BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **232,** 69–73 (1997)
ARTICLE NO. RC976235

A Novel Mutation Which Enhances the Fluorescence of Green Fluorescent Protein at High Temperatures

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Received January 13, 1997

Green fluorescent protein (GFP) from *Aequorea victoria* is widely used as a marker of gene expression and protein localization in living cells from prokaryotes to eukaryotes. However, the total fluorescent signal from wild-type GFP is very weak when expressed in cells cultured at 37°C compared to 30°C or below. This characteristic makes GFP poorly suited to use as a marker in mammalian cells. Here we describe a new variant of GFP which carries a substitution of Ser^{147} to Pro (S147P GFP) and which emits a stronger fluorescent signal than the wild-type GFP at high temperature. When S147P is combined with the Ser^{65} to Thr mutation (S65T GFP), the resulting double mutant emits fluorescence which is several-fold stronger than GFP with a single S65T modification in both bacterial or mammalian cells. This S147P mutation should be useful for constructing new GFP variants which stably emit strong fluorescence at a wide range of culturing temperatures.

The green fluorescent protein (GFP) of *Aequorea victoria* is a unique protein which emits strong fluorescence as a result of fluorophore formed by the post-translational modification of 3 amino acid residues (-Ser^{65}-Tyr-Gly-) within the primary structure of the protein [1,2]. Newly-synthesized GFP molecules need to be processed into the mature fluorescent form, i.e. the protein properly folded and the fluorophore moiety completely formed, before being able to emit fluorescence [3,4]. Successful heterologous expression of GFP indicated that maturation of GFP occurs equally well in prokaryotic and eukaryotic organisms, and this finding subsequently extended the application of GFP as a reporter of gene expression or as a fusion tag to monitor protein localization to a wide range of living organisms [4-6]. Recently, there have been attempts to alter the properties of GFP to meet practical applications in the laboratory, and as a result, some useful genetically-engineered GFP variants have been created [4,7-11]. Among them, Ser^{65}→Thr (S65T) GFP is the most widely-used due to its stronger fluorescence and faster maturation rate compared to the wild-type [9].

Nevertheless, a major shortcoming is found in both wild-type and the mutant S65T GFPs; viz., the intensity of their fluorescent signal in cells is highly dependent on the culture temperature [4,12,13]. For example, when yeast or mammalian cells expressing GFP were cultured at 37°C, which is the optimum growth temperature for most mammalian cells, only a faint green fluorescence could be detected, whereas when they were cultured at temperatures lower than 30°C, a steady bright green fluorescence was detected [12]. We previously showed that temperature is the main cause of this phenomenon, inhibiting the maturation of newly-synthesized molecules but not the fluorescence of pre-existing properly-matured molecules [12].

We, therefore, attempted to generate a mutant GFP variants which stably emit strong fluorescence at a wide range of culture temperatures. Here we report the discovery of a new GFP variant which carries a substitution of Ser^{147} to Pro (S147P GFP). *Escherichia coli* cells carrying GFP variants with both this mutation and the S65T mutation were found to emit approximately 6-fold brighter fluorescence at 37°C than those carrying the single S65T mutation. The utility of the GFP variant carrying the S147P mutation was demonstrated by its ability to fluoresce strongly in mammalian cells cultured at 37°C.

MATERIALS AND METHODS

Preparation of clones of randomly mutagenized GFP gene. For low fidelity PCR amplification [14] of the cDNA containing the complete open reading frame of GFP, a 100μl reaction mixture containing

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Abbreviations used: GFP, green fluorescent protein; GST, glutathione S-transferase; Amp, ampicillin; IPTG, isopropylthio-β-D-galactoside.

