Minireview

'Green mice' and their potential usage in biological research

Masahito Ikawa, Shuichi Yamada, Tomoko Nakanishi, Masaru Okabe*

Genome Information Research Center, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan

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1. Introduction

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a single peptide of 238 amino acids [1]. *Aequorea* bioluminescence is first activated when Ca^{2+} binds to aequorin following emission of blue fluorescence. Blue fluorescence and subsequent energy transfer from activated aequorin cause GFP to emit green fluorescence. Therefore, unlike enzymes, GFP needs no substrate to emit fluorescence. Rather, it requires only energy to excite the fluorophore. The fluorophore of GFP is formed by post-translational auto-cyclization of Ser^{65}, Tyr^{66}, and Gly^{67} following oxidation. This conformational change requires no substrate(s) or cofactor(s). Therefore, GFP can be fluorescent even when expressed in heterologous species. After the formation of fluorophore that locates in the center of a barrel-like basket composed of 11 β-sheets [2], GFP becomes quite stable and remains fluorescent up to 65°C, pH 11, 1% SDS or 6 M guanidinium chloride [3–5].

Since the first report of expression of GFP in *Caenorhabditis elegans* [6], this novel and simple reporter has attracted much interest for its potential as an in vivo marker of gene expression, because other reporter genes that are used frequently encode enzymes, e.g. β-galactosyltransferase (lacZ), chloramphenicol acetyltransferase (CAT), and firefly luciferase (luc), and require substrates to detect the gene expression. Recently, several substrates have been developed that penetrate the plasma membrane and are processed in the cytoplasm for use in living cells [7]. However, it is still necessary to load the substrate with these markers. In this context, GFP has a significant advantage over other commonly used reporters. Since GFP emits green fluorescence without any substrates or cofactors, one can monitor the presence of GFP by illuminating living cells. GFP may open the door for the use of intact cells and organisms as experimental systems [4,5,8]. Here we describe our trials in expressing GFP in a transgenic mouse system and give an overview of the application of GFP as a vital marker in mammals.

2. 'Dark' ages

Wild-type GFP used in the early reports was not as bright as mutant GFPs available today. The emission spectrum of wild-type (wt) GFP (maximum at 508 nm) is proportional to that of the well-known dye fluorescein isothiocyanate (FITC; maximum at 515 nm), but its excitation spectrum (maximum at 395 nm) differs considerably from that of FITC (maximum at 493.5 nm). Therefore, a special filter set is necessary for optimized observation. Moreover, it was reported that the formation of the wt-GFP chromophore takes a while (about 4 h at 22°C [9]), and this makes it difficult to determine the exact point at which gene expression begins. Although culturing at a low temperature (30–33°C) facilitates chromophore formation [10], this presents obvious problems in using the GFP in culturing mammalian cells and in transgenic mice.

Our first trial in expressing GFP in transgenic mouse lines was performed using elongation factor 1 promoter [11]. Despite its reputation of ubiquitous expression ability, weak green fluorescence was observed only in livers of several transgenic mouse lines. We then used the CAG promoter, which was composed of the CMV enhancer, a fragment of the chicken β-actin promoter and rabbit β-globin exons [12]. Wild-type GFP was amplified by PCR to introduce Kozac sequence and the *Eco*RI fragment of the PCR product covering the coding sequence was inserted into the vector. Using this construct, we could produce a first version of the transgenic 'green mouse' lines [13]. In all of the transgenic lines produced, muscle and pancreas were bright green fluorescent as a rule (more than 20 lines were produced). It was possible to tell the transgenic pups on the day of birth. We even could separate the transgenic embryos in their preimplantation stages [14].

On the other hand, brain and blood vessels always remained negative. With this construct, kidney was one of the GFP expressing organs. However, renal tubules, arteries and interstitium were always silent in GFP expression and only glomeruli becomes green fluorescent. To confirm the cellular localization in the glomerulus, glomerular basement membrane (GBM) was stained with laminin antibody. When GFP was stained with rhodamine conjugated antibody, the GFP positive cells were determined as podocytes, which were present just outside the GBM. The podocyte specific expression with the CAG promoter in kidney was also a rule without exceptions among many transgenic mouse lines that expressed GFP. Transgenic human CD4 expressed with the same promoter was also limited in the glomerular region in the kidney. Therefore, we thought the vector could be used as a podocyte oriented expression promoter [15]. However, to our surprise, we found a very different expression pattern when we used mutant GFP as a coding sequence in the same vector.

3. Mutant GFPs

Recently, genetically engineered GFPs have been reported to show different characteristics such as spectra, magnitude, solubility, etc. [9,16,17]. Many of the GFP variants contain mutations within the chromophore region itself. Replacement

*Corresponding author. Fax: (81) (6) 879-8339.
E-mail: okabe@gen-info.osaka-u.ac.jp

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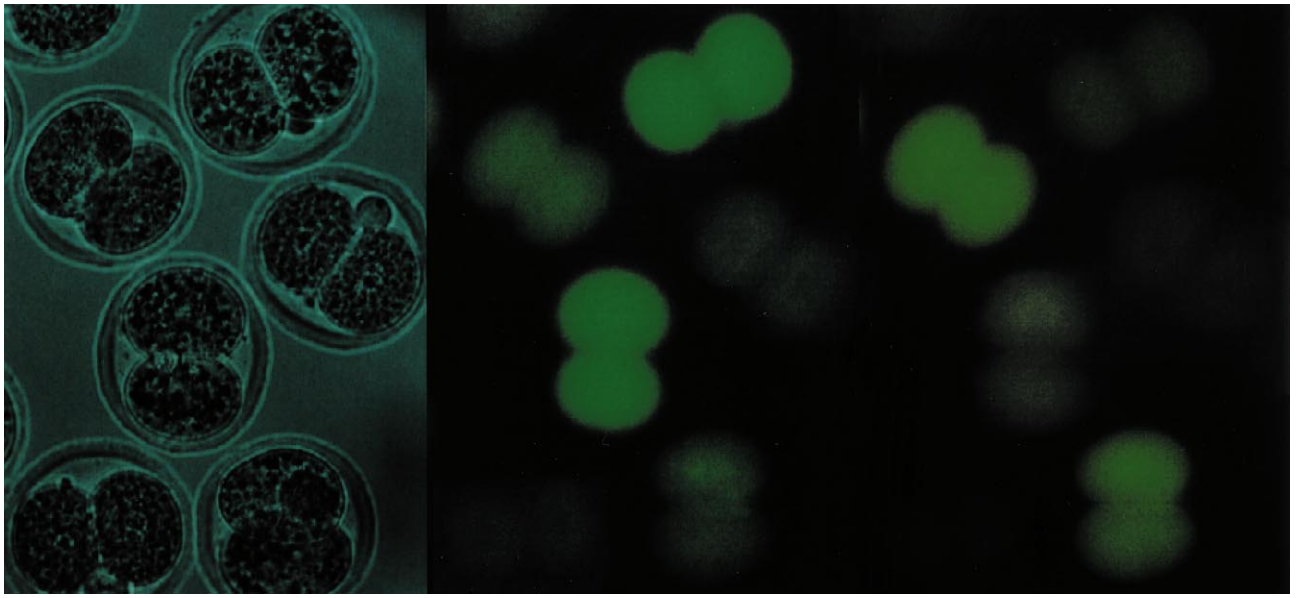

Fig. 1. Eggs labeled with wt-GFP or RS-GFP were distinguishable by shedding the different excitation light. The eggs expressing wt-GFP emit green when excited with V-filter sets (middle), while RS-GFP expressing eggs (right) are fluorescent with B-filter sets.

of Ser^{65} by Thr (S65T) shifts the excitation spectrum to a slightly longer wavelength (maximum at 489 nm) and results in optimal spectra for observation with FITC filter sets or for analysis using flow cytometry [18]. The S65T mutant was also reported to form a chromophore about four times more rapidly than wt-GFP [18]. Some mutations were reported to improve the thermosensitivity of GFP [19,20]. The double amino acid substitutions (P64L and S65T) in GFPmut1 (EGFP) caused about 35-fold brighter fluorescence compared to wt-GFP due to an increase in its extinction coefficient (EM) [17]. To date, various GFPs not only emitting green but also blue (BFP) or yellow (YFP) fluorescence have been created. With or without mutation, GFP can emit red fluorescence with green excitation under conditions of low oxygen [21]. This implies that a fluorescent protein emitting red fluorescence might also be available in the near future. Combining these variants, the separation of specific GFP expressing cells is possible (Fig. 1). The characteristics of GFP and its typical variants are listed in Table 1.

4. Ubiquitous 'green mice'

We kept producing 'green mice' in an attempt to have the GFP transgene integrated in the sex chromosome. Meanwhile, as described in Section 3, various mutants with characteristic natures have appeared and we always used a newer and brighter version of GFP to make 'green mice'. We found no difference when we switched from wt-GFP to RS-GFP (not listed in Table 1 but similar to S65T) [22] in the expression pattern except the latter has brighter fluorescence. However, to our surprise, when EGFP was expressed using the same CAG promoter, all the organs of all the transgenic mouse lines were uniformly green with a few exception of hair and red blood cells. The brain, liver, kidney, adrenal gland, lung, muscle, heart, intestine, adipose tissue, thymus, spleen and testicular cells of all transgenic mouse lines were naturally green when irradiated with excitation light [23]. Since a matching of genetic background is important, we have also recently produced a ubiquitous 'green mice' with C57BL/6 background. The mice are available to the scientific field upon request.

In jellyfish, GFP is normally sequestered in microbody-like lumisomes. However, in the transgenic experiment described here, the EGFP was designed to be expressed in the cytosol and the soluble nature of the protein allowed its distribution throughout the cytosol. This situation could cause a toxic effect if EGFP were expressed at high levels. However, all of