Fluorescent proteins from nonbioluminescent Anthozoa species

Mikhail V. Matz, Arkady F. Fradkov, Yulii A. Labas[1], Aleksandr P. Savitsky[2], Andrey G. Zaraisky, Mikhail L. Markelov, and Sergey A. Lukyanov*

*Institute of Bioorganic Chemistry, Russian Acadmy of Science, 117871 Moscow, Russia. [1]Institute of Ecology and Evolution, and [2]Institute of Biochemistry Russian Academy of Science, 17071 Moscow, Russia. *Corresponding author (e-mail: luk@ibch.siobc.ras.ru).*

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We have cloned six fluorescent proteins homologous to the green fluorescent protein (GFP) from *Aequorea victoria*. Two of these have spectral characteristics dramatically different from GFP, emitting at yellow and red wavelengths. All the proteins were isolated from nonbioluminescent reef corals, demonstrating that GFP-like proteins are not always functionally linked to bioluminescence. The new proteins share the same β-can fold first observed in GFP, and this provided a basis for the comparative analysis of structural features important for fluorescence. The usefulness of the new proteins for in vivo labeling was demonstrated by expressing them in mammalian cell culture and in mRNA microinjection assays in *Xenopus* embryos.

Keywords: green fluorescent protein, fluorophore, Anthozoa

Green fluorescent protein (GFP) from the luminescent jellyfish *Aequorea victoria* has become an excellent marker of gene expression and protein localization in various biological systems[1]. Despite this advance, the mechanism of GFP fluorescence and the structural features that determine its parameters are not fully understood. Cloning and comparative analysis of GFP homologs from other species could further our understanding of these proteins. Since the first reports on GFP[2], it has generally been thought that any homologs would also function in bioluminescence systems as secondary emitters converting the short-wavelength light of primary emission into light of a longer wavelength[3,4]. Thus, the search has been limited to species exhibiting bioluminescence[5–7]. Moreover, it has been generally supposed that GFP homologs should also emit green light. A single GFP homolog—the green fluorescent protein from *Renilla reniformis* has been sequenced and purified[8], but its sequence has not been reported.

We hypothesized that GFP-like proteins are not necessarily linked to bioluminescence. Although the basic physical principles of the phenomenon are the same throughout the animal kingdom, there are wide variations in its molecular implementation[9]. This suggests that bioluminescence evolved independently and relatively recently in different phylogenetic groups, most probably by combination and adjustment of preexisting functional domains[10]. If so, such ancestral domains could still be present in nonbioluminescent organisms.

Our consideration of possible original functions of GFP domains led us to the enormous variety of colors present in coral reefs. In particular, many Anthozoa species exhibit bright fluorescent colors. The most common color is green, but other hues are also found[11,12]. This fluorescence is assumed to provide protection from strong solar radiation[13]—quite an important consideration in shallow tropical waters. For organisms living in deeper habitats, where light is significantly depleted of low-energy components and thus is mostly blue, Schlichter et al.[14] suggested a different function of the fluorescence: conversion of the blue light into a longer wavelength more suitable for photosynthesis by algal endosymbionts. The fluorescence of reef corals has generally been attributed to unidentified pigments; however, we hypothesized that this fluorescence may be due to GFP-like fluorescent proteins, and therefore attempted to isolate homologous cDNAs.

Results and discussion

Isolation of target cDNAs. Five brightly fluorescent Anthozoa species from the Indo-Pacific area were studied (Table 1; images of the animals can be found in ref. 15). We used a modified method for Rapid Amplification of 3′ cDNA ends (3′-RACE) employing degenerate primers to amplify cDNA fragments from samples prepared from the colored body parts. Sets of 3′-directed degenerate primers were synthesized, corresponding to amino acid sequences of *A. victoria* GFP that, in our view, were more likely to be conserved among GFP-like proteins. The primers covered several regions of the fluorophore-bearing α-helix and tight turns between β-strands. A specific cDNA fragment was amplified from *Anemonia majano* cDNA using a primer for the tight turn region at positions 19–25 of *A. victoria* GFP. After this, additional sets of degenerate primers targeted for the actual conserved regions were designed as the project progressed. The 5′ ends of the cDNA fragments were amplified using a step-out RACE strategy[16]. The full-length proteins (with the exception of the protein from *Clavularia*, see below) were then expressed in *Escherichia coli* with His_6 tags at their N termini and purified using metal affinity chromatography.

