U.S. applications and provisional applications is herein incorporated by reference in its entirety.

Claims

What is claimed is:

1. An isolated nucleic acid fragment, comprising a sequence of nucleotides encoding a Renilla mulleri luciferase, a Gaussia luciferase or a Pleuromamma luciferase, wherein the sequence of nucleotides is selected from the group consisting of

a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; or

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No.19 or SEQ ID No. 28.

2. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 19 or 28.

3. The nucleic acid probe or primer of claim 2, comprising at least 16 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 19 or 28.

4. The nucleic acid probe or primer of claim 2, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in the coding portion of SEQ ID Nos. 17, 19 or 28.

5. A plasmid, comprising the nucleic acid fragment of claim 1.

6. The plasmid of claim 5 that is an expression vector.

7. The plasmid of claim 6, comprising a sequence of nucleotides encoding:

a promoter region;

a Gaussia, Pleuromamma or Renilla mulleri luciferase; and

a selectable marker;

wherein the sequence of nucleotides encoding the luciferase is operatively linked to the promoter, whereby the luciferase is expressed.

8. The plasmid of claim 6, further comprising a sequence of nucleotides encoding a green fluorescent protein (GFP).

9. A recombinant host cell, comprising the plasmid of claim 6.

10. The cell of claim 9, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

11. A method for producing a Gaussia, Renilla mulleri or Pleuromamma luciferase protein, comprising growing the recombinant host cell of claim 9, wherein the luciferase protein is expressed by the cell, and recovering the expressed luciferase protein.

12. An isolated nucleic acid fragment encoding a green fluorescent protein (GFP), comprising the sequence of nucleotides selected from the group consisting of

a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; or

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

13. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30.

14. The probe or primer of claim 13, comprising at least 16 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30.

15. The probe or primer of claim 13, comprising at least 30 contiguous nucleotides selected from the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30.

16. A plasmid, comprising the sequence of nucleotides of claim 12.

17. An expression vector, comprising:

the plasmid of claim 16,

a promoter element;

a multiple cloning site for the introduction of nucleic acid; and

a selectable marker;

wherein the nucleic acid encoding the multiple cloning site is positioned between nucleic acids encoding the promoter element and the green fluorescent protein and wherein the nucleic acid encoding the green fluorescent protein is operatively linked to the promoter element.

18. The plasmid of claim 16, further comprising a sequence of nucleotides encoding:

a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the green fluorescent protein is operatively linked to the promoter element, whereby the green fluorescent protein is expressed.

19. The plasmid of claim 18, further comprising a sequence of nucleotides encoding a luciferase.

20. A recombinant host cell, comprising the plasmid of claim 16.

21. The cell of claim 20, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

22. A nucleic acid construct, comprising a sequence of nucleoides encoding a luciferase and a sequence of nucleotides encoding a green fluorescent protein (GFP), wherein the luciferase is encoded by:

a sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19, or SEQ ID No. 28;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 18, SEQ ID No. 20 or SEQ ID No. 29; and

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, SEQ ID No. 19 or SEQ ID No. 28.

23. The nucleic acid construct of claim 22, wherein the GFP is a Renilla green fluorescent protein (GFP) or a Ptilosarcus GFP.

24. The nucleic acid construct of claim 23, wherein the Renilla GFP is a Renilla reniformis, Renilla kollokeri or Renilla mulleri GFP.

25. The nucleic acid construct of claim 22, wherein the GFP is encoded by:

a sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31;

a sequence of nucleotides encoding the amino acid sequence set forth in SEQ ID No. 16 or SEQ ID No. 32; and

a sequence of nucleotides that hybridizes under high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, SEQ ID No. 30 or SEQ ID No. 31.

26. The nucleic acid construct of claim 25, wherein the luciferase and GFP are from Renilla.

27. The nucleic acid construct of claim 26, wherein the Renilla luciferase and GFP are from Renilla mulleri.

28. The nucleic acid construct of claim 22, wherein the nucleotide sequence encoding the luciferase and GFP are linked contiguously.

29. The nucleic acid construct of claim 22 that is DNA.

30. The nucleic acid construct of claim 22 that is RNA.

31. A plasmid, comprising the nucleic acid construct of claim 22.

32. The plasmid of claim 31, further comprising a sequence of nucleotides encoding:

a promoter element;

a selectable marker;

wherein, the sequence of nucleotides encoding the luciferase and GFP is operatively linked to the promoter element, whereby a fusion protein of the luciferase and GFP is expressed.

33. A recombinant host cell, comprising the plasmid of claim 31.

34. The cell of claim 33, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

35. The nucleic acid construct of claim 22, wherein the nucleotide sequence encoding the luciferase and GFP are not contiguous.

36. The nucleic acid construct of claim 35, further comprising a sequence of nucleotides that encodes a ligand binding domain of a protein.

37. A nucleic acid probe or primer, comprising at least 14 nucleotides that encode amino acids 51 to 68, 82 to 98 or 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 or amino acids 39-53 set forth in SEQ

ID No. 27.

38. The probe or primer of claim 37, comprising a sequence of nucleic acids in SEQ ID No.15 that that encode amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16.

39. A method of isolating nucleic acid that encodes a Renilla green fluorescent protein (GFP), comprising:

screening a Renilla nucleic acid library with a probe or plurality of probes of claim 37; and

identifying and isolating nucleic acid that encodes a GFP.

40. A method of isolating nucleic acid that encodes a Renilla green fluorescent protein (GFP), comprising:

amplifying nucleic acid in a Renilla nucleic acid library with a primer or plurality of primers of claim 37; and

isolating the amplified nucleic acids, whereby nucleic acid encoding a GFP is identified and isolated.

41. The isolated nucleic acid fragment of claim 1, wherein the a sequence of nucleotides encodes a Renilla mulleri luciferase.

42. The isolated nucleic acid fragment of claim 1, wherein the a sequence of nucleotides encodes a Gaussia luciferase.

43. The isolated nucleic acid fragment of claim 1, wherein the a sequence of nucleotides encodes a Pleuromamma luciferase.

44. The isolated nucleic acid fragment of claim 42, wherein the Gaussia is a member of the species of princeps.

45. The isolated nucleic acid fragment of claim 1, wherein the nucleic acid is DNA.

46. The isolated nucleic acid fragment of claim 1, wherein the nucleic acid is RNA.

47. A host cell, comprising a plasmid of claim 5.

48. The isolated nucleic acid fragment of claim 12, wherein the nucleic acid is DNA.

49. The isolated nucleic acid fragment of claim 12, wherein the nucleic acid is RNA.

50. The isolated nucleic acid fragment of claim 12, wherein the sequence of nucleotides encodes a Renilla green fluorescent protein (GFP).

51. The isolated nucleic acid fragment of claim 12, wherein the sequence of nucleotides encodes a Ptilocarpus green fluorescent protein.

52. The isolated nucleic acid fragment of claim 50, wherein the Renilla species is selected from the group consisting of Renilla reniformis, Renilla kollokeri and Renilla mulleri.

53. The construct of claim 23, wherein the GFP is a Renilla green fluorescent protein (GFP).

54. The construct of claim 23, wherein the GFP is a Ptilosarcus GFP.

55. The nucleic acid construct of claim 22, wherein the luciferase is a Renilla mulleri luciferase.

56. The nucleic acid construct of claim 22, wherein the luciferase is a Gaussia luciferase.

57. The nucleic acid construct of claim 22, wherein the luciferase is a Pleuromamma luciferase.

58. The nucleic acid construct of claim 56, wherein the Gaussia luciferase is a Gaussia princepes luciferase.

59. A plasmid, comprising the nucleic acid construct of claim 25.

60. The nucleic acid construct of claim 25, wherein the nucleotide sequence encoding the luciferase and GFP are contiguous.

61. The nucleic acid construct of claim 25, wherein the nucleotide sequence encoding the luciferase and GFP are not contiguous.

62. A nucleic acid probe or primer, comprising at least 16 contiguous nucleotides that encode on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 or amino acids 39-53

set forth in SEQ ID No. 27.

63. A nucleic acid probe or primer, comprising at least 30 contiguous nucleotides that encode on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 or amino acids 39-53 set forth in SEQ ID No. 27.

Description

FIELD OF INVENTION

The present invention relates to isolated and purified nucleic acids and encoded proteins from the genera Renilla, Gaussia, Philocarpus and Pleuromamma. More particularly, nucleic acids encoding luciferase and fluorescent proteins from species of these genera are provided.

BACKGROUND OF THE INVENTION

Luminescence is a phenomenon in which energy is specifically channeled to a molecule to produce an excited state. Return to a lower energy state is accompanied by release of a photon (hy). Luminescence includes fluorescence, phosphorescence, chemiluminescence and bioluminescence. Bioluminescence is the process by which living organisms emit light that is visible to other organisms. Luminescence may be represented as follows:

where X* is an electronically excited molecule and hy represents light emission upon return of X* to a lower energy state. Where the luminescence is bioluminescence, creation of the excited state derives from an enzyme catalyzed reaction. The color of the emitted light in a bioluminescent (or chemiluminescent or other luminescent) reaction is characteristic of the excited molecule, and is independent from its source of excitation and temperature.

An essential condition for bioluminescence is the use of molecular oxygen, either bound or free in the presence of a luciferase. Luciferases, are oxygenases, that act on a substrate, luciferin, in the presence of molecular oxygen and transform the substrate to an excited state. Upon return to a lower energy level, energy is released in the form of light [for reviews see, e.g., McElroy et al. (1966) in Molecular Architecture in Cell Physiology, Hayashi et al., eds., Prentice-Hall, Inc., Englewood Cliffs, N.J., pp. 63-80; Ward et al., Chapter 7 in Chemi-and Bioluminescence, Burr, ed., Marcel Dekker, Inc. NY, pp.321-358; Hastings, J. W. in (1995) Cell Physiology:Source Book, N. Sperelakis (ed.), Academic Press, pp 665-681; Luminescence, Narcosis and Life in the

Deep Sea, Johnson, Vantage Press, NY, see, esp. pp. 50-56].

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria [primarily marine bacteria including Vibrio species], fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial organism including annelid worms and insects.

Assays employing bioluminescence

During the past twenty years, high-sensitivity biochemical assays used in research and in medicine have increasingly employed luminescence and fluorescence rather than radioisotopes. This change has been driven partly by the increasing expense of radioisotope disposal and partly by the need to find more rapid and convenient assay methods. More recently, the need to perform biochemical assays in situ in living cells and whole animals has driven researchers toward protein-based luminescence and fluorescence. The uses of firefly luciferase for ATP assays, aequorin and obelin as calcium reporters, Vargula luciferase as a neurophysiological indicator, and the Aequorea green fluorescent protein as a protein tracer and pH indicator show the potential of bioluminescence-based methods in research laboratories.

Bioluminescence is also beginning to directly impact medicine and biotechnology; for example, Aequorea GFP is employed to mark cells in murine model systems and as a reporter in high throughput drug screening. Renilla luciferase is under development for use in diagnostic platforms.

Bioluminescence generating systems

Bioluminescence, as well as other types of chemiluminescence, is used for quantitative determinations of specific substances in biology and medicine. For example, luciferase genes have been cloned and exploited as reporter genes in numerous assays, for many purposes. Since the different luciferase systems have different specific requirements, they may be used to detect and quantify a variety of substances. The majority of commercial bioluminescence applications are based on firefly [Photinus pyralis] luciferase. One of the first and still widely used assays involves the use of firefly luciferase to detect the presence of ATP. It is also used to detect and quantify other substrates or co-factors in the reaction. Any reaction that produces or utilizes

NAD(H), NADP(H) or long chain aldehyde, either directly or indirectly, can be coupled to the light-emitting reaction of bacterial luciferase.