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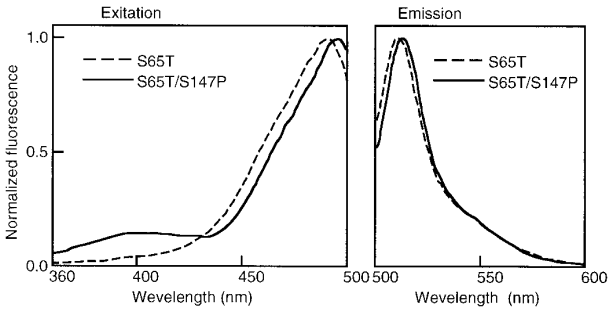

FIG. 1. Excitation and emission spectra of purified GFP variants. GST-tagged S65T and S65T/S147P GFPs were purified from bacterial cells. Excitation (left) and emission (right) spectra of each sample when the emission peak is fixed at 510nm (left) or the excitation peak is fixed at 480nm (right). All spectra were arbitrarily normalized to a maximum value of 1.0.

100ng pAGN1 (encoding wild-type GFP [12]) as the DNA template, 1μM of each oligonucleotide primer [5′ sense primer: 5′-GGGCCC-**GGATCC**ATGAGTAAAGGAGAAGAACTTTTC-3′ (*Bam*HI site just before the first codon ATG of GFP is in bold) and 3′ antisense primer: 5′-GCGCAC**GGTACC**TTATTTGTATAGTTCATCCATGCCATG-3′ (*Kpn*I site is in bold)], 1mM of each dNTP, 2.5mM Tris-Cl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 0.5mM $MnCl_2$ and 1μl TaKaRa Taq (heat-stable DNA polymerase; Takara Shuzo, Otsu, Japan) was subjected to 30 cycles of amplification: 94°C for 1min, 55°C for 2min, 72°C for 3min (72°C for 10min in the last cycle). These PCR products were digested with *Bam*HI and *Kpn*I, and ligated with similarly digested pQE30 (see below). Then, resultant plasmids were introduced into the *E. coli* strain XL1-blue [15].

Plasmids. Plasmids used in this study were derived from either pQE vectors (pQE30 and pQE31; bacterial expression vectors for proteins tagged with 6 consecutive His residues; Qiagen, Chatsworth, CA), pGEX5X-2 (a bacterial expression vector for proteins tagged with *Schistosoma japonicum* glutathione S-transferase (GST); Pharmacia LKB Biotechnology, Uppsala, Sweden) or pCAGGS (an eukaryotic expression vector containing a chicken β-actin promoter and a human cytomegalovirus enhancer; a generous gift from Drs. Niwa and Miyazaki (Osaka University Medical School) [16]). Plasmids pQA1 and pQA2 are for bacterial expression of wild-type and S147P GFPs, respectively. To construct pQB1 and pQB2, GFP genes from pQA1 and pQA2 were modified to carry the S65T mutation, which is derived from pRSETB-GFP, a generous gift of Dr. R. Y. Tsien (University of California, San Diego), and then inserted individually into pQE31, generating the sequence **GGTACC**CGG-GATGAGTAAA------AAATAAGGTAC**CCCGGG** (*Kpn*I and *Sma*I sites of pQE31 are in bold, and GFP coding region is underlined). To construct pGG1 and pGG2 for expression of GST-GFP fusion protein in *E. coli*, GFP genes derived from pQB1 and pQB2 were re-inserted individually into pGEX5X-2, generating the sequence as **CCCGGG**-ATGAGTAAA------AAATAAGGTACCCCGGGTCGA**GCGGCCGC** (*Sma*I and *Not*I sites of pGEX5X-2 are in bold, and the GFP coding region is underlined). To construct pMG1 and pMG2 for expression of GFP in mouse L cells, GFP genes derived from pQB1 and pQB2 were modified to carry Kozak's consensus sequence [17] and re-inserted individually into pCAGGS, generating the sequence **GAA-TTC**CACCATGGGTAGTAAA------AAATAA**GAATTC** (*EcoRI* sites of pCAGGS are in bold, and GFP coding region is underlined; GGT(Gly) sequence is inserted just after the initiation codon to create Kozak's consensus sequence).