Primary structures of the isolated proteins. Our approach yielded six remote homologs of GFP. The overall identity with GFP was 26–30%. To facilitate discussion, we introduced the following nomenclature. Each protein was designated by lowercase letters identifying the species, FP for fluorescent protein, and a number identifying the major emission maximum. Using this nomenclature, GFP from *A. victoria* would be named avFP509.

The alignment of the amino acid sequences of the novel proteins with GFP (Fig. 1A) was constructed manually, paying special attention to the following specific features of the GFP fold. First, there is a clear tendency for amino acids to alternate between hydrophobic and hydrophilic along β-strands. Second, the "inside" (mostly hydrophobic) positions are less variable as they constitute the pro-

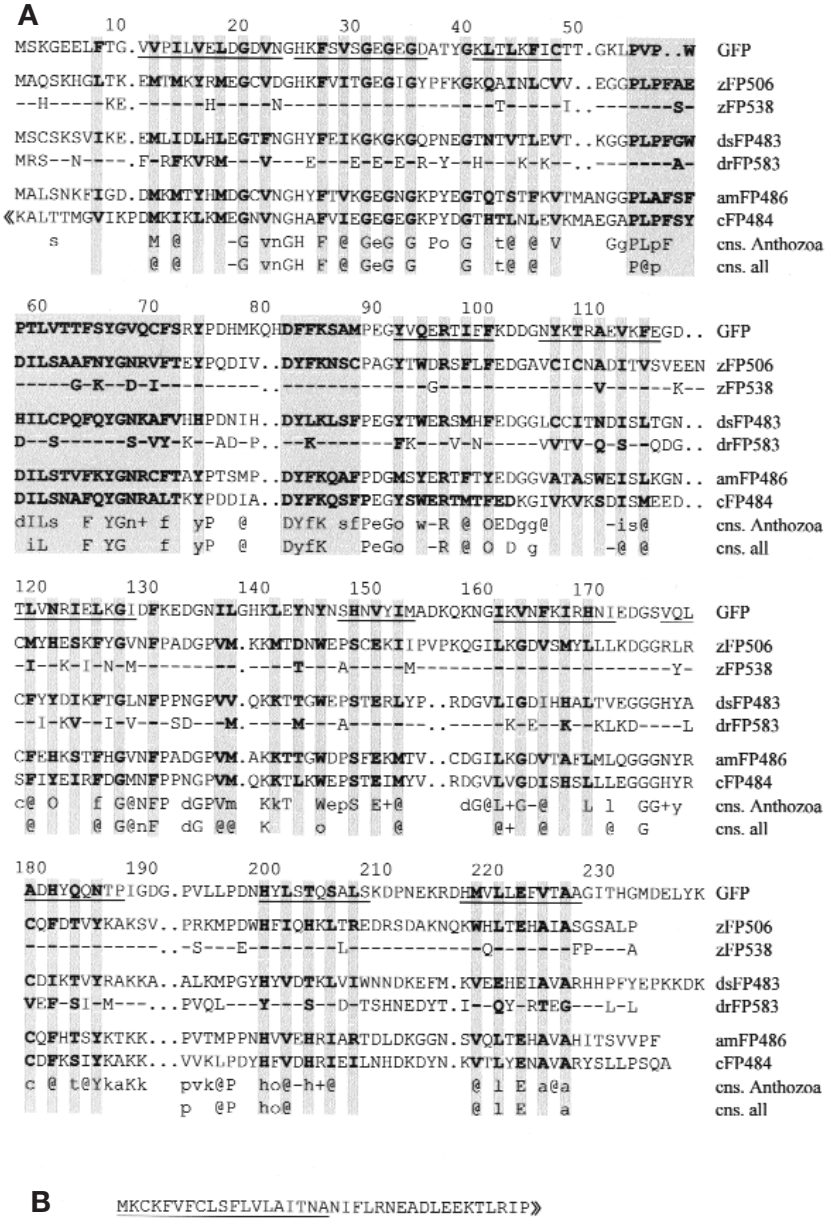

Figure 1. (A) Multiple alignment of fluorescent proteins. The numbering is based on *A. victoria* GFP. Two proteins from *Zoanthus* and two from *Discosoma* are compared pairwise: in the second protein of the pair the residues identical to the corresponding ones in the first protein are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β-sheets[18] are underlined; the residues whose side chains form the interior of the β-can[18] are shaded. In the consensus sequences ("cns."), "O" marks an aromatic residue (Phe, Tyr, Trp, His); "@" = bulky hydrophobic residues (Val, Leu, Ile, Met, Phe, Trp); "+" = positively charged residues (His, Arg, Lys); "–" = negatively charged residues (Asp, Glu). (B) The N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

tein core. In addition, good landmarks for correct alignment were provided by turn motifs (stretches of one to two Asp, Glu, or Asn residues and one to three Gly). On the basis of the alignment, it appears that the overall β-can fold[17,18] is well conserved, as all the key secondary structure elements observed in GFP can be easily detected in the newly isolated proteins in the same arrangement. Remarkable similarity can be observed in the stretches forming the "caps" of the can: positions 82–91 form the "top" cap (corresponding to the side where both N and C termini of the protein are located), and positions 129–140 form the "bottom" cap. The key sites for degenerate primers used in this work corresponded to the tight turn formed by residues 19–25, the "void" β-strand (positions 31–35; the "inside" positions of this stretch are occupied by glycines only in all the fluorescent proteins), and the bottom cap (positions 127–131 and 133–137).

Five of the novel proteins have N termini exactly as long as the N terminus of GFP. One, cFP484 from *Clavularia*, has 37 additional residues at its N terminus (Fig. 1B), of which the first 19 constitute a putative signal peptide[19]. Truncation of either the signal peptide alone or the entire 37 additional residues did not affect the spectral properties of the protein.

Fluorescent features. The excitation-emission spectra of the proteins expressed in *E. coli* are shown in Figure 2. The corresponding fluorescence characteristics are summarized in Table 2. The emission spectra closely matched the fluorescence observed in vivo in the corresponding organisms (data not shown). The only exception was dsFP483 from *Discosoma striata*: The protein is mostly green, and the living organism exhibits both cyan and green fluorescence. This suggests the presence of a second fluorescent protein (like in *Zoanthus* sp.) that was not detected in our experiment. All the proteins exhibited fluorescence that was more similar to *Renilla* GFP[5] and mutant variants of *Aequorea* GFP than to the wild-type GFP. Specifically, there was always a single (though sometimes quite structured) excitation peak in the visible band, so the excitation and emission spectra were more or less mirror images.

Fluorophore and its environment. There are two functional features of the GFP-like proteins that make them particularly interesting for protein science. The first is the ability to form a fluorophore autocatalytically without the help of any external agents other than molecular oxygen. It was expected that this mechanism and the functional groups involved should exhibit the highest degrees of conservation. In GFP, the fluorophore is formed by residues 65–67 (Ser-Tyr-Gly) as a result of condensation between the carbonyl carbon of Ser65 and the amino nitrogen of Gly67, followed by the dehydrogenation of the Tyr66 methyl-

Table 1. Anthozoa species used in this study.

Species	Taxonomy	Fluorescent color
Anemonia majano	*Zoantharia, Actiniaria, Actiniidae*	bright green tentacle tips
Zoanthus sp.	*Zoantharia, Zoanthidea, Zoanthidae*	yellow-green tentacles and oral disk
Discosoma striata	*Zoantharia, Corallimorpharia, Discosomatidae*	blue-green stripes on oral disk
Discosoma sp. "red"	same as above	orange-red spots on oral disk
Clavularia sp.	*Alcyonaria, Stolonifera, Clavulariidae*	bright green tentacles and oral disk

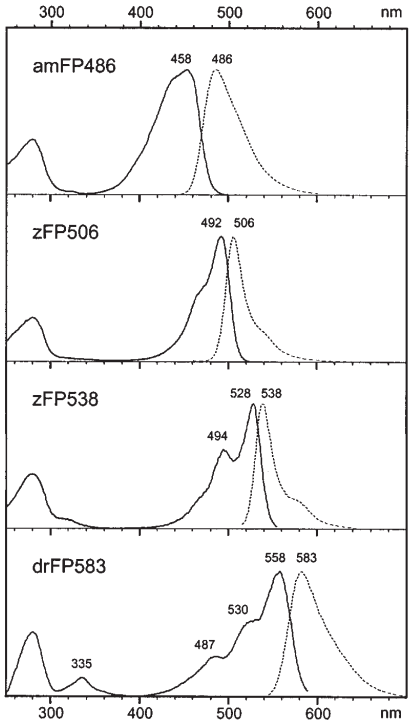

Figure 2. The absorption (solid lines) and emission (dotted lines) spectra for the identified proteins (as determined for the recombinant products of expression in *E. coli*). The data for amFP486 is representative of three proteins (amFP486, dsFP483, and cFP484) that have almost identical spectra.

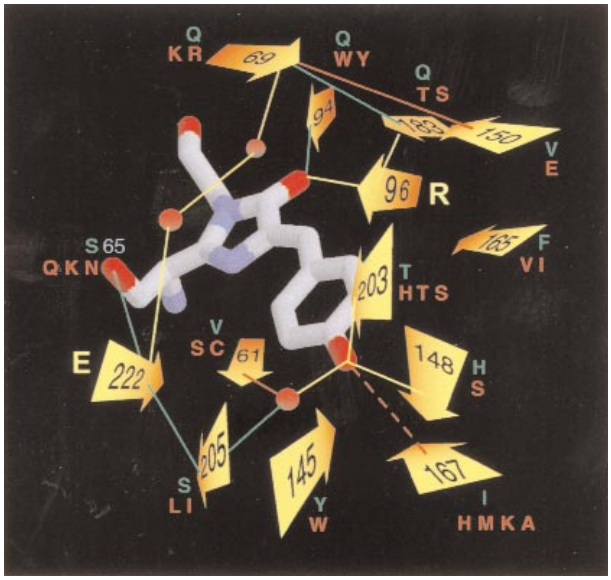

Figure 3. Schematic outline of the fluorophore environment in GFP and its putative variations in new *Anthozoa* proteins. The fluorophore is represented as a "sticks" model; carbons are gray, nitrogens are blue, and oxygens are red. Arrows represent side chains at positions corresponding to the numbers on the arrows. An arrow points in the approximate direction from the alpha carbon toward the distal side chain atoms. Red balls represent buried water molecules. A green letter near an arrow identifies the particular residue in GFP; red letters identify equivalent residues in the newly identified proteins; yellow letters identify strictly conserved residues (Glu222 and Arg96). Lines represent polar interactions: Conserved interactions are yellow; interactions found only in GFP are green; and the putative interactions in the new proteins are red.

ene bridge[17,18,20]. The details of these events are not completely understood. Ormö et al.[17] have suggested that Arg96, which interacts with the carbonyl of the imidazolidinone ring in GFP, may be one of the key catalytic residues promoting ring closure. This idea is supported by the fact that Arg96 is present in all the newly isolated fluorescent proteins. As for the fluorophore-forming triad (positions 65–67), only the last two residues (66 and 67, Tyr-Gly) were conserved in the new fluorescent proteins. In particular, the perfect conservation of the Gly67 is consistent with the previous mutational studies[21,22], supporting the idea that its rather nucleophilic and sterically unrestricted NH group is essential for proper cyclization. Meanwhile, in the new proteins, Ser65 is replaced by Gln, Lys, or Asn. In GFP, substitution of Ser65 by Asn causes a dramatic drop in fluorescence, although a number of other substitutions are tolerated (except the change of the ratio between excitation peaks[21,22]). This suggests that the identity of the residue at position 65 is dictated mostly by the structural peculiarities of the particular protein core.

Another strictly conserved residue of GFP is Glu222. Its proposed roles are maintaining the equilibrium between neutral and anionic forms of the fluorophore (which absorb at 395 and 475 nm, respectively) and serving as the acceptor in the excited-state proton transfer mechanism[23,24]. However, the presence of a single absorption band analogous to the 475 nm band of GFP in the spectra of the newly identified proteins (Fig. 2) suggests that their fluorophores are always deprotonated. Indeed, in *Anthozoa* fluorescent proteins, Glu222 is unlikely to accept a hydrogen bond from a side chain at position 65. In GFP this leads to almost complete fluorophore ionization[17,21,24]. Moreover, in GFP, the Glu222 carboxylate and the fluorophore hydroxyl group are bridged into a single hydrogen-bonding network with the help of Ser205 (ref. 24), whereas in the new pro-

Table 2. Spectral properties of identified fluorescent proteins.

Species	Protein name	Number of amino acids	Calculated molecular mass kDa	Absorption maximum nm	Emission maximum nm	Maximum extinction coefficient	Quantum yield	Relative brightness[a]
Anemonia majano	amFP486	229	25.4	458	486	40,000	0.24	0.43
Zoanthus sp.	zFP506	231	26.1	496	506	35,600	0.63	1.02
	zFP538	231	26.2	528	538	20,200	0.42	0.38
Discosoma striata	dsFP483	232	26.4	443	483	23,900	0.46	0.50
Discosoma sp. "red"	drFP583	225	25.9	558	583	22,500	0.23	0.24
Clavularia sp.	cFP484	266	30.4	456	484	35,300	0.48	0.77
Aequorea victoria[b]	GFP	238	27	397	509	27,600	0.80	1.0

[a]As compared to the brightness (maximal extinction coefficient multiplied by quantum yield) of *A. victoria* GFP.
[b]The data for *A. victoria* GFP are from ref. 3.

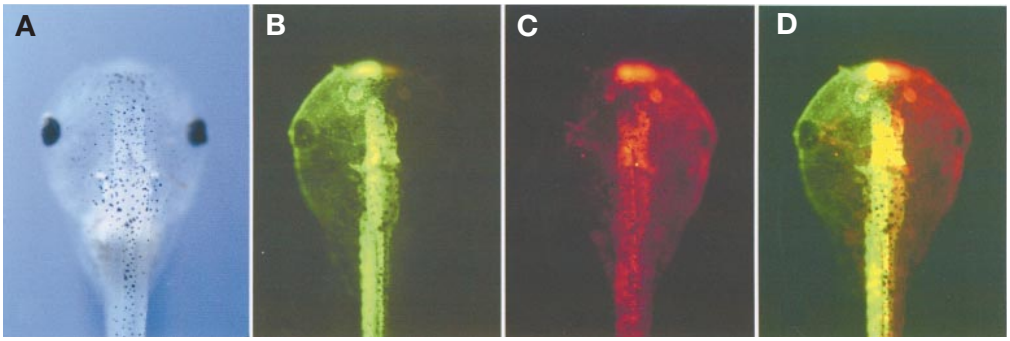

Figure 4. Results of synthetic mRNA microinjection assay. The *Xenopus* embryo was injected into the right and left dorsal blastomeres (eight-cell stage) with amFP486 and drFP583 mRNAs, respectively, and was photographed at the tadpole stage (one week of development) under white light (A), epifluorecsence with FITC (B), TRITC (C), or successive FITC and TRITC (D) filter sets. The embryo is shown from the dorsal side, anterior to the top.

teins this residue is replaced by isoleucine or leucine. Therefore, in the new proteins, Glu222 and the fluorophore hydroxyl are probably always separated, and Glu222 probably cannot be involved in photochemical reactions. Nevertheless, this residue is conserved, suggesting an essential role in protein folding or function. One possibility is that this residue, along with Arg96, is involved in catalysis of fluorophore formation, specifically in cyclization and/or oxidation. A possible arguement against this hypothesis is that Ehrig et al.[25] demonstrated that the substitution of Glu222 by glycine (i.e., complete removal of the side chain) does not abolish GFP fluorescence. However, if the side chain is involved in autocatalysis, such substitution could result in much slower fluorophore maturation, but not necessarily slow enough as to cause the complete loss of fluorescence. In other words, the mutation should primarily affect the yield of the active protein, which was not teted by Ehrig et al.[25]

The factors determining fluorescence itself are expected to be much more tunable (and therefore variable) than the components of the autocatalytic reaction. On the basis of the alignment and the crystal structure of GFP[17,18], we predicted the variations in the fluorophore environment in the newly isolated proteins (Fig. 3). Notable conservation is observed in the key residues stabilizing the ionized fluorophore state, since they are found at positions 148 and 203, similar to GFP[17,24]. The proteins from *Discosoma* and *Clavularia* have one more side chain that may participate in this interaction—histidine or lysine residue at position 167.

Two specific features of the fluorophore environment can be observed in the newly identified proteins. First, residue 69, located very close to the fluorophore, is arginine or lysine instead of the glutamine found in GFP. A possible counterpart for this buried positive charge may be a carboxyl group of Glu150, which is also present in all the new proteins. A salt bond between these residues would not disrupt—and might even favor—the function attributed to residue 69 in GFP, which is to anchor the buried water molecules that solvate the Glu222 carboxylate[17,18,24]. Second, in contrast to GFP, the new proteins contain up to four tryptophan residues, two of which (positions 94 and 145) are located in the immediate vicinity of the fluorophore. This may reflect the proposed ancestral function of these proteins, namely protection from ultraviolet light, which could be absorbed by tryptophan, transferred to the fluorophore, and emitted in the form of nonhazardous, long-wavelength light. Indeed, in all the new proteins, the major emission band has a pronounced ultraviolet excitation peak corresponding to tryptophan absorption (Fig. 2).

It is difficult to correlate the spectral properties of each protein with the amino acid composition of the fluorophore environment, despite the fact that there are two pairs of proteins in the series that seem especially suitable for comparative analysis. These are the two proteins from *Zoanthus* (zFP506 and zFP538) and the two proteins from *Discosoma* (dsFP483 and drFP583). Within each pair, there are only a few amino acid replacements, but the spectra differ dramatically. drFP583, in particular, has an excitation-emission maximum shifted by 54 nm toward the red even in comparison with the most red-shifted mutant of GFP (Q69K/T203Y/S65G/V68L/S72A, which emits at 529 nm; ref. 26). As the analysis of the putative fluorophore environment gives no clear explanation for such a great red shift, it is tempting to speculate that in red fluorescent proteins an additional autocatalytic reaction, presumably inhibited in GFP, takes place during fluorophore maturation and leads to the extension of its conjugated π-system. This issue should be resolved by further structural studies of the proteins.

Applications of new proteins as in vivo markers. To evaluate the usefulness of the new proteins for biotechnological applications, we tested some of them in eukaryotic expression systems. First, amFP486 and drFP583 were expressed in the human adenovirus 5-transformed embryonic kidney cell line 293 (American Type Culture Collection CRL 1573). The genes were cloned into the retroviral vector pLNCX (ref. 27) under the control of the cytomegalovirus immediate-early enhancer/promoter region. The fluorescence of amFP486 and drFP583 was easily detected in two to three days after transfection (data not shown). Furthermore, the proteins did not show any toxic effect on the cells during selection of stable cell lines expressing either amFP486 or drFP583.

Three of the new proteins (amFP486, zFP538, and drFP583) were used in mRNA microinjection assays (performed exactly as described[28]). Synthetic mRNAs were microinjected into *Xenopus laevis* embryos at the eight-cell stage, and the fluorescence produced by the proteins was inspected in the developing tadpoles. In all cases, bright fluorescence was observed. An example is shown in Figure 4: left and right dorsal blastomeres of the same embryo were injected with amFP486 and drFP583 mRNAs, respectively. The green- (Fig. 4B) or red-fluorescing (Fig. 4C) descendants of the injected blastomeres were easily detected in the developed tadpole using epifluorescence microscopy with commonly used filter sets. The regions marked with both proteins appeared yellow (Fig. 4D). Thus, these two proteins are well suited for double-labeling experiments. As in the cell culture experiments, no visible toxic effects were detected during at least the first three weeks of development, even though in some cases as much as 1 ng of mRNA was injected per blastomere. Moreover, the new proteins (or their mRNAs) seem to be more stable than EGFP (Enhanced GFP; Clontech, Palo Alto, CA) in *Xenopus* cells. In the control microinjection assay with EGFP, the signal began to decrease after the first week of embryonic development, whereas the fluorescence produced by amFP486, zFP538, and drFP583 was stable until at least the fourth week.

Experimental protocol

cDNA preparation. Total RNA was isolated as described[29]. First-strand cDNA was synthesized from 1–3 μg of total RNA using the SMART PCR cDNA Synthesis Kit (Clontech). The protocol was followed with a single alteration: The TN3 primer (5′-CGCAGTCGACCG$(T)_{13}$) was used in place of the cDNA synthesis primer (CDS) provided in the kit. The cDNA samples were amplified by PCR using the provided protocol and the TS (5′-AAGCAGTGGTATCAACGCAGAGT) and TN3 primers, both in 0.1 μM concentration. We performed 18–25 PCR cycles to amplify each cDNA sample. The amplified cDNA was diluted 20-fold in water, and 1 μl of this dilution was used in the following procedures.

Design of oligonucleotides. Four degenerate primers were designed to isolate cDNA fragments of GFP homologs: 5′-GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA for Asp-Gly-Cys-Val-Asn-Gly-His (positions 19–25); 5′-GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG, 5′-GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG, 5′-GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG and 5′-GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG for Val-Thr-Gly-Glu-Gly-(Glu/Asn)-Gly (positions 29–35); 5′-TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC for Phe-His-Gly-(Met-Val)-Asn-Phe-Pro (positions 125–131); and 5′-CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG and 5′-CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG for Pro-Ala-(Asp/Asn)-Gly-Pro-Val-Met (positions 131–137). All oligos were gel-purified before use.

Isolation of 3′-cDNA fragments of fluorescent proteins. The target fragments were isolated using a modified 3′-RACE strategy with two consecutive PCR steps. The first PCR used a degenerate primer and the T7-TN3 primer (5′-GTAATACGACTCACTATAGGGCCGCAGTCGACCG$[T]_{13}$; the 3′ half of this primer is identical to the TN3 primer used for cDNA synthesis). The second PCR involved the TN3 primer and nested degenerate primers.

The first PCR was performed as follows: 1 µl of 20-fold dilution of the amplified cDNA sample was added into the reaction mixture containing 1x Advantage KlenTaq Polymerase Mix (Clontech), the manufacturer's 1× reaction buffer, 200 µM dNTPs, 0.3 µM of first degenerate primer (for Asp-Gly-Cys-Val-Asn-His-Gly), and 0.1 µM of the T7-TN3 primer in a total volume of 20 µl. Cycling was performed in a Hybaid OmniGene Thermocycler in tube control mode: 1 cycle for 95°C, 10 s; 55°C, 1 min; 72°C, 40 s; 24 cycles at 95°C, 10 s; 62°C, 30 s; 72°C, 40 s. The reaction was then diluted 20-fold in water, and 1 µl of the dilution was added to the second PCR, which contained 1 × Advantage KlenTaq Polymerase Mix, the manufacturer's 1 × reaction buffer, 200 uM dNTPs, 0.3 µM of second (nested) degenerate primer, and 0.1 uM of the TN3 primer in a total volume of 20 ul. The cycling profile was: 1 cycle at 95°C, 10 s; 55°C (for Val-Thr-Gly-Glu-Gly-[Glu-Asn]-Gly or Pro-Ala-(Asp-Asn)-Gly-Pro-Val-Met series) or 52°C (for Phe-His-Gly-[Met-Val]-Asn-Phe-Pro primer), 1 min; 72°C, 40 s; 13 cycles at 95°C, 10 s; 62°C (for Val-Thr-Gly-Glu-Gly-(Glu-Asn)-Gly or Pro-Ala-(Asp-Asn)-Gly-Pro-Val-Met series) or 58°C (for Phe-His-Gly-(Met-Val)-Asn-Phe-Pro primer), 30 s; 72°C, 40 s.

Purification of proteins and fluorescence spectroscopy. Proteins were expressed in *E. coli* with His_6 tags at their N termini and purified using TALON metal affinity resin (Clontech). Proteins were at least 95% pure according to SDS–PAGE. Concentrations of the proteins were determined as described[30], using the average extinction coefficients of tryptophan, tyrosine, and cysteine. We verified three described models for such calculation (those of Wetlaufer[31], Gill and Hippel[30], and Mach et al.[32]) by applying them to *A.victoria* GFP and comparing the results to the published data. Not excluding Tyr66 from the calculation, we observed 6.4% (Wetlaufer), 9.0% (Gill), and 0.4% (Mach) deviation of calculated extinction coefficient at 280 nm from the one published (22,000 $M^{-1}cm^{-1}$, [ref. 3]). To calculate the extinction coefficients at 280 nm for new proteins, the model by Mach et al.[32] was used. These values were then used to determine the concentrations of proteins and therefore the molar extinction coefficients in the visible band. Quantum yields for novel proteins were determined relative to wild-type GFP (Clontech). Perkin-Elmer LS50B spectrometer (Beaconsfield, UK) was used for quantitative measurements. All samples were excited at 470 nm, absorbance at this wavelength was 0.02, and excitation and emission slits were 5 nm. The spectra were corrected for photomultiplier response and monochromator transmittance, transformed to wave number and integrated.

Accession numbers. The accession numbers of genes described in this report are: drFP583 (AF168419); dsFP483 (AF168420); amFP486 (AF168421); zFP506 (AF168422); zFP538 (AF168423); cFP484 (AF168424).

Acknowledgments

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