Another luciferase system that has been used commercially for analytical purposes is the Aequorin system. The purified jellyfish photoprotein, aequorin, is used to detect and quantify intracellular Ca.sup.2+ and its changes under various experimental conditions. The Aequorin photoprotein is relatively small [.about.20 kDa], nontoxic, and can be injected into cells in quantities adequate to detect calcium over a large concentration range [3.times.10.sup.-7 to 10.sup.-4 M].

Because of their analytical utility, luciferases and substrates have been studied and well-characterized and are commercially available [eq., firefly luciferase is available from Sigma, St. Louis, Mo., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.; recombinantly produced firefly luciferase and other reagents based on this gene or for use with this protein are available from Promega Corporation, Madison, Wis.; the aequorin photoprotein luciferase from jellyfish and luciferase from Renilla are commercially available from Sealite Sciences, Bogart, Ga.; coelenterazine, the naturally-occurring substrate for these luciferases, is available from Molecular Probes, Eugene, Oreg.]. These luciferases and related reagents are used as reagents for diagnostics, quality control, environmental testing and other such analyses.

Because of the utility of luciferases as reagents in analytical systems and the potential for use in high throughput screening systems, there is a need to identify and isolated a variety of luciferases that have improved or different spectral properties compared to those presently available. For all these reasons, it would be advantageous to have luciferases from a variety of species, such as Gaussia and various Renilla species available.

Fluorescent Proteins

Reporter genes, when co-transfected into recipient cells with a gene of interest, provide a means to detect transfection and other events. Among reporter genes are those that encode fluorescent proteins. The bioluminescence generating systems described herein are among those used as reporter genes. To increase the sensitivity bioluminescence generating systems have been combined with fluorescent compounds and proteins, such as naturally fluorescent phycobiliproteins. Also of interest are the fluorescent proteins that are present in a variety of marine invertebrates, such as the green and blue fluorescent proteins, particularly the green fluorescent protein (GFP) of Aequorea victoria.

The green fluorescent proteins (GFP) constitute a class of chromoproteins found only among

certain bioluminescent coelenterates. These accessory proteins are fluorescent and function as the ultimate bioluminescence emitter in these organisms by accepting energy from enzyme-bound, excited-state oxyluciferin (e.g., see Ward et al. (1979) J. Biol. Chem. 254:781-788; Ward et al. (1978) Photochem. Photobiol. 27:389-396; Ward et al. (1982) Biochemistry 21:4535-4540).

The best characterized GFPs are those isolated from the jellyfish species Aequorea, particularly Aequorea victoria (A. victoria) and Aequorea forskalea (Ward et al. (1982) Biochemistry 21:4535-4540; Prendergast et al. (1978) Biochemistry 17:3448-3453). Purified A. victoria GFP is a monomeric protein of about 27 Kda that absorbs blue light with excitation wavelength maximum of 395 nm, with a minor peak at 470 nm, and emits green fluorescence with an emission wavelength of about 510 nm and a minor peak near 540 nm (Ward et al. (1979) Photochem. Photobiol. Rev 4:1-57). This GFP has certain limitations. The excitation maximum of the wildtype GFP is not within the range of wavelengths of standard fluorescein detection optics.

The detection of green fluorescence does not require any exogenous substrates or co-factors. Instead, the high level of fluorescence results from the intrinsic chromophore of the protein. The chromophore includes modified amino acid residues within the polypeptide chain. For example, the fluorescent chromophore of A. victoria GFP is encoded by the hexapeptide sequence, FSYGVQ, encompassing amino acid residues 64-69. The chromophore is formed by the intramolecular cyclization of the polypeptide backbone at residues Ser65 and Gly67 and the oxidation of the .alpha.-.beta. bond of residue Tyr66 (e.g., see Cody et al. (1993) Biochemistry 32:1212-1218; Shimomura (1978) FEBS Letters 104:220-222; Ward et al. (1989) Photochem. Photobiol. 49:62S). The emission spectrum of the isolated chromophore and the denatured protein at neutral Ph do not match the spectrum of the native protein, suggesting that chromophore formation occurs post-translationally (e.g., see Cody et al. (1993) Biochemistry 32:1212-1218).

In addition, the crystal structure of purified A. victoria GFP has been determined (e.g., see Ormo (1996) Science 273:1392-1395). The predominant structural features of the protein are an 11-stranded .beta. barrel that forms a nearly perfect cylinder wrapping around a single central .alpha.-helix, which contains the modified p-hydroxybenzylideneimadaxolidinone chromophore. The chromophore is centrally located within the barrel structure and is completely shielded from exposure to bulk solvent.

DNA encoding an isotype of A. victoria GFP has been isolated and its nucleotide sequence has been determined (e.g., see Prasher (1992) Gene 111:229-233). The A. victoria CDNA contains a 714 nucleotide open reading frame that encodes a 238 amino acid polypeptide of a calculated

M.sub.r of 26,888 Da. Recombinantly expressed A. victoria GFPs retain their ability to fluoresce in vivo in a wide variety organisms, including bacteria (e.g., see Chalfie et al. (1994) Science 263:802-805; Miller et al. (1997) Gene 191:149-153), yeast and fungi (Fey et al. (1995) Gene 165:127-130; Straight et al. (1996) Curr. Biol. 6:1599-1608; Cormack et al. (1997) Microbiology 143:303-311), Drosophila (e.g., see Wang et al. (1994) Nature 369:400-403; Plautz (1996) Gene 173:83-87), plants (Heinlein et al. (1995); Casper et al. (1996) Gene 173:69-73), fish (Amsterdam et al. (1995)), and mammals (Ikawa et al. (1995). Aequorea GFP vectors and isolated Aequorea GFP proteins have been used as markers for measuring gene expression, cell migration and localization, microtubule formation and assembly of functional ion channels (e.g., see Terry et al. (1995) Biochem. Biophys. Res. Commun. 217:21-27; Kain et al. (1995) Biotechniques 19:650-655).The A. victoria GFP, however, is not ideal for use in analytical and diagnostic processes. Consequently GFP mutants have been selected with the hope of identifying mutants that have single excitation spectral peaks shifted to the red.

In fact a stated purpose in constructing such mutants has been to attempt to make the A. victoria GFP more like the GFP from Renilla, which has thus far not been cloned, but which has properties that make it far more ideal for use as an analytical tool. For many practical applications, the spectrum of Renilla GFP would be preferable to that of the Aequorea GFP, because wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and narrow rather than low and broad [see, U.S. Pat. No. 5,625,048]. Furthermore, the longer wavelength excitation peak (475 nm) of Renilla GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching [Chalfie et al. (1994) Science 263:802-805].

There exists a phylogenetically diverse and largely unexplored repertoire of bioluminescent proteins that are a reservoir for future development. Many of these, such as nucleic acid encoding Renilla GFPs have not, despite concentrated efforts to do so.

For these reasons, it would be desirable to have a variety of new luciferases and fluorescent proteins, particularly, Renilla GFP available rather than use muteins of A. victoria GFP. It has, not, however, been possible to clone the gene encoding any Renilla GFPs. It would also be desirable to have a variety of GFPs and luciferases available in order to optimize systems for particular applications and to improve upon existing methods. Therefore, it is an object herein to provide isolated nucleic acids encoding heretofore unavailable luciferases and the protein encoded thereby. It is also an object herein to provide isolated nucleic acids encoding Renilla GFPs, GFPs from other species, and luciferases from a variety of species, and the proteins encoded thereby. It is also an object herein to provide bioluminescence generating systems that

include the luciferases, luciferins, and also include GFPs.

SUMMARY OF THE INVENTION

Isolated nucleic acids that encode fluorescent proteins and nucleic acids that encode luciferases are provided. Nucleic acid molecules encoding GFPs from Renilla and from Ptilosarcus are provided. Nucleic acid molecules that encode the Renilla mulleri luciferase, a Gaussia species luciferase and a Pleuromamma species luciferase are provided. Nucleic acid probes derived therefrom are also provided. Functionally equivalent nucleic acids, such as those that hybridize under conditions of high stringency to the disclosed molecules, are also contemplated.

Host cells, including bacterial, yeast and mammalian host cells, and plasmids for expression of the nucleic acids encoding each luciferase and GFP and combinations of luciferases and GFPs are also provided in these hosts are also provided. The genes can be modified by substitution of codons optimized for expression in selected host cells or hosts, such as humans and other mammals, or can be mutagenized to alter the emission properties.

Luciferases

Recombinant host cells, including bacterial, yeast and mammalian cells, containing heterologous nucleic acid encoding a Renilla mulleri luciferase and the nucleic acid are provided. In preferred embodiments, the heterologous nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 18. In more preferred embodiments, the heterologous nucleic acid encodes the sequence of nucleotides set forth in SEQ ID No. 17. Also provided are functionally equivalent nucleic acids, such as nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 17, particularly when using the probes provided herein.

Isolated nucleic acids that encode luciferases from Gaussia are provided herein. In particular, nucleic acid fragments that encode Gaussia princeps luciferase, and nucleic acid probes derived therefrom are provided. In a particular embodiment, the luciferase is encoded by the sequence of nucleotides set forth in SEQ ID No. 19. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 19, particularly when using probes provided herein. Probes derived from this nucleic acid that can be used in methods provided herein to isolate luciferases from any Gaussia species are provided. In an exemplary embodiment, nucleic acid encoding Gaussia princeps luciferase is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 20.

Nucleic acids that encode Pleuromamma luciferase are provided. In particular, a nucleic acid molecule that encodes a Pleuromamma luciferase and the encoded luciferase are set forth in SEQ ID Nos. 28 and 29, respectively. Nucleic acid encoding a Pleuromamma luciferase has also been isolated.

Expression vectors that contain DNA encoding a Renilla mulleri, Gaussia or Pleuromamma luciferase linked in operational association with a promoter element that allows for the constitutive or inducible expression of the luciferase are provided. In preferred embodiments, the vectors are capable of expressing the Renilla mulleri luciferase in a wide variety of host cells. Vectors for producing chimeric Renilla mulleri luciferase fusion proteins, preferably chimeric antibody-luciferase or acetylcholine esterase fusion proteins, containing a promoter element and a multiple cloning site located upstream or downstream of DNA encoding Renilla mulleri luciferase are also provided.

Recombinant cells containing heterologous nucleic acid encoding a Gaussia luciferase are also provided. Purified Gaussia luciferases and compositions containing a Gaussia luciferase alone or in combination with at other components of a bioluminescence-generating system, such as a Renilla green fluorescent protein, are provided. The Gaussia luciferase can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, the Gaussia luciferase may be used in conjunction with suitable fluorescent proteins in assays provided herein.

Methods using the probes for the isolation and cloning of luciferase-encoding DNA in Gaussia, Pleuromamma and other species are also provided. In preferred embodiments, the nucleic acid probes are degenerate probes of at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids set forth in SEQ ID No. 19 and or the sequence of nucleotides set forth in SEQ ID No. 29.

Vectors containing DNA encoding a Gaussia luciferase or Pleuromamma luciferase are provided. In particular, expression vectors that contain DNA encoding the luciferase linked in operational association with a promoter element that allows for the constitutive or inducible expression of luciferase are provided. In preferred embodiments, the vectors are capable of expressing the luciferase in a wide variety of host cells. Vectors for producing chimeric luciferase fusion proteins (see, e.g., U.S. Pat. No. 5,464,745, which describes the use of protein binding domains; see SEQ ID Nos. 21 and 22, which set forth the sequences of a cellulose binding domain-

luciferase fusion protein; and which are depicted in FIGS. 1 and 2) containing a promoter element and a multiple cloning site located upstream or downstream from DNA encoding Gaussia or Pleuromamma luciferase are also provided. In a particular embodiment, DNA encoding the luciferase is linked to DNA encoding the N-terminal portion of the cellulose binding domain (CBD.sub.clos ; see, SEQ ID Nos. 21 and 22) in a PET vector (Novagen; see, U.S. Pat. Nos. 5,719,044 and 5,738,984, 5,670,623 and 5,496,934 and the Novagen catalog; complete sequences of each PET vector are provided with purchase of the vector).

Fusions of the nucleic acid, particularly DNA, encoding a Gaussia or Pleuromamma luciferase with DNA encoding a GFP or phycobiliprotein are also provided herein. Also provided are fusions of Renilla luciferase and a Renilla GFP. In these fusions the luciferase and GFP encoding DNA can be contiguous or separated by a spacer peptide. The fusions are used to produce fusion proteins, which by virtue of the interaction between the luciferase and GFP pair have a variety of unique analytical applications. The interaction is assessed by the emission spectrum of the luciferase-GFP protein pair in the presence of a luciferin and appropriate binding factors.

Recombinant host cells containing heterologous nucleic acid encoding a Gaussia or Pleuromamma luciferase are provided. In certain embodiments, the recombinant cells that contain the heterologous DNA encoding the luciferase are produced by transfection with DNA encoding a luciferase or by introduction of RNA transcripts of DNA encoding the protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

The cells that express functional luciferase may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein. Presently preferred host cells for expressing the luciferase are bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

Purified Gaussia, Pleuromamma and Renilla mulleri luciferases are provided. These luciferases are preferably obtained by expression of the nucleic acid provided herein in prokaryotic or eukaryotic cells that contain the nucleic acid that encodes the luciferase protein; and isolation of the expressed protein.

Compositions containing the luciferases are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a Gaussia luciferase, Gaussia luciferase peptide or Gaussia luciferase fusion protein, formulated for use in luminescent novelty items, immunoassays, donors in FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer]

assays, HTRF [homogeneous time-resolved fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

In more preferred embodiments, the bioluminescence-generating system includes, in addition to the luciferase a Renilla mulleri or Ptilosarcus GFP. These compositions can be used in a variety of methods and systems, such as included in conjunction with diagnostic systems for the in vivo detection of neoplastic tissues and other tissues, such as those methods described herein.

Combinations containing a first composition containing a luciferase and a second composition containing one or more additional components of a bioluminescence-generating system for use with articles of manufacture to produce novelty items are provided. These novelty items are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as cosmetics, bath powders, body lotions, gels, powders and creams, nail polishes, make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. The novelty in the novelty item derives from its bioluminescence.

GFPS

Isolated nucleic acids that encode GFPs from Renilla are provided herein. Also provided are isolated and purified nucleic acids that encode a component of the bioluminescence generating

system and a green fluorescent protein (GFP) of a member of the genus Renilla, and the proteins encoded thereby are provided. In particular, nucleic acid fragments that encode Renilla green fluorescent protein (GFPs) and the Renilla mulleri luciferase, and nucleic acid probes derived therefrom are provided.

Nucleic acid molecules encoding Renilla GFP are provided. In particular, nucleic acid molecules encoding a Renilla GFP that includes the coding portion of the sequence of nucleotides set forth in SEQ ID No. 15 or that hybridizes under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID No. 15, particularly when using probes provided herein, are provided. Probes derived from this nucleic acid that can be used in methods provided herein to isolated GFPs from any Renilla species. In an exemplary embodiment, nucleic acid encoding Renilla mulleri GFP is provided. This nucleic acid encodes the sequence of amino acids set forth in SEQ ID No. 16.

Nucleic acid probes can be labeled, which if needed, for detection, containing at least about 14, preferably at least about 16, or, if desired, 20 or 30 or more, contiguous nucleotides of sequence of nucleotides encoding a Renilla GFP, particularly Renilla mulleri. In preferred embodiments, the nucleic acid probes for the Renilla GFP are selected from the sequence of nucleotides set forth in SEQ ID No. 15.

Methods using the probes for the isolation and cloning of GFP-encoding DNA in Renilla and other species are also provided. In preferred embodiments, the nucleic acid probes are degenerate probes based upon the conserved regions between the Renilla species of GFP as set forth in FIG. 3. Such degenerate probes contain at least 14 nucleotides, preferably 16 to 30 nucleotides, that are based on amino acids 51 to 68, 82 to 98 and 198 to 208 set forth in SEQ ID No. 16, amino acid sequence set forth in SEQ ID No. 24, amino acids 9-20 set forth in SEQ ID No. 25 and amino acids 39-53 set forth in SEQ ID No. 27. In other preferred embodiments, the nucleic acid probes encoding the above-described preferred amino acid regions are selected among the sequence of nucleotides encoding these regions as set forth in SEQ ID NO. 15 . Alternatively, nucleic acids, particularly those set forth in SEQ ID No. 15 that encode the noted regions may be used as primers for PCR amplification of libraries of a selected Renilla species, whereby DNA comprising that encodes a Renilla GFP is isolated.

Nucleic acids that encode a Ptilosarcus GFP are set forth in SEQ ID Nos. 30 and 31; the encoded GFP is set forth in SEQ ID No. 32. Also provided are nucleic acid molecules that hybridize under moderate or high stringency to the sequence of nucleotides set forth in SEQ ID Nos. 28, 30 and 31.

Vectors containing DNA encoding a Renilla or Ptilosarcus GFP are provided. In particular, expression vectors that contain DNA encoding a Renilla or Ptilosarcus GFP linked in operational association with a promoter element that allows for the constitutive or inducible expression of Renilla or Ptilosarcus GFP are provided. Native Renilla GFP has been expressed.

The vectors are capable of expressing the Renilla GFP in a wide variety of host cells. Vectors for producing chimeric Renilla GFP fusion proteins containing a promoter element and a multiple cloning site located upstream or downstream of DNA encoding Renilla GFP are also provided.

Recombinant cells containing heterologous nucleic acid encoding a Ptilosarcus GFP, Renilla GFP, Renilla mulleri luciferase, Gaussia luciferase, and Pleuromamma luciferase are also provided. Purified Renilla mulleri GFP, Renilla reniformis GFP peptides and compositions containing a Renilla GFPs and GFP peptides alone or in combination with at least one component of a bioluminescence-generating system, such as a Renilla mulleri luciferase, are provided. The Renilla GFP and GFP peptide compositions can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and In vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, these proteins may be used in FP [fluorescence polarization] assays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays and also in the BRET assays and sensors provided herein.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:8790-8794; Peerce et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8092-8096; U.S. Pat. No. 4,777,128; U.S. Pat. No. 5,162,508; U.S. Pat. No. 4,927,923; U.S. Pat. No. 5,279,943; and International PCT Application No. WO 92/01225]. Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261]. Non-radioactive energy transfer reactions using GFPs and luciferases, such as a luciferase and its cognate GFP (or multimers thereof), such as in a fusion protein, are contemplated herein.

Nucleic acids that exhibit substantial sequence identity with the nucleic acids provided herein are also contemplated. These are nucleic acids that can be produced by substituting codons that encode conservative amino acids and also nucleic acids that exhibit at least about 80%,

preferably 90 or 95% sequence identity. Sequence identity refers to identity as determined using standard programs with default gap penalties and other defaults as provided by the manufacturer thereof.

The nucleic acids provide an opportunity to produce luciferases and GFPs, which have advantageous application in all areas in which luciferase/luciferins and GFPs have application. The nucleic acids can be used to obtain and produce GFPs and GFPs from other, particularly Renilla species using the probes described herein that correspond to conserved regions (see, e.g., FIG. 3). These GFPs have advantageous application in all areas in which GFPs and/or luciferase/luciferins have application. For example, The GFP's provide a means to amplify the output signal of bioluminescence generating systems. Renilla GFP has a single excitation absorbance peak in blue light (and around 498 nm) and a predominantly single emission peak around 510 nm (with a small shoulder near 540). This spectra provides a means for it to absorb blue light and efficiently convert it to green light. This results in an amplification of the output. When used in conjunction with a bioluminescence generating system that yields blue light, such as Aequorea or Renilla or Vargula (Cypridina), the output signal for any application, including diagnostic applications, is amplified. In addition, this green light can serve as an energy donor in fluorescence-based assays, such as fluorescence polarization assays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays and HTRF [homogeneous time-resolved fluorescence] assays. Particular assays, herein referred to as BRET [bioluminescence resonance energy transfer assays in which energy is transferred from a bioluminescence reaction of a luciferase to a fluorescent protein], are provided.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer that are carried out between a donor luminescent label and an acceptor label [see, e.g., Cardullo et a. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:8790-8794; Peerce et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8092-8096; U.S. Pat. Nos. 4,777,128; 5,162,508; 4,927,923; 5,279,943; and International PCT Application No. WO 92/01225]. Non-radioactive energy transfer reactions using GFPs have been developed [see, International PCT application Nos. WO 98/02571 and WO 97/28261].

Mutagenesis of the GFPs is contemplated herein, particularly mutagenesis that results in modified GFPs that have red-shifted excitation and emission spectra. The resulting systems have higher output compared to the unmutagenized forms. These GFPs may be selected by random mutagenesis and selection for GFPs with altered spectra or by selected mutagenesis of the chromophore region of the GFP.

Recombinant host cells containing heterologous nucleic acid encoding a Renilla or Ptilosarcus

GFP are also provided. In certain embodiments, the recombinant cells that contain the heterologous DNA encoding the Renilla or Ptilosarcus GFP are produced by transfection with DNA encoding a Renilla or Ptilosarcus GFP or by introduction of RNA transcripts of DNA encoding a Renilla or Ptilosarcus protein. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the encoding DNA.

In certain embodiments, the cells contain DNA or RNA encoding a Renilla mulleri GFP or a Ptilosarcus GFP (particularly from a species other than P. gurneyi) also express the recombinant Renilla mulleri GFP or Ptilosarcus polypeptide. It is preferred that the cells are selected to express functional GFPs that retain the ability to fluorescence and that are not toxic to the host cell. In some embodiments, cells may also include heterologous nucleic acid encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species Aequorea, Vargula, Pleuromamma, Ptilosarcus or Renilla. In more preferred embodiments, the bioluminescence-generating system component is a Renilla mulleri luciferase including the amino acid sequence set forth in SEQ ID No. 18 or the Pleuromamma luciferase set forth in SEQ ID No. 28, or the Gaussia luciferase set forth in SEQ ID No. 19 .

The GFPs provided herein may be used in combination with any suitable bioluminescence generating system, but is preferably used in combination with a Renilla or Aequorea, Pleuromamma or Gaussia luciferase.

Purified Renilla GFPs, particularly Renilla mulleri GFP, and purified Renilla reniformis GFP peptides are provided. Presently preferred Renilla GFP for use in the compositions herein is Renilla mulleri GFP including the sequence of amino acids set forth in SEQ ID No. 16. Presently preferred Renilla reniformis GFP peptides are those containing the GFP peptides selected from the amino acid sequences set forth in SEQ ID Nos 19-23.

The Renilla GFP, GFP peptides and luciferase can be isolated from natural sources or isolated from a prokaryotic or eukaryotic cell transfected with nucleic acid that encodes the Renilla GFP and/or luciferase protein.

Fusions of the nucleic acid, particularly DNA, encoding Renilla or Ptilosarcus GFP with DNA encoding a luciferase are also provided herein.

The cells that express functional luciferase and/or GFP, which may be used alone or in

conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein.

Presently preferred host cells for expressing GFP and luciferase are bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

The luciferases and GFPs or cells that express them also may be used in methods of screening for bacterial contamination and methods of screening for metal contaminants. To screen for bacterial contamination, bacterial cells that express the luciferase and/or GFP are put in autoclaves or in other areas in which testing is contemplated. After treatment or use of the area, the area is tested for the presence of glowing bacteria. Presence of such bacteria is indicative of a failure to eradicate other bacteria. Screening for heavy metals and other environmental contaminants can also be performed with cells that contain the nucleic acids provided herein, if expression is linked to a system that is dependent upon the particular heavy metal or contaminant.

The systems and cells provided herein can be used for high throughout screening protocols, intracellular assays, medical diagnostic assays, environmental testing, such as tracing bacteria in water supplies, in conjunction with enzymes for detecting heavy metals, in spores for testing autoclaves in hospital, foods and industrial autoclaves. Non-pathogenic bacteria containing the systems can be included in feed to animals to detect bacterial contamination in animal products and in meats.

Compositions containing a Renilla or Ptilosarcus GFP are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a Renilla GFP or GFP peptide, preferably Renilla mulleri GFP or Renilla reniformis GFP peptide, formulated for use in luminescent novelty items, immunoassays, FET [fluorescent energy transfer] assays, FRET [fluorescent resonance energy transfer] assays, HTRF [homogeneous time-resolved fluorescence] assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein. In other instances, the GFPs are used in beverages, foods or cosmetics.

Compositions that contain a Renilla mulleri GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence-generating system is selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Bioluminescence-generating systems include those isolated from Renilla, Aequorea, and Vargula, Gaussia and Pleuromamma.

Combinations containing a first composition containing a Renilla mulleri GFP or Ptilosarcus GFP or mixtures thereof and a second composition containing a bioluminescence- generating system for use with inanimate articles of manufacture to produce novelty items are provided. These novelty items, which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as bath powders, body lotions, gels, powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, cosmetics, body paints, and bubble bath, bubbles made from non-detergent sources, particularly proteins such as albumin and other non-toxic proteins; in fishing lures, particularly crosslinked polyacrylamide containing a fluorescent protein and/or components of a bioluminescence generating system, which glow upon contact with water; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles.

Methods for diagnosis and visualization of tissues in vivo or in situ using compositions containing a Renilla mulleri GFP and/or a Renilla mulleri luciferase or others of the luciferases and/or GFPs provided herein are provided. For example, the Renilla mulleri GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues in situ. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a Renilla mulleri GFP, a luciferase or luciferin. The

systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the Renilla mulleri GFP. In some embodiments, all components, except for activators, which are provided in situ or are present in the body or tissue, are included in a single composition.

Methods for diagnosis and visualization of tissues in vivo or in situ using compositions containing a Gaussia luciferase are provided. For example, the Gaussia luciferase or Gaussia luciferase peptide can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues in situ. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a Gaussia luciferase, a GFP or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the Gaussia luciferase. In some embodiments, all components, except for activators, which are provided in situ or are present in the body or tissue, are included in a single composition.

In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include antibodies directed against tumor antigens conjugated to a component of the bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other embodiments, tumor-specific targeting agents are linked to microcarriers that are coupled with, preferably more than one of the bioluminescence generating components, preferably more than one luciferase molecule.

The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin are linked to a protein, such as a serum albumin, or other protein carrier. The carrier and time release formulations, permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase.

Methods for diagnosing diseases, particularly infectious diseases, using chip methodology (see, e.g., copending U.S. application Ser. No. 08/990,103) a luciferase/luciferin bioluminescence-generating system and a Renilla mulleri or Ptilosarcus GFP are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system, particularly using luciferase encoded by the nucleic acids provided herein

and/or Renilla mulleri or Ptilosarcus GFP.

In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for a bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted with a second antibody linked to a Renilla or Pleuromamm GFP, a chimeric antibody-Renilla GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a luciferase or luciferin, that are specific for the antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.

Methods for generating chimeric GFP fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP coding region in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream of the GFP coding sequence to produce chimeric GFP proteins.

Methods for identifying compounds using recombinant cells that express heterologous DNA encoding a GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify novel compounds or ligands that modulate the level of transcription from the promoter of interest by measuring GFP-mediated fluorescence. Recombinant cells expressing the chimeric Renilla or Ptilosarcus GFPs may also be used for monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

Other assays using the GFPs and/or luciferases are contemplated herein. Any assay or diagnostic method known used by those of skill in the art that employ Aequora GFPs and/or other luciferases are contemplated herein.

Kits containing the GFPs for use in the methods, including those described herein, are provided. In one embodiment, the kits containing an article of manufacture and appropriate reagents for generating bioluminescence are provided. The kits containing such soap compositions, with preferably a moderate Ph [between 5 and 8] and bioluminescence generating reagents, including luciferase and luciferin and the GFP are provided herein. These kits, for example, can be used with a bubble-blowing or producing toy. These kits can also include a reloading or charging cartridge or can be used in connection with a food.

In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains the GFP and at least one component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

Thus, these kits will typically include two compositions, a first composition containing the GFP formulated for systemic administration (or in some embodiments local or topical application), and a second composition containing the components or remaining components of a bioluminescence generating system, formulated for systemic, topical or local administration depending upon the application. Instructions for administration will be included.

In other embodiments, the kits are used for detecting and identifying diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a secondary antibody, such as an antibody specific for the infectious agent that is linked to a Renilla mulleri or Ptilosarcus GFP protein, a chimeric antibody-Renilla mulleri (or Ptilosarcus) GFP fusion protein or F(Ab).sub.2 antibody fragment-Renilla mulleri GFP fusion protein. A second composition containing a bioluminescence generating system that emits a wavelength of light within the excitation range of the Renilla mulleri GFP, such as species of Renilla or Aequorea, for exciting the Renilla mulleri, which produces green light that is detected by the photodetector of the device to indicate the presence of the agent.

As noted above, fusions of nucleic acid encoding the luciferases and or GFPs provided herein with other luciferases and GFPs are provided. Of particular interest are fusions that encode pairs of luciferases and GFPs, such as a Renilla luciferase and a Renilla GFP (or a homodimer or other multiple of a Renilla GFP). The luciferase and GFP bind and in the presence of a luciferin will produced fluroescence that is red shifted compared to the luciferase in the absence of the GFP.

This fusion or fusions in which the GFP and luciferase are linked via a target, such as a peptide, can be used as a tool to assess anything that interacts with the linker.

Muteins of the GFPs and luciferases are provided. Of particular interest are muteins, such as temperature sensivitive muteins, of the GFP and luciferases that alter their interaction, such as mutations in the Renilla luciferase and Renilla GFP that alters their interaction at a critical temperature.

Antibodies, polyclonal and monoclonal antibodies that specifically bind to any of the proteins encoded by the nucleic acids provided herein are also provided. These antibodies, monoclonal or polyclonal, can be prepared employing standard techniques, known to those of skill in the art. In particular, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure preparation of a luciferase or GFP provided herein or an or epitope-containing fragment thereof are provided. Monoclonal antibodies are also provided. The immunoglobulins that are produced have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a GFP or luciferase, particularly a Renilla or Ptsilocarpus GFP or a Pleuromamma, Gaussia or Renilla mulleri luciferase, that may be present in a biological sample or a solution derived from such a biological sample.

DESCRIPTION OF THE FIGURES

FIG. 1 depicts the components of the commercially available PET-34 vector (EK is enterokinase).

FIG. 2 shows a portion of the vector with the inserted Gaussia-encoding luciferase.

FIG. 3 displays an alignment of the deduced amino acid sequence of Renilla mulleri green fluorescent protein and the amino acid sequence of isolated Renilla reniformis GFP peptides obtained by proteolytic digestion of purified Renilla reniformis GFP. Positions in the amino acid sequence of direct identity are marked by the solid vertical lines (.vertline.) between the two Renilla species.

FIG. 4 shows the fluorescence emission and excitation spectrum for the Renilla mulleri GFP, with a peak emission at 506 nm.

FIG. 5 shows the fluorescence emission and excitation spectrum for the Ptilosarcus GFP, with a peak emission at 508.

EXHIBIT "B"

United States Patent **7,109,315**
Bryan , et al. **September 19, 2006**

Renilla reniformis fluorescent proteins, nucleic acids encoding the fluorescent proteins and the use thereof in diagnostics, high throughput screening and novelty items

Abstract

Isolated and purified nucleic acids encoding green fluorescent proteins from Renilla reniformis and the green fluorescent protein encoded thereby are also provided. Mutants of the nucleic acid molecules and the modified encoded proteins are also provided. Compositions and combinations comprising the green fluorescent proteins and/or the luciferase are further provided.

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Field of Search:	536/23.1 435/69.1,325,252.3,320.1

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Parent Case Text

RELATED APPLICATIONS

Benefit of priority under 35 U.S.C. .sctn.119(e) is claimed to U.S. provisional application Ser. No. 60/189,691, filed Mar. 15, 2000, to Bryan et al., entitled "RENILLA RENIFORMIS FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS" is claimed.

This application is related to allowed U.S. application Ser. No. 09/277,716, filed Mar. 26, 1999, to Bruce Bryan and Christopher Szent-Gyorgyi, entitled "LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS." This application is related to International PCT application No. WO 99/49019 to Bruce Bryan and Prolume, LTD., entitled "LUCIFERASES, FLUORESCENT PROTEINS, NUCLEIC ACIDS ENCODING THE LUCIFERASES AND

FLUORESCENT PROTEINS AND THE USE THEREOF IN DIAGNOSTICS, HIGH THROUGHPUT SCREENING AND NOVELTY ITEMS."

This application is also related to subject matter in U.S. application Ser. No. 08/757,046, filed Nov. 25, 1996, to Bruce Bryan entitled "BIOLUMINESCENT NOVELTY ITEMS", now U.S. Pat. No. 5,876,995, issued Mar. 2, 1999, and in U.S. application Ser. No. 08/597,274, filed Feb. 6, 1996, to Bruce Bryan, entitled "BIOLUMINESCENT NOVELTY ITEMS". This application is also related to U.S. application Ser. No. 08/908,909, filed Aug. 8, 1997, to Bruce Bryan entitled "DETECTION AND VISUALIZATION OF NEOPLASTIC TISSUE AND OTHER TISSUES". The application is also related to U.S. application Ser. No. 08/990,103, filed Dec. 12, 1997, to Bruce Bryan entitled "APPARATUS AND METHODS FOR DETECTING AND IDENTIFYING INFECTIOUS AGENTS".

Where permitted, the subject matter of each of the above noted applications and patents is herein incorporated by reference in its entirety.

Claims

What is claimed is:

1. An isolated nucleic acid molecule encoding a Renilla reniformis green fluorescent protein, comprising a nucleotide sequence that encodes the protein of SEQ ID NO. 27 or a green fluorescent protein encoded by a nucleic acid molecule of Renilla reniformis having at least 80% sequence identity thereto.

2. An isolated nucleic acid molecule of claim 1 that encodes a protein having at least 90% sequence identity to the protein of SEQ ID NO. 27.

3. The isolated nucleic acid molecule of claim 1, comprising a nucleotide sequence selected from the group consisting of: (a) the coding portion of the nucleotide sequence set forth in any of SEQ ID NOs. 23-25; (b) a nucleotide sequence that hybridizes under conditions of 0.1.times. standard saline phosphate EDTA buffer, 0.1% SDS at 65.degree. C. to the nucleotide sequence of (a); and (c) a nucleotide sequence comprising degenerate codons of (a) or (b).

4. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid is DNA.

5. The isolated nucleic acid molecule of claim 1, wherein the nucleic acid is RNA.

6. A nucleic acid probe or primer, comprising at least 14 contiguous nucleotides selected from the nucleotide sequence set of claim 1.

7. The probe or primer of claim 6, comprising at least 16 contiguous nucleotides selected from the nucleotide sequence in claim 1.

8. The probe or primer of claim 7, comprising at least 30 contiguous nucleotides.

9. A plasmid, comprising the nucleotide sequence of claim 1.

10. A cell comprising the plasmid of claim 9, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

11. The plasmid of claim 9 that is an expression vector, further comprising: a promoter element; a cloning site for the introduction of the nucleic acid molecule; and a marker gene; wherein the nucleic acid comprising the cloning site is positioned between nucleic acids encoding the promoter element and the green fluorescent protein and wherein the nucleic acid encoding the green fluorescent protein is operatively linked to the promoter element.

12. The plasmid of claim 11, wherein the marker gene comprises a nucleotide sequence encoding a luciferase.

13. A recombinant host cell, comprising the plasmid of claim 9.

14. The cell of claim 13, wherein the cell is selected from the group consisting of a bacterial cell, a yeast cell, a fungal cell, a plant cell, an insect cell and an animal cell.

15. A reporter gene construct, comprising the nucleic acid molecule of claim 1.

16. A nucleic acid construct, comprising the nucleotide sequence encoding a luciferase and a nucleotide sequence of claim 1 that encodes a Renilla reniformis fluorescent protein (GFP).

17. The nucleic acid construct of claim 16, wherein the luciferase is a Renilla mulleri luciferase, a Gaussia luciferase or a Pleuromamma luciferase.

18. The nucleic acid construct of claim 17, wherein the Gaussia luciferase is a Gaussia princeps

luciferase.

19. The nucleic acid construct of claim 16, wherein the luciferase is encoded by: a nucleotide sequence set forth in SEQ ID NO. 17, SEQ ID NO. 19, or SEQ ID NO. 28; a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO. 18, SEQ ID NO. 20 or SEQ ID NO. 29; and a nucleotide sequence that hybridizes under the condition of claim 3 to the nucleotide sequence set forth in SEQ ID NO. 17, SEQ ID NO. 19 or SEQ ID NO. 28.

20. The nucleic acid construct of claim 16, that is DNA.

21. The nucleic acid construct of claim 16, that is RNA.

22. A plasmid, comprising the nucleic acid construct of claim 16.

23. The plasmid of claim 22, further comprising a nucleotide sequence encoding: a promoter element; and a selectable marker; wherein, the nucleotide sequence encoding the luciferase and Renilla reniformis GFP is operatively linked to the promoter element, whereby the luciferase and Renilla reniformis GFP are expressed.

24. The construct of claim 16, wherein the luciferase and the GFP are encoded by a polycistronic message.

25. The construct of claim 16, wherein the encoded luciferase and Renilla reniformis GFP comprise a fusion protein.

26. The nucleic acid construct of claim 25, wherein the nucleotide sequence that encoding the luciferase and GFP are not contiguous.

27. The nucleic acid construct of claim 26, comprising a nucleotide sequence that encodes a ligand binding domain of a target protein.

28. The construct of claim 16, wherein the luciferase is Renilla reniformis luciferase.

29. A recombinant host cell, comprising the plasmid of claim 22.

Description

FIELD OF INVENTION

Provided herein are isolated and purified nucleic acids and encoded fluorescent proteins from Renilla reniformis and uses thereof.

BACKGROUND OF THE INVENTION

Luminescence is a phenomenon in which energy is specifically channeled to a molecule to produce an excited state. Return to a lower energy state is accompanied by release of a photon (h.gamma.). Luminescence includes fluorescence, phosphorescence, chemiluminescence and bioluminescence. Bioluminescence is the process by which living organisms emit light that is visible to other organisms. Luminescence may be represented as follows: A+B.fwdarw.X*+Y X*.fwdarw.X+hv, where X* is an electronically excited molecule and h.gamma. represents light emission upon return of X* to a lower energy state. Where the luminescence is bioluminescence, and creation of the excited state is derived from an enzyme catalyzed reaction. The color of the emitted light in a bioluminescent (or chemiluminescent or other luminescent) reaction is characteristic of the excited molecule, and is independent from its source of excitation and temperature.

An essential condition for bioluminescence is the use of molecular oxygen, either bound or free in the presence of a luciferase. Luciferases, are oxygenases, that act on a substrate, luciferin, in the presence of molecular oxygen and transform the substrate to an excited state. Upon return to a lower energy level, energy is released in the form of light (for reviews see, e.g., McElroy et al. (1966) in Molecular Architecture in Cell Physiology, Hayashi et al., eds., Prentice-Hall, Inc., Englewood Cliffs, N.J., pp. 63-80; Ward et al., Chapter 7 in Chemi-and Bioluminescence, Burr, ed., Marcel Dekker, Inc. NY, pp. 321-358; Hastings, J. W. in (1995) Cell Physiology Source Book, N. Sperelakis (ed.), Academic Press, pp 665-681; Luminescence, Narcosis and Life in the Deep Sea, Johnson, Vantage Press, NY, see, esp. pp. 50-56).

Though rare overall, bioluminescence is more common in marine organisms than in terrestrial organisms. Bioluminescence has developed from as many as thirty evolutionarily distinct origins and, thus, is manifested in a variety of ways so that the biochemical and physiological mechanisms responsible for bioluminescence in different organisms are distinct. Bioluminescent species span many genera and include microscopic organisms, such as bacteria (primarily marine bacteria including Vibrio species), fungi, algae and dinoflagellates, to marine organisms, including arthropods, mollusks, echinoderms, and chordates, and terrestrial organisms including annelid worms and insects.

Assays Employing Bioluminescence

During the past twenty years, high-sensitivity biochemical assays used in research and in medicine have increasingly employed luminescence and fluorescence rather than radioisotopes. This change has been driven partly by the increasing expense of radioisotope disposal and partly by the need to find more rapid and convenient assay methods. More recently, the need to perform biochemical assays in situ in living cells and whole animals has driven researchers toward protein-based luminescence and fluorescence. The uses of firefly luciferase for ATP assays, aequorin and obelin as calcium reporters, Vargula luciferase as a neurophysiological indicator, and the Aequorea green fluorescent protein as a protein tracer and pH indicator show the potential of bioluminescence-based methods in research laboratories.

Bioluminescence is also beginning to directly impact medicine and biotechnology; for example, Aequorea green fluorescent protein (GFP) is employed to mark cells in murine model systems and as a reporter in high throughput drug screening. Renilla luciferase is under development for use in diagnostic platforms.

Bioluminescence Generating Systems

Bioluminescence, as well as other types of chemiluminescence, is used for quantitative determinations of specific substances in biology and medicine. For example, luciferase genes have been cloned and exploited as reporter genes in numerous assays, for many purposes. Since the different luciferase systems have different specific requirements, they may be used to detect and quantify a variety of substances. The majority of commercial bioluminescence applications are based on firefly (Photinus pyralis) luciferase. One of the first and still widely used assays involves the use of firefly luciferase to detect the presence of ATP. It is also used to detect and quantify other substrates or co-factors in the reaction. Any reaction that produces or utilizes NAD(H), NADP(H) or long chain aldehyde, either directly or indirectly, can be coupled to the light-emitting reaction of bacterial luciferase.

Another luciferase system that has been used commercially for analytical purposes is the Aequorin system. The purified jellyfish photoprotein, aequorin, is used to detect and quantify intracellular Ca.sup.2+ and its changes under various experimental conditions. The Aequorin photoprotein is relatively small (.about.20 kDa), nontoxic, and can be injected into cells in quantities adequate to detect calcium over a large concentration range (3.times.10.sup.-7 to 10.sup.-4 M).

Because of their analytical utility, luciferases and substrates have been studied and well-

characterized and are commercially available (e.g., firefly luciferase is available from Sigma, St. Louis, Mo., and Boehringer Mannheim Biochemicals, Indianapolis, Ind.; recombinantly produced firefly luciferase and other reagents based on this gene or for use with this protein are available from Promega Corporation, Madison, Wis.; the aequorin photoprotein luciferase from jellyfish and luciferase from Renilla are commercially available from Sealite Sciences, Bogart, Ga.; coelenterazine, the naturally-occurring substrate for these luciferases, is available from Molecular Probes, Eugene, Oreg.). These luciferases and related reagents are used as reagents for diagnostics, quality control, environmental testing and other such analyses.

Because of the utility of luciferases as reagents in analytical systems and the potential for use in high throughput screening systems, there is a need to identify and isolate a variety of luciferases that have improved or different spectral properties compared to those presently available. For all these reasons, it would be advantageous to have luciferases from a variety of species, such as Gaussia and various Renilla species available.

Fluorescent Proteins

Reporter genes, when co-transfected into recipient cells with a gene of interest, provide a means to detect transfection and other events. Among reporter genes are those that encode fluorescent proteins. The bioluminescence generating systems described herein are among those used as reporter genes. To increase the sensitivity bioluminescence generating systems have been combined with fluorescent compounds and proteins, such as naturally fluorescent phycobiliproteins. Also of interest are the fluorescent proteins that are present in a variety of marine invertebrates, such as the green and blue fluorescent proteins, particularly the green fluorescent protein (GFP) of Aequorea victoria.

The green fluorescent proteins (GFP) constitute a class of chromoproteins found only among certain bioluminescent coelenterates. These accessory proteins are fluorescent and function as the ultimate bioluminescence emitter in these organisms by accepting energy from enzyme-bound, excited-state oxyluciferin (e.g., see Ward et al. (1979) J. Biol. Chem. 254:781-788; Ward et al. (1978) Photochem. Photobiol. 27:389-396; Ward et al. (1982) Biochemistry 21:4535-4540).

The best characterized GFPs are those isolated from the jellyfish species Aequorea, particularly Aequorea victoria (A. victoria) and Aequorea forskalea (Ward et al. (1982) Biochemistry 21:4535-4540; Prendergast et al. (1978) Biochemistry 17:3448-3453). Purified A. victoria GFP is a monomeric protein of about 27 kDa that absorbs blue light with excitation wavelength maximum of 395 nm, with a minor peak at 470 nm, and emits green fluorescence with an emission wavelength of about 510 nm and a minor peak near 540 nm (Ward et al. (1979)

Photochem. Photobiol. Rev 4:1-57). This GFP has certain limitations. The excitation maximum of the wildtype GFP is not within the range of wavelengths of standard fluorescein detection optics.

The detection of green fluorescence does not require any exogenous substrates or co-factors. Instead, the high level of fluorescence results from the intrinsic chromophore of the protein. The chromophore includes modified amino acid residues within the polypeptide chain. For example, the fluorescent chromophore of A. victoria GFP is encoded by the hexapeptide sequence, FSYGVQ, encompassing amino acid residues 64-69. The chromophore is formed by the intramolecular cyclization of the polypeptide backbone at residues Ser65 and Gly67 and the oxidation of the .alpha.-.beta. bond of residue Tyr66 (e.g., see Cody et al. (1993) Biochemistry 32:1212-1218; Shimomura (1978) FEBS Letters 104:220-222; Ward et al. (1989) Photochem. Photobiol. 49:62S). The emission spectrum of the isolated chromophore and the denatured protein at neutral pH do not match the spectrum of the native protein, suggesting that chromophore formation occurs post-translationally (e.g., see Cody et al. (1993) Biochemistry 32:1212-1218).

In addition, the crystal structure of purified A. victoria GFP has been determined (e.g., see Ormo (1996) Science 273:1392-1395). The predominant structural features of the protein are an 11-stranded .beta. barrel that forms a nearly perfect cylinder wrapping around a single central .alpha.-helix, which contains the modified p-hydroxybenzylideneimadaxolidinone chromophore. The chromophore is centrally located within the barrel structure and is completely shielded from exposure to bulk solvent.

DNA encoding an isotype of A. victoria GFP has been isolated and its nucleotide sequence has been determined (e.g., see Prasher (1992) Gene 111:229-233). The A. victoria cDNA contains a 714 nucleotide open reading frame that encodes a 238 amino acid polypeptide of a calculated M.sub.r of 26,888 Da. Recombinantly expressed A. victoria GFPs retain their ability to fluoresce in vivo in a wide variety organisms, including bacteria (e.g., see Chalfie et al. (1994) Science 263:802-805; Miller et al. (1997) Gene 191:149-153), yeast and fungi (Fey et al. (1995) Gene 165:127-130; Straight et al. (1996) Curr. Biol. 6:1599-1608; Cormack et al. (1997) Microbiology 143:303-311), Drosophila (e.g., see Wang et al. (1994) Nature 369:400-403; Plautz (1996) Gene 173:83-87), plants (Heinlein et al. (1995); Casper et al. (1996) Gene 173:69-73), fish (Amsterdam et al. (1995), and mammals (Ikawa et al. (1995). Aequorea GFP vectors and isolated Aequorea GFP proteins have been used as markers for measuring gene expression, cell migration and localization, microtubule formation and assembly of functional ion channels (e.g., see Terry et al. (1995) Biochem. Biophys. Res. Commun. 217:21-27; Kain et al. (1995) Biotechniques 19:650-655). The A. victoria GFP, however, is not ideal for use in analytical and diagnostic

processes. Consequently GFP mutants have been selected with the hope of identifying mutants that have single excitation spectral peaks shifted to the red.

In fact a stated purpose in constructing such mutants has been to attempt to make the A. victoria GFP more like the GFP from Renilla, but which has properties that make it far more ideal for use as an analytical tool. For many practical applications, the spectrum of Renilla GFP is preferable to that of the Aequorea GFP, because wavelength discrimination between different fluorophores and detection of resonance energy transfer are easier if the component spectra are tall and narrow rather than low and broad (see, U.S. Pat. No. 5,625,048). Furthermore, the longer wavelength excitation peak (475 nm) of Renilla GFP is almost ideal for fluorescein filter sets and is resistant to photobleaching, but has lower amplitude than the shorter wavelength peak at 395 nm, which is more susceptible to photobleaching (Chalfie et al. (1994) Science 263:802-805).

There exists a phylogenetically diverse and largely unexplored repertoire of bioluminescent proteins that are a reservoir for future development. For these reasons, it would be desirable to have a variety of new luciferases and fluorescent proteins, particularly, Renilla reniformis GFP available rather than use muteins of A. victoria GFP. Published International PCT application No. WO 99/49019 (see, also, allowed U.S. application Ser. No. 09/277,716) provides a variety of GFPs including those from Renilla species. It remains desirable to have a variety of GFPs and luciferases available in order to optimize systems for particular applications and to improve upon existing methods. Therefore, it is an object herein to provide isolated nucleic acid molecules encoding Renilla reniformis GFP and the protein encoded thereby. It is also an object herein to provide bioluminescence generating systems that include the luciferases, luciferins, and also include Renilla reniformis GFP.

SUMMARY OF THE INVENTION

"Isolated nucleic acid molecules that encode Renilla reniformis fluorescent proteins are provided. Nucleic acid probes and primers derived therefrom are also provided. Functionally equivalent nucleic acids, such as those that hybridize under conditions of high stringency to the disclosed molecules and those that have high sequence identity, are also contemplated. Nucleic acid molecules and the encoded proteins are set forth in SEQ ID. NOs. 23-27, an exemplary mutein is set forth in SEQ ID. NO. 33. Also contemplated are nucleic acid molecules that encode the protein set forth in SEQ ID. NO. 27."

Host cells, including bacterial, yeast and mammalian host cells, and plasmids for expression of the nucleic acids encoding the Renilla reniformis green fluorescent protein (GFP), are also provided. Combinations of luciferases and the Renilla reniformis GFP are also provided.

The genes can be modified by substitution of codons optimized for expression in selected host cells or hosts, such as humans and other mammals, or can be mutagenized to alter the emission properties. Mutations that alter spectral properties are also contemplated.

Such mutations may be identified by substituting each codon with one encoding another amino acid, such as alanine, and determining the effect on the spectral properties of the resulting protein. Particular regions of interest are those in which corresponding the sites mutated in other GFPs, such Aequora to produce proteins with altered spectral properties are altered.

The Renilla reniformis GFP may be used in combination with nucleic acids encoding luciferases, such as those known to those of skill in the art and those that are described in copending allowed U.S. application Ser. No. 09/277,716 (see, also, Published International PCT application No. WO 99/49019).

Compositions containing the Renilla reniformis GFP or the Renilla reniformis GFP and luciferase combination are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a Gaussia luciferase, Gaussia luciferase peptide or Gaussia luciferase fusion protein, formulated for use in luminescent novelty items, immunoassays, donors in FET (fluorescent energy transfer) assays, FRET (fluorescent resonance energy transfer) assays, HTRF (homogeneous time-resolved fluorescence) assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein.

The bioluminescence-generating system includes, in addition to the luciferase, a Renilla reniformis GFP or mutated form thereof. These compositions can be used in a variety of methods and systems, such as those included in conjunction with diagnostic systems for the in vivo detection of neoplastic tissues and other tissues, such as those methods described herein.

Combinations of the Renilla reniformis GFP with articles of manufacture to produce novelty items are provided. These novelty items are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as cosmetics, bath powders, body lotions, gels, powders and creams, nail polishes, make-up, toothpastes and other dentifrices, soaps, body paints, and bubble bath; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins

and transgenic fish, particularly transgenic fish that express a luciferase; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase; and beverages, such as beer, wine, champagne, soft drinks, and ice cubes and ice in other configurations; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable form. The combinations optionally include a bioluminescence generating system. The bioluminescence generating systems can be provided as two compositions: a first composition containing a luciferase and a second composition containing one or more additional components of a bioluminescence generating system.

Any article of manufacture that can be combined with a bioluminescence-generating system as provided herein and thereby provide entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles. The novelty in the novelty item derives from its bioluminescence.

GFPS

"Isolated nucleic acids that encode GFP from Renilla reniformis are provided herein. Also provided are isolated and purified nucleic acids that encode a component of the bioluminescence generating system and the green fluorescent protein (GFP) (see SEQ ID NOs. 23-27). In particular, nucleic acid molecules that encode Renilla reniformis green fluorescent protein (GFPs) and nucleic acid probes and primers derived therefrom are provided (see SEQ ID NOs. 23-26).

Nucleic acid probes and primers containing 14, 16, 30, 100 or more contiguous nucleotides from any of SEQ ID NOs. 23-26 are provided. Nucleic acid probes can be labeled, if needed, for detection, containing at least about 14, preferably at least about 16, or, if desired, 20 or 30 or more, contiguous nucleotides of the sequence of nucleotides encoding the Renilla reniformis GFP."

Methods using the probes for the isolation and cloning of GFP-encoding DNA in Renilla reniformis are also provided. Vectors containing DNA encoding the Renilla reniformis GFP are provided. In particular, expression vectors that contain DNA encoding a Renilla reniformis or in operational association with a promoter element that allows for the constitutive or inducible

expression of Renilla reniformis are provided.

The vectors are capable of expressing the Renilla reniformis GFP in a wide variety of host cells. Vectors for producing chimeric Renilla reniformis GFP/luciferase fusion proteins and/or polycistronic mRNA containing a promoter element and a multiple cloning site located upstream or downstream of DNA encoding Renilla reniformis GFP are also provided.

Recombinant cells containing heterologous nucleic acid encoding a Renilla reniformis GFP are also provided. Purified Renilla reniformis GFP peptides and compositions containing the Renilla GFPs and GFP peptides alone or in combination with at least one component of a bioluminescence-generating system, such as a Renilla luciferase, are provided. The Renilla GFP and GFP peptide compositions can be used, for example, to provide fluorescent illumination of novelty items or used in methods of detecting and visualizing neoplastic tissue and other tissues, detecting infectious agents using immunoassays, such homogenous immunoassays and in vitro fluorescent-based screening assays using multi-well assay devices, or provided in kits for carrying out any of the above-described methods. In particular, these proteins may be used in FP (fluorescence polarization) assays, FET (fluorescent energy transfer) assays, FRET (fluorescent resonance energy transfer) assays and HTRF (homogeneous time-resolved fluorescence) assays and also in the BRET assays and sensors provided herein.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer and are carried out between a donor luminescent label and an acceptor label (see, e.g., Cardullo et al. (1988) Proc. Natl. Acad. Sd. U.S.A. 85:8790-8794; Peerce et al. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:8092-8096; U.S. Pat. No. 4,777,128; U.S. Pat. No. 5,162,508; U.S. Pat. No. 4,927,923; U.S. Pat. No. 5,279,943; and International PCT Application No. WO 92/01 225). Non-radioactive energy transfer reactions using GFPs have been developed (see, International PCT application Nos. WO 98/02571 and WO 97/28261). Non-radioactive energy transfer reactions using GFPs and luciferases, such as a luciferase and its cognate GFP (or multimers thereof), such as in a fusion protein, are contemplated herein.

Nucleic acids that exhibit substantial sequence identity with the nucleic acids provided herein are also contemplated. These are nucleic acids that can be produced by substituting codons that encode conservative amino acids and also nucleic acids that exhibit at least about 80%, preferably 90 or 95% sequence identity. Sequence identity refers to identity as determined using standard programs with default gap penalties and other defaults as provided by the manufacturer thereof.

The nucleic acids provide an opportunity to produce luciferases and GFPs, which have advantageous application in all areas in which luciferase/luciferins and GFPs have application. The nucleic acids can be used to obtain and produce GFPs and GFPs from other, particularly Renilla, species using the probes described herein that correspond to conserved regions. These GFPs provide a means to amplify the output signal of bioluminescence generating systems. Renilla GFP has a single excitation absorbance peak in blue light (and around 498 nm) and a predominantly single emission peak around 510 nm (with a small shoulder near 540 nm). This spectrum provides a means for it to absorb blue light and efficiently convert it to green light. This results in an amplification of the output. When used in conjunction with a bioluminescence generating system that yields blue light, such as Aequorea or Renilla or Vargula (Cypridina), the output signal for any application, including diagnostic applications, is amplified. In addition, this green light can serve as an energy donor in fluorescence-based assays, such as fluorescence polarized assays, FET (fluorescent energy transfer) assays, FRET (fluorescent resonance energy transfer) assays and HTRF (homogeneous time-resolved fluorescence) assays. Particular assays, herein referred to as BRET (bioluminescence resonance energy transfer assays in which energy is transferred from a bioluminescence reaction of a luciferase to a fluorescent protein), arc provided.

Non-radioactive energy transfer reactions, such as FET or FRET, FP and HTRF assays, are homogeneous luminescence assays based on energy transfer that are carried out between a donor luminescent label and an acceptor label (see, e.g., Cardullo et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:8790-8794; Peerce et al. (1986) Proc. Natl. Acad. Sc. U.S.A. 83:8092-8096; U.S. Pat. No. 4,777,128; U.S. Pat. No. 5,162,508; U.S. Pat. No. 4,927,923; U.S. Pat. No. 5,279,943; and International PCT Application No. WO 92/01225). Non-radioactive energy transfer reactions using GFPs have been developed (see, International PCT application Nos. WO 98/02571 and WO 97/28261).

Mutagenesis of the GFPs is contemplated herein, particularly mutagenesis that results in modified GFPs that have red-shifted excitation and emission spectra. The resulting systems have higher output compared to the unmutagenized forms. These GFPs may be selected by random mutagenesis and selection for GFPs with altered spectra or by selected mutagenesis of the chromophore region of the GFP.

"The DNA may be introduced as a linear DNA molecule (fragment) or may be included in an expression vector for stable or transient expression of the encoding DNA. In certain embodiments, the cells that contain DNA or RNA encoding a Renilla GFP also express the recombinant Renilla GFP or polypeptide. It is preferred that the cells selected to express functional GFPs that retain the ability to fluorescence and that are not toxic to the host cell. In

some embodiments, cells may also include heterologous nucleic acid encoding a component of a bioluminescence-generating system, preferably a photoprotein or luciferase. In preferred embodiments, the nucleic acid encoding the bioluminescence-generating system component is isolated from the species Aequorea, Vargula, Pleuromamma, Ptilosarcus or Renilla. In more preferred embodiments, the bioluminescence-generating system component is a Renilla reniformis luciferase or mullen including the amino acid sequence set forth in SEQ ID NO. 19 or the Pleuromamma luciferase set forth in SEQ ID NO. 28, or the Gaussia luciferase set forth in SEQ ID NO. 19."

The GFPs provided herein may be used in combination with any suitable bioluminescence generating system, but is preferably used in combination with a Renilla or Aequorea, Pleuromamma or Gaussia luciferase.

Purified Renilla GFPs, particularly purified Renilla reniformis GFP peptides are provided. Presently preferred Renilla GFP for use in the compositions herein is Renilla reniformis GFP including the sequence of amino acids set forth above and in the Sequence Listing.

Fusions of the nucleic acid, particularly DNA, encoding Renilla GFP with DNA encoding a luciferase are also provided herein.

The cells that express functional luciferase and/or GFP, which may be used alone or in conjunction with a bioluminescence-generating system, in cell-based assays and screening methods, such as those described herein.

Presently preferred host cells for expressing GFP and luciferase are bacteria, yeasts, fungi, plant cells, insect cells and animal cells.

The luciferases and GFPs or cells that express them also may be used in methods of screening for bacterial contamination and methods of screening for metal contaminants. To screen for bacterial contamination, bacterial cells that express the luciferase and/or GFP are put in autoclaves or in other areas in which testing is contemplated. After treatment or use of the area, the area is tested for the presence of glowing bacteria. Presence of such bacteria is indicative of a failure to eradicate other bacteria. Screening for heavy metals and other environmental contaminants can also be performed with cells that contain the nucleic dependent upon the particular heavy metal or contaminant.

The systems and cells provided herein can be used for high throughout screening protocols, intracellular assays, medical diagnostic assays, environmental testing, such as tracing bacteria in

water supplies, in conjunction with enzymes for detecting heavy metals, in spores for testing autoclaves in hospital, foods and industrial autoclaves. Non-pathogenic bacteria containing the systems can be included in feed to animals to detect bacterial contamination in animal products and in meats.

Compositions containing a Renilla GFP are provided. The compositions can take any of a number of forms, depending on the intended method of use therefor. In certain embodiments, for example, the compositions contain a Renilla GFP or GFP peptide, preferably Renilla mulleri GFP or Renilla reniformis GFP peptide, formulated for use in luminescent novelty items, immunoassays, FET (fluorescent energy transfer) assays, FRET (fluorescent resonance energy transfer) assays, HTRF (homogeneous time-resolved fluorescence) assays or used in conjunction with multi-well assay devices containing integrated photodetectors, such as those described herein. In other instances, the GFPs are used in beverages, foods or cosmetics.

Compositions that contain a Renilla reniformis GFP or GFP peptide and at least one component of a bioluminescence-generating system, preferably a luciferase, luciferin or a luciferase and a luciferin, are provided. In preferred embodiments, the luciferase/luciferin bioluminescence-generating system is selected from those isolated from: an insect system, a coelenterate system, a ctenophore system, a bacterial system, a mollusk system, a crustacea system, a fish system, an annelid system, and an earthworm system. Bioluminescence-generating systems include those isolated from Renilla, Aequorea, and Vargula, Gaussia and Pleuromamma.

Combinations containing a first composition containing a Renilla reniformis GEP or Ptilosarcus GFP or mixtures thereof and a second composition containing a bioluminescence-generating system for use with inanimate articles of manufacture to produce novelty items are provided. These novelty items, which are articles of manufacture, are designed for entertainment, recreation and amusement, and include, but are not limited to: toys, particularly squirt guns, toy cigarettes, toy "Halloween" eggs, footbags and board/card games; finger paints and other paints, slimy play material; textiles, particularly clothing, such as shirts, hats and sports gear suits, threads and yarns; bubbles in bubble making toys and other toys that produce bubbles; balloons; figurines; personal items, such as bath powders, body lotions, gels, powders and creams, nail polishes, cosmetics including make-up, toothpastes and other dentifrices, soaps, cosmetics, body paints, and bubble bath, bubbles made from non-detergent sources, particularly proteins such as albumin and other non-toxic proteins; in fishing lures and glowing transgenic worms, particularly crosslinked polyacrylamide containing a fluorescent protein and/or components of a bioluminescence generating system, which glow upon contact with water; items such as inks, paper; foods, such as gelatins, icings and frostings; fish food containing luciferins and transgenic animals, such as transgenic fish, worms, monkeys, rodents, ungulates, ovine, ruminants and

others, that express a luciferase and/or Renilla reniformis GFP; transgenic worms that express Renilla reniformis GFP and are used as lures; plant food containing a luciferin or luciferase, preferably a luciferin for use with transgenic plants that express luciferase and Renilla reniformis GFP, transgenic plants that express Renilla reniformis GFP, particularly ornamental plants, such as orchids, roses, and other plants with decorative flowers; transgenic plants and animals in which the Renilla reniformis GFP is a marker for tracking introduction of other genes; and beverages, such as beer, wine, champagne, soft drinks, milk and ice cubes and ice in other configurations containing Renilla reniformis GFP; fountains, including liquid "fireworks" and other such jets or sprays or aerosols of compositions that are solutions, mixtures, suspensions, powders, pastes, particles or other suitable forms.

Any article of manufacture that can be combined with a bioluminescence-generating system and Renilla reniformis GFP or with just a Renilla reniformis GFP, as provided herein, that thereby provides entertainment, recreation and/or amusement, including use of the items for recreation or to attract attention, such as for advertising goods and/or services that are associated with a logo or trademark is contemplated herein. Such uses may be in addition to or in conjunction with or in place of the ordinary or normal use of such items. As a result of the combination, the items glow or produce, such as in the case of squirt guns and fountains, a glowing fluid or spray of liquid or particles.

Methods for diagnosis and visualization of tissues in vivo or in situ using compositions containing a Renilla reniformis GFP and/or a Renilla reniformis or mulleri luciferase or others of the luciferases and/or GFPs provided herein are provided. For example, the Renilla reniformis GFP protein can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues in situ. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a Renilla reniformis GFP, a luciferase or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the Renilla reniformis GFP. In some embodiments, all components, except for activators, which are provided in situ or are present in the body or tissue, are included in a single composition.

Methods for diagnosis and visualization of tissues in vivo or in situ using compositions containing a Gaussia luciferase are provided. For example, the Gaussia luciferase or Gaussia luciferase peptide can be used in conjunction with diagnostic systems that rely on bioluminescence for visualizing tissues in situ. The systems are particularly useful for visualizing and detecting neoplastic tissue and specialty tissue, such as during non-invasive and invasive

procedures. The systems include compositions containing conjugates that include a tissue specific, particularly a tumor-specific, targeting agent linked to a targeted agent, a Gaussia luciferase, a GFP or luciferin. The systems also include a second composition that contains the remaining components of a bioluminescence generating reaction and/or the Gaussia luciferase. In some embodiments, all components, except for activators, which are provided in situ or are present in the body or tissue, are included in a single composition.

In particular, the diagnostic systems include two compositions. A first composition that contains conjugates that, in preferred embodiments, include antibodies directed against tumor antigens conjugated to a component of the bioluminescence generating reaction, a luciferase or luciferin, preferably a luciferase are provided. In certain embodiments, conjugates containing tumor-specific targeting agents are linked to luciferases or luciferins. In other embodiments, tumor-specific targeting agents are linked to microcarriers that are coupled with, preferably more than one of the bioluminescence generating components, preferably more than one luciferase molecule.

The second composition contains the remaining components of a bioluminescence generating system, typically the luciferin or luciferase substrate. In some embodiments, these components, particularly the luciferin are linked to a protein, such as a serum albumin, or other protein carrier. The carrier and time release formulations permit systemically administered components to travel to the targeted tissue without interaction with blood cell components, such as hemoglobin that deactivates the luciferin or luciferase.

Methods for diagnosing diseases, particularly infectious diseases, using chip methodology (see, e.g., copending U.S. application Ser. No. 08/990,103) a luciferase/luciferin bioluminescence-generating system and a Renilla reniformis GFP are provided. In particular, the chip includes an integrated photodetector that detects the photons emitted by the bioluminescence-generating system, particularly using luciferase encoded by the nucleic acids provided herein and/or Renilla reniformis GFP.

In one embodiment, the chip is made using an integrated circuit with an array, such as an X-Y array, of photodetectors. The surface of circuit is treated to render it inert to conditions of the diagnostic assays for which the chip is intended, and is adapted, such as by derivatization for linking molecules, such as antibodies. A selected antibody or panel of antibodies, such as an antibody specific for a bacterial antigen, is affixed to the surface of the chip above each photodetector. After contacting the chip with a test sample, the chip is contacted with a second antibody linked to a Renilla GFP, a chimeric antibody-Renilla GFP fusion protein or an antibody linked to a component of a bioluminescence generating system, such as a luciferase or luciferin,

that are specific for the antigen. The remaining components of the bioluminescence generating reaction are added, and, if any of the antibodies linked to a component of a bioluminescence generating system are present on the chip, light will be generated and detected by the adjacent photodetector. The photodetector is operatively linked to a computer, which is programmed with information identifying the linked antibodies, records the event, and thereby identifies antigens present in the test sample.

Methods for generating chimeric GFP fusion proteins are provided. The methods include linking DNA encoding a gene of interest, or portion thereof, to DNA encoding a GFP coding region in the same translational reading frame. The encoded-protein of interest may be linked in-frame to the amino- or carboxyl-terminus of the GFP. The DNA encoding the chimeric protein is then linked in operable association with a promoter element of a suitable expression vector. Alternatively, the promoter element can be obtained directly from the targeted gene of interest and the promoter-containing fragment linked upstream of the GFP coding sequence to produce chimeric GFP proteins or to produce polycistronic mRNAs that encode the Renilla reniformis GFP and a luciferase, preferably a Renilla luciferase, more preferably Renilla reniformis luciferase.

Methods for identifying compounds using recombinant cells that express heterologous DNA encoding a Renilla reniformis GFP under the control of a promoter element of a gene of interest are provided. The recombinant cells can be used to identify compounds or ligands that modulate the level of transcription from the promoter of interest by measuring Renilla reniformis GFP-mediated fluorescence. Recombinant cells expressing the chimeric Renilla reniformis GFP or polycistronic mRNA encoding Renilla reniformis and a lucifierase, may also be used for monitoring gene expression or protein trafficking, or determining the cellular localization of the target protein by identifying localized regions of GFP-mediated fluorescence within the recombinant cell.

Other assays using the GFPs and/or luciferases are contemplated herein. Any assay or diagnostic method known used by those of skill in the art that employ Aequora GFPs and/or other luciferases are contemplated herein.

Kits containing the GFPs for use in the methods, including those described herein, are provided. In one embodiment, the kits containing an article of manufacture and appropriate reagents for generating bioluminescence are provided. The kits containing such soap compositions, with preferably a moderate pH (between 5 and 8) and bioluminescence generating reagents, including luciferase and luciferin and the GFP are provided herein. These kits, for example, can be used with a bubble-blowing or producing toy. These kits can also include a reloading or charging

cartridge or can be used in connection with a food.

In another embodiment, the kits are used for detecting and visualizing neoplastic tissue and other tissues and include a first composition that contains the GFP and at least one component of a bioluminescence generating system, and a second that contains the activating composition, which contains the remaining components of the bioluminescence generating system and any necessary activating agents.

Thus, these kits will typically include two compositions, a first composition containing the GFP formulated for systemic administration (or in some embodiments local or topical application), and a second composition containing the components or remaining components of a bioluminescence generating system, formulated for systemic, topical or local administration depending upon the application. Instructions for administration will be included.

In other embodiments, the kits are used for detecting and identifying diseases, particularly infectious diseases, using multi-well assay devices and include a multi-well assay device containing a plurality of wells, each having an integrated photodetector, to which an antibody or panel of antibodies specific for one or more infectious agents are attached, and composition containing a secondary antibody, such as an antibody specific for the infectious agent that is linked to a Renilla reniformis GFP protein, a chimeric antibody-Renilla reniformis GFP fusion protein or F(Ab).sub.2 antibody fragment-Renilla reniformis GFP fusion protein. A second composition contains a bioluminescence generating system that emits a wavelength of light within the excitation range of the Renilla mulleri GFP, such as species of Renilla or Aequorea, for exciting the Renilla reniformis, which produces light that is detected by the photodetector of the device to indicate the presence of the agent.

As noted above, fusions of nucleic acid encoding the luciferases and or GFPs provided herein with other luciferases and GFPs are provided. Of particular interest are fusions that encode pairs of luciferases and GFPs, such as a Renilla luciferase and a Renilla GFP (or a homodimer or other multiple of a Renilla GFP). The luciferase and GFP bind and in the presence of a luciferin will produced fluorescence that is red shifted compared to the luciferase in the absence of the GFP. This fusion or fusions in which the GFP and luciferase are linked via a target, such as a peptide, can be used as a tool to assess anything that interacts with the linker.

Muteins of the GFPs and luciferases are provided. Of particular interest are muteins, such as temperature sensitive muteins, of the GFP and luciferases that alter their interaction, such as mutations in the Renilla luciferase and Renilla GFP that alters their interaction at a critical temperature.

Antibodies, polyclonal and monoclonal antibodies that specifically bind to any of the proteins encoded by the nucleic acids provided herein are also provided. These antibodies, monoclonal or polyclonal, can be prepared employing standard techniques, known to those of skill in the art. In particular, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure preparation of a luciferase or GFP provided herein or an or epitope-containing fragment thereof are provided. Monoclonal antibodies are also provided. The immunoglobulins that are produced have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a GFP or luciferase, particularly a Renilla or Ptilosarcus GFP or a Pleuromamma, Gaussia or Renilla mulleri luciferase, that may be present in a biological sample or a solution derived from such a biological sample.

DESCRIPTION OF THE FIGURES

FIG. 1 depicts phylogenetic relationships among the anthozoan GFPs.

FIGS. 2A-D illustrate the underlying principle of Bioluminescent Resonance Energy Transfer (BRET) and its use as sensor: A) in isolation, a luciferase, preferably an anthozoan luciferase, emits blue light from the coelenterazine-derived chromophore; B) in isolation, a GFP, preferably an anthozoan GFP that binds to the luciferase, that is excited with blue-green light emits green light from its integral peptide based fluorophore; C) when the luciferase and GFP associate as a complex in vivo or in vitro, the luciferase non-radiatively transfers its reaction energy to the GFP flurophore, which then emits the green light; D) any molecular interaction that disrupts the luciferase-GEP complex can be quantitatively monitored by observing the spectral shift from green to blue light.

FIG. 3 illustrates exemplary BRET sensor architecture.

"FIG. 4 depicts the substitution of altered fluorophores into the background of Ptilosarcus, Renilla mulleri and Renilla reniformis GFPs (the underlined regions correspond to amino acids 56-75 of SEQ ID NO. 27 Renilla reniformis GFP; amino acids 59-78 of SEQ ID NO. 16 Renilla mulleri GFP; and amino acids 59-78 of SEQ ID NO. 32 for Ptilosarcus GFP)."

FIG. 5 depicts the three anthozoan fluorescent proteins for which a crystal structure exists; another is available commercially from Clontech as dsRed (from Discosoma striata; also known as drFP583, as in this alignment); a dark gray background depicts amino acid conservation, and a light gray background depicts shared physicochemical properties.

FIG. 6 compares the sequences of a variety of GFPs, identifying sites for mutation to reduce multimerization; abbreviations are as follows: Amemonia majona is amFP486; Zoanthus sp. zFP506 and zFP538; Discosoma sp. "red" is drFP583; Clavularia sp. is cFP484; and the GFP from the anthozoal A. sulcata is designated FP595.

DETAILED DESCRIPTION OF THE INVENTION

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Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, applications and publications of referred to throughout the disclosure are incorporated by reference in their entirety.

As used herein, chemiluminescence refers to a chemical reaction in which energy is specifically channeled to a molecule causing it to become electronically excited and subsequently to release a photon thereby emitting visible light. Temperature does not contribute to this channeled energy. Thus, chemiluminescence involves the direct conversion of chemical energy to light energy.

"As used herein, luminescence refers to the detectable electromagnetic (EM) radiation, generally, ultraviolet (UV), infrared (IR) or visible EM radiation that is produced when the excited product of an exergic chemical process reverts to its ground state with the emission of light. Chemiluminescence is luminescence that results from a chemical reaction. Bioluminescence is chemiluminescence that results from a chemical reaction using biological molecules (or synthetic versions or analogs thereof) as substrates and/or enzymes.

As used herein, bioluminescence, which is a type of chemiluminescence, refers to the emission of light by biological molecules, particularly proteins. The essential condition for bioluminescence is molecular oxygen, either bound or free in the presence of an oxygenase, a luciferase, which acts on a substrate, a luciferin. Bioluminescence is generated by an enzyme or other protein

(luciferase) that is an oxygenase that acts on a substrate luciferin (a bioluminescence substrate) in the presence of molecular oxygen and transforms the substrate to an excited state, which upon return to a lower energy level releases the energy in the form of light.

As used herein, the substrates and enzymes for producing bioluminescence are generically referred to as luciferin and luciferase, respectively. When reference is made to a particular species thereof, for clarity, each generic term is used with the name of the organism from which it derives, for example, bacterial luciferin or firefly luciferase.

As used herein, luciferase refers to oxygenases that catalyze a light emitting reaction. For instance, bacterial luciferases catalyze the oxidation of flavin mononucleotide (FMN) and aliphatic aldehydes, which reaction produces light. Another class of luciferases, found among marine arthropods, catalyzes the oxidation of Cypridina (Vargula) luciferin, and another class of luciferases catalyzes the oxidation of Coleoptera luciferin.

Thus, luciferase refers to an enzyme or photoprotein that catalyzes a bioluminescent reaction (a reaction that produces bioluminescence). The luciferases, such as firefly and Gaussia and Renilla luciferases, that are enzymes which act catalytically and are unchanged during the bioluminescence generating reaction. The luciferase photoproteins, such as the aequorin photoprotein to which luciferin is non-covalently bound, are changed, such as by release of the luciferin, during bioluminescence generating reaction. The luciferase is a protein that occurs naturally in an organism or a variant or mutant thereof, such as a variant produced by mutagenesis that has one or more properties, such as thermal stability, that differ from the naturally-occurring protein. Luciferases and modified mutant or variant forms thereof are well known. For purposes herein, reference to luciferase refers to either the photoproteins or luciferases.

Thus, reference, for example, to "Gaussia luciferase" means an enzyme isolated from member of the genus Gaussia or an equivalent molecule obtained from any other source, such as from another related copepod, or that has been prepared synthetically. It is intended to encompass Gaussia luciferases with conservative amino acid substitutions that do not substantially alter activity. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. Molecular Biology of the Gene, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p. 224).

"Renilla GFP" refers to GFPs from the genus Renilla and to mutants or variants thereof. It is intended to encompass Renilla GFPs with conservative amino acid substitutions that do not substantially alter activity and physical properties, such as the emission spectra and ability to shift the spectral output of bioluminescence generating systems.

Such substitutions are preferably made in accordance with those set forth in TABLE 1 as follows:

TABLE-US-00001 TABLE 1 Original residue Conservative substitution Ala (A) Gly; Ser Arg (R) Lys Asn (N) Gln; His Cys (C) Ser Gln (Q) Asn Glu (E) Asp Gly (G) Ala; Pro His (H) Asn; Gln Ile (I) Leu; Val Leu (L) Ile; Val Lys (K) Arg; Gln; Glu Met (M) Leu; Tyr; Ile Phe (F) Met; Leu; Tyr Ser (S) Thr Thr (T) Ser Trp (W) Tyr Tyr (Y) Trp; Phe Val (V) Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions.

The luciferases and luciferin and activators thereof are referred to as bioluminescence generating reagents or components. Typically, a subset of these reagents will be provided or combined with an article of manufacture. Bioluminescence will be produced upon contacting the combination with the remaining reagents. Thus, as used herein, the component luciferases, luciferins, and other factors, such as O.sub.2, Mg.sup.2+, Ca.sup.2+ are also referred to as bioluminescence generating reagents (or agents or components).

"As used herein, a Renilla reniformis green fluorescent protein (GEP) refers to a fluorescent protein that is encoded by a sequence of nucleotides that encodes the protein of SEQ ID NO. 27 or to a green fluorescent protein from Renilla reniformis having at least 80%, 90% or 95% or greater sequence identity thereto; or that is encoded by a sequence of nucleotides that hybridizes under high stringency along its full length to the coding portion of the sequence of nucleotides set forth in any of SEQ ID NOs. 23-25. A Renilla reniformis GEP is protein that is fluorescent and is produced in a Renilla reniformis."

As used herein, bioluminescence substrate refers to the compound that is oxidized in the presence of a luciferase, and any necessary activators, and generates light. These substrates are referred to as luciferins herein, are substrates that undergo oxidation in a bioluminescence reaction. These bioluminescence substrates include any luciferin or analog thereof or any synthetic compound with which a luciferase interacts to generate light. Preferred substrates are those that are oxidized in the presence of a luciferase or protein in a light-generating reaction. Bioluminescence substrates, thus, include those compounds that those of skill in the art recognize as luciferins.