Quick preparation of E. coli cell extracts. XL1-blue cells transformed with each of the GFP variant expression plasmids were cultured in LB medium containing 50μg/ml ampicillin (LB+Amp medium). Next, cells were harvested, washed, and resuspended in 100μl of buffer A (50mM Tris-Cl pH7.5, 20mM EDTA, 2mM phenylmethylsulfonyl fluoride) to a final OD_{600} of 5.0. After addition of SDS (0.1% final), the cell suspension was vortexed at top speed for 10 sec and centrifuged at 15,000G for 10min to obtain the supernatant.

Preparation of purified GFP variants. GST-tagged GFP variants were expressed from pGG1 and pGG2 in XL1-blue and purified with Bulk GST Purification Module (Pharmacia LKB Biotechnology).

Mammalian cells and their transient transfection. 10^5 mouse L cells were seeded in a 35-mm plastic dish and allowed to adhere to the glass coverslips. After 24 hr, 2μg of DNA (pMG1 or pMG2) coprecipitated with calcium phosphate was added to the culture and incubated for 18 hr. DNA precipitates were then washed out and the medium was replaced by growth medium (αMEM+10% fetal bovine serum). After an additional 48 hr incubation, cells were fixed with 10% formalin in phosphate buffered saline, and GFP fluorescence was observed by a fluorescent microscope.

Fluorescent and immunoblot analysis of GFP variants. Fluorescence of the GFP variants were detected using either a fluorescent image scanner FluorImager (Molecular Dynamics Japan, Tokyo, Japan), a fluorescence microscope Axiophoto (Carl Zeiss Vision, Tokyo, Japan) or a spectrofluorometer [12]. Microscopic images obtained by Axiophoto were processed with a digital image analyzer ATTO

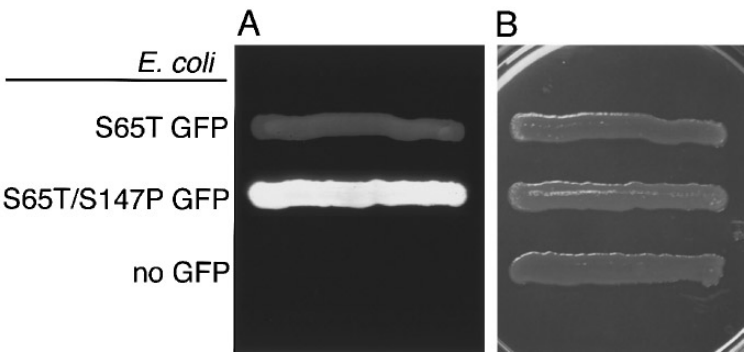

FIG. 2. Fluorescent emission (A) and normal light (B) images of *E. coli* plate cultures expressing GFP variants. *E. coli* XL1-blue carrying plasmids pQB1 (top; for expression of S65T GFP), pQB2 (middle; for expression of S65T/S147P GFP), or pQE30 (bottom; a control vector) were inoculated on an LB agar plate and incubated at 37°C for 12 hr. (A) When the plate was irradiated by a 488nm Argon ion laser beam, fluorescent signals were detected through a low-fluorescence filter (passing wavelengths >515nm) and images were defined using a fluorescent image scanner.

TABLE 1

Fluorescence Intensity Assay of GFP Variants

Expression plasmid	Mutations in GFP	Cell lysate: Relative fluorescence intensity[a]	Cell lysate: GFP conc[b]	Specific fluorescence intensity[c]
pQB1	S65T	244	40	6.10
pQB2	S65T/S147P	1321	73	18.09
pQE31	no GFP	11	<3	

Note. E. coli XL1-blue cells containing expression plasmids for GFP variants were grown logarithmically at 37°C in LB+Amp medium, and their lysates were prepared as described in Materials and Methods. Data are presented as means of duplicate determinations. Individual values did not vary by more than 5% from the mean.

[a] Fluorospectrometric analysis was performed at room temperature with excitation at 488nm and emission at 510nm.

[b] Quantitative immunoblot detection of GFP was performed as described in Materials and Methods.

[c] Value obtained by dividing relative fluorescence intensity with relative GFP concentration in each cell lysate.

Densitograph (Atto, Tokyo, Japan). Detection of GFP variants by immunoblotting was performed as described in Harlow and Lane [18] using Anti-rGFP antiserum (Clontech Laboratories, Palo Alto, CA) and an ECL Western blotting detection set (Amersham, Buckinghamshire, England), and the intensity of the luminescent signals were estimated by Densitograph Lumino-CCD (Atto, Tokyo, Japan).

RESULTS AND DISCUSSION

To generate randomly mutagenized variants of the GFP genes, low fidelity PCR amplification of the GFP cDNA was performed. The PCR products obtained were cloned into pQE30, a bacterial expression vector, and the resultant plasmids were introduced into *E. coli* cells. Transformation gave rise to about 6,000 colonies, and each colony was illuminated directly on agar by a standard UV light box (365nm). The colony which exhibited the strongest fluorescence was isolated, and its plasmid (pQA2) was recovered. Sequencing of the pQA2 DNA revealed a T to C transition at nucleotide position 439, which resulted in a substitution of Ser^{147} to Pro (S147P) in the protein. When pQA2 was re-introduced into bacterial cells transformants consistently exhibited fluorescence brighter than that of the control cells transformed with pQA1, a plasmid equivalent to pQA2 but carrying the wild-type GFP gene.

To further evaluate the effects of the S147P mutation, we combined this mutation with the commonly-used S65T mutation and compared this novel GFP variant (carrying both S65T and S147P mutation) to the S65T GFP variant. The excitation and emission spectra of both are shown in Fig. 1. The emission spectrum of S65T/S147P GFP was almost identical to that of S65T GFP, with its peak located at 512nm. On the other hand, unlike the wild-type GFP [5], neither variant exhibited any excitation bands in the UV zone. Compared to S65T GFP, the single excitation peak of S65T/S147P GFP had shifted to a slightly longer wavelength; i.e. the excitation peaks of S65T GFP and S65T/S147P GFP were 490nm and 496nm, respectively. Hence we determined that the fluorescence of S65T/S147P GFP could be assayed in a manner similar to that of S65T GFP, i.e. by excitation in the blue wavelength zone and monitoring emission in the green wavelength zone.

To compare the brightness of fluorescence in living organisms, bacterial cells expressing GFP variants were inoculated separately on agar, incubated at 37°C,

TABLE 2

Fluorescence Intensity Assay of GFP Variants Fused with GST

Expression plasmid	Mutations in GFP	Culturing condition: Inducer	Culturing condition: Temp. (°C)	Cell lysate: Relative fluorescence intensity[a]	Cell lysate: GFP conc[b]	Specific fluorescence intensity[c]
pGG1	S65T	−[d]	37	36	ND[f]	
pGG2	S65T/S147	−	37	99	ND	
pGG1	S65T	+[e]	37	164	344	0.48
pGG2	S65T/S147	+	37	751	436	1.72
pGG1	S65T	+	23	2744	556	4.94
pGG2	S65T/S147	+	23	2239	572	3.91
pGEX5X-2	no GFP	+	37	13	<3	

Note. E. coli XL1-blue cells containing expression plasmids for GFP variants were cultured according to the indicated conditions, and their lysates were prepared as described in Materials and Methods. Data are presented as means of duplicate determinations. Individual values did not vary by more than 5% from the mean.

[a–c] See Table 1, footnotes a, b, and c.

[d] Cells were grown logarithmically at 37°C in LB+Amp medium.

[e] Cells were brought to logarithmic growth at 37°C in LB+Amp medium before IPTG was added to a final 1mM and further cultured at the indicated temperature for 4 hours.

[f] Not determined.

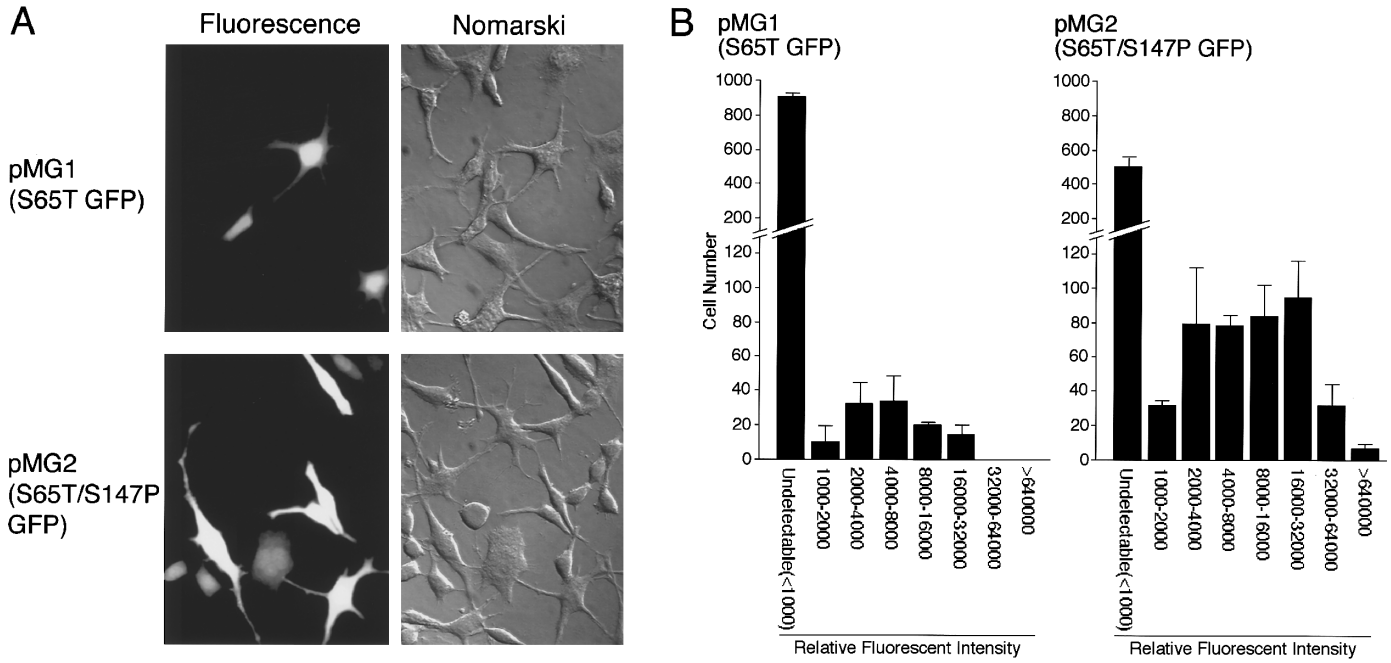

FIG. 3. Fluorescent emission of mammalian cells transfected with expression plasmids encoding GFP variants. After transfecting L cells with the plasmids pMG1 or pMG2, expressing S65T or S65T/S147P GFPs respectively, cells were further incubated at 37°C for 48 hr. (A) These cells were then illuminated by blue light (450-490nm) and observed under a fluoromicroscope through a low-fluorescence filter (passing wavelengths >520nm). (B) The fluoromicroscopic images of the transfected cells were subjected to image processing, and the intensity of fluorescent signals was estimated and quantified for 1000 individual cells per transfection. Data are presented as means ± SD of 3 independent experiments.

and scanned for fluorescence with a fluorescent image scanner (Fig. 2). From this assay we estimated that the bacterial cultures expressing S65T/S147P GFP emitted fluorescence approximately 6-fold brighter than those expressing S65T GFP.

To measure the immediate fluorescent intensity of GFP variants produced in bacteria cells under various culturing conditions, we developed a quick assay method which does not require purification of the GFP variants. This quicker method reduces the opportunity for the continued maturation of GFP following cell lysis. Cells were lysed quickly in 0.1% SDS, which is able to dissolve cell walls swiftly without affecting the fluorescence of GFP (data not shown). The pQB2 transformants (expressing S65T/S147P GFP), cultured at 37°C and then lysed, exhibited a 5.4-fold stronger fluorescence than the pQB1 transformants (expressing S65T GFP)(Table 1). This value measured by our method correlated well with the whole cell fluorescence intensity of living bacteria as described before. To compare fluorescence intensities, we introduced a new unit, specific fluorescence intensity, which is determined by dividing the relative fluorescence intensity by the relative GFP concentration in each cell lysate (Table 1, 2). By this determination the specific fluorescence intensity of S65T/S147P GFP was estimated to be 3-fold stronger than that of S65T GFP (Table 1).

Since GFP is widely used as a protein tag, we compared the intensity of fluorescence of the S65T and S65T/S147P GFP variants fused to the carboxyl terminus of the GST protein, derived from plasmids pGG1 and pGG2, respectively. As shown in Table 2, GFP fluorescence was very faint in the absence of the inducer IPTG. After adding IPTG to the culture and incubating at 23°C for 4 hr, S65T/S147P GFP emitted a slightly weaker fluorescence than S65T GFP. However, when the culture temperature was maintained at 37°C for 4 hr after IPTG induction, the specific fluorescence intensity of S65T/S147P GFP was 3.6-fold stronger than that of S65T GFP. Of particular interest we found that the specific fluorescence intensity of S65T GFP incubated at 37°C was less than one tenth of that of the same protein incubated at 23°C. This indicates that the fluorescence intensity of S65T GFP is highly thermosensitive, similar to wild-type GFP. In contrast, the specific fluorescence intensity of S65T/S147P GFP incubated at 37°C was about 45% of the value obtained at 23°C. These results clearly show that this new GFP variant stably expresses strong fluorescence through a wide range of temperatures.

Since mammalian cells are usually cultured at 37°C, we tested these GFP variants to find out whether the observations made in bacterial cells could be reproduced in a mammalian cell system as well. Mouse L

cells were transiently transfected using the standard calcium phosphate method with the plasmids pMG1 or PMG2, for the expression of S65T GFP or S65T/S147P GFP respectively, and then further incubated at 37°C for 2 days. As shown in Fig. 3, cells transfected with pMG2 exhibited a brighter fluorescent signal than those transfected with pMG1.

How does the S147P mutation enhance the fluorescence of GFP in the cells cultured at 37°C? We believe that the S147P mutation has changed the properties of GFP in several ways. First, we found that the S65T/S147P GFP protein was present at a higher concentration than S65T GFP when expressed in *E. coli*. This implies that the S147P mutation might have improved the stability of GFP at 37°C in this overexpression system. Second, even at equal amounts of proteins, S65T/S147P GFP was found to have a higher fluorescence intensity than S65T GFP. As this enhancement of fluorescence could be observed only when GFP was expressed at 37°C, one possibility is that the S147P mutation exerted its effect via increasing the efficiency of maturation. That is, the efficiency of maturation deteriorates when GFP is expressed at 37°C resulting in a lower ratio of fluorescently active GFP to total GFP protein. Another possibility is that the S147P mutation alters the fluorescent properties of the mature form of the GFP molecule. In fact, the excitation peak of the S65T/S147P protein was hyperchromically shifted by 5nm, although we do not know if the specific fluorescent activity of the mature form of this protein was altered. In addition, the 3D structural analysis of GFP shows that the Tyr^{145} and His^{148} amino acid residues neighboring Ser^{147} closely contact the fluorophore [19,20]. Hence, it is likely that the environment around the fluorophore is greatly affected by the S147P mutation. Recently, some GFP variants which carry mutations within the fluorophore region (-Ser^{65}-Tyr-Gly-), such as S65T GFP, were created to alter its fluorescent properties [7-10]. As demonstrated here in the context of both the wild-type and S65T GFPs, the S147P mutation may have the potential to improve the fluorescence of other GFP variants as well.

ACKNOWLEDGMENTS

We are grateful to Dr. R. Y. Tsien for a plasmid pRSETB-GFP and Drs. H. Niwa and J. Miyazaki for a plasmid pCAGGS. We also thank Rieko Ando for her technical support and Dr. M. Lamphier (University of Tokyo) for critical reading of the manuscript. This work was supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan (07458254 and 08274212) and partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society. C. R. Lim is a Fellow from the Japan Society for the Promotion Science for Japanese Junior Scientists.

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