Expressing a Novel Fluorescent Protein from a Mid-water Siphonophore

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ABSTRACT

GFP-like fluorescent proteins (FPs) are used as molecular markers in a variety of studies. The demand for novel FPs is rising as bioengineering of FPs to make different color variants becomes increasingly important in molecular studies. The identification of a novel FP from the siphonophore, *Diphyes dispar*, is a particularly interesting find, as there is a modification within the chromophore structure. All FPs have a conserved chromophore; this new mutation could lead to interesting and unprecedented properties. To determine these properties, the FP must first be sequenced and expressed to test for fluorescence.

INTRODUCTION

When Shimomura et. al (1) purified aquorin, a bioluminescent protein from *Aequorea*, they stumbled upon a companion protein that fluoresced green. Surely, in 1962, these scientists had no idea just how serendipitous the discovery of the first fluorescent protein (FP) was and how it would revolutionize science. Thirty years later, Prasher et al (2) published the sequence of green fluorescent protein (GFP), setting the stage for advances in fluorescence research methods. However, GFP would not be successfully expressed until two years later in 1994, when Chalfie et al (3) used PCR to isolate only the coding region of the GFP gene, and subsequently was able to transform it into both E. coli and C. elegans cells, successfully expressing the protein. The ability of GFP to express fluorescence in non-jellyfish cells stipulates that the gene must encode for the synthesis of the chromophore without excess enzymes provided by the jellyfish. This breakthrough launched GFP to the star it is now as a fluorescent molecular marker.

The potential of GFP as a molecular marker skyrocketed with the discovery of varied wavelength emissions, introducing variety to fluorescence imaging studies. Advancements in GFP modifications yielded yellow to cyan to blue fluorescence, simply by tweaking the FP chromophore (4). The chromophore consists of three proteins at positions 65, 66, 67; corresponding to X-Tyr-Gly, where X is any amino acid (5).

Tyrosine and Glyceine are conserved in nature, but biosynthesis research has shown that replacing the Tyrosine can yield various color modifications (6).

The particular fluorescent protein of interest belongs to a mid-water jelly called *Diphyes dispar,* a species of siphonophore. Siphonophores are colonial animals consisting of polyps and medusae, or zooids, each specialized for a different functional purpose (7). Latz et al. (8) measured the spectral emission of Diphyes dispar at a maximum wavelength of 464 nm. Since this initial reading in 1988, however, no one has characterized the bioluminescence of *Diphyes dispar*. My goal this summer was to fill in some of this gap in knowledge about Diphyes bioluminescence, with a specific focus on the Diphyes FP.

The first step was to find the sequence of the gene encoding the FP. To do this cDNA libraries were sequenced using 454 sequencing. This method sequences short sub-sequences of the transcribed protein-coding cDNA sequence. This short sub-sequence is called an expressed sequence tag, or EST. Overlapping ESTs were then put together to make contiguous sequences, or contigs, of the whole FP gene. However, there were a few different sequences that showed up. The most common sequence of the FP had a Cysteine in spot 66 in the chromophore where Tyrosine usually is. No FP previously described has had a Cysteine in this spot in the chromophore and still fluoresced, so to test for fluorescence the gene was amplified and transformed into E. coli for expression. There were also two contigs with the traditional Tyrosine in position 66, but the 5' and 3' ends were not the same as the Cysteine version of the FP. Another goal was to elucidate these ends of the Tyrosine FP, and again amplify and express in E. coli.

MATERIALS AND METHODS

PCR AMPLIFICATION OF FP GENE

The cDNA used for PCR was synthesized from RNA isolated from *Diphyes dispar* sample collected by Steve Haddock in the Gulf of California. cDNA was prepared from the RNA by Meghan Powers. To obtain preliminary sequences, the cDNA library was sent for 454 sequencing. Contiguous sequences were assembled and PCR primers designed based on these sequences. For isolation of the gene containing the cysteine in the chromophore, two sets of primers were used, which corresponded to either Sfi1 or Nco1 and Not1 restriction sites. PCR was carried out using Phusion High-Fidelity DNA Polymerase from Finnzymes. PCR reactions consisted of 2-6 μl cDNA, 1x Phusion HF Buffer, 0.5 μM primers, 10 mM dNTPs, 1 U polymerase, and water up to 50 μl total volume. Cycling parameters were: 98°C for 30 s, 30 cycles of 98°C for 10 s, 62°C for 30 s, and 72°C for 15 s, followed by 72°C for 10 min.

PLASMID DIGESTION AND LIGATION

Two different expression vectors were used: pBAD and pQE. The pBAD vector, engineered by Nathan Shaner, has restriction sites for the Sfi1 enzyme. The pQE vector

has restriction sites for the Not1 and Nco1 enzymes. After the PCR product is purified, using a Quaigen purification kit, it is digested. The digestion reaction consists of 20 μl purified PCR product, 1 μl BSA, 5 μl NEB Buffer 3 or 4 for pQE and pBAD vectors respectively, 1-2 μl enzyme: Sfi1 for pBAD and Not1 and Nco1 for pQE. The reaction containing the Sfi1 enzyme sits at 50°C for 2-2.5 hours. The reaction containing Not1 and Nco1 enzymes sits at 37°C for 2-2.5 hours. The digest is again purified using a Qiagen kit. The ligation reaction consists of 2 μl 5x buffer, 1μl vector DNA (either pBAD or pQE), 6 μl purified, digested insert DNA, and 1 μl of T4 ligase. The reaction is mixed and allowed to sit at room temperature for 15 minutes.

TRANSFORMATION INTO EXPRESSION VECTOR

After ligation, the vector was transformed into competent cells. Electro-competent LMG and DH10B cells were used for the pBAD vector. Electroporation was carried out according to protocol. 20 and 100 μl were plated on LB plates with ampicillin and charcol. Plates were grown in the incubator overnight at 37°C and induced the next morning with 20% Arabinose. Blue light irradiation with a yellow filter was used to screen for fluorescence. Chemically competent XL1-Blue, XL2-Blue, and NEB-shuffle cells were used with the pQE vector. The vector was transformed using the XL1-Blue Competent Cell Transformation Protocol. 20% IPTG was used for induction.

TRANSFORMATION INTO CLONING VECTOR FOR SEQUENCING

The StrataClone PCR Cloning Kit from Stratagene was used for cloning the DNA insert into a sequencing vector. PCR, ligation and transformation were carried out according to the Stratagene instruction manual. Sequencing was carried out according to the Plasmid Sequencing, 1/16 reactions with BDTv3.1 protocol. Sequences were aligned and analyzed using Codon Code Aligner.

RESULTS

Original PCR primers were designed based on a complete 454 contig containing the GCG chromophore. It was originally thought that these primers would isolate the FP with both the GCG and GYG chromophore. After transforming the FP in many kinds of competent cells, there was no expression of the FP. To determine if the lack of expression was caused by unsuccessful transformation, insert DNA was sequenced. After alignment it was apparent that the inserts were indeed the FP. However, they all had the GCG chromophore; there was not a single tyrosine version in the multiple batches of sequencing. This was also confirmed by the non-fluorescing colonies.

Once it was determined that the GCG chromophore would not fluoresce under normal conditions, the 454 contigs containing the GYG chromophore were examined to make new primers. A new methyanine start codon was found before the original methyanine, so it was thought that this could be the real start of the GYG gene. Another GYG contig showed an extended 3' end as well. Analysis of the PCR product using these new primers showed a band of the apparent size of the FP. Expression and sequencing followed in

tandem. No colonies expressed the FP, and sequencing of colonies containing the insert revealed sequence that did not resemble the FP.

DISCUSSION

All natural FPs isolated have conserved the traditional tyrosine in position 66 of the chromophore. Even color variations, including blue, red, orange, and lavender FPs, contain the tyrosine in position 66 (9, 10). It is therefore not surprising that the FP variation with the cysteine in position 66 does not fluoresce. However, it is interesting that the siphonophore would be expressing a non-fluorescent FP along with a supposedly fluorescent FP. No research has shown why an organism would possess and express a non-fluorescent protein with a structure so similar to a FP. The siphonophore must have some reason for conserving this non-fluorescent FP. Perhaps it is a genetic relic of the FP before it evolved to have the tyrosine in position 66. Or it may have some unknown use to the siphonophore that hasn't been discovered yet. Alternatively, the cysteine chromophre may in fact fluoresce but under conditions not previously tested.

CONCLUSIONS/RECOMMENDATIONS

Further research is needed to isolate, express, and characterize the fluorescent GYG protein. The best way to do this would be to use the new primers on cDNA and express in many different competent cell types and do a mass screening for fluorescence. The fluorescent colonies can then be grown in culture and sequenced for the exact sequence of the FP. Another interesting experiment would be to replace the cysteine in the chromophore with a tyrosine to see if this construct fluoresces.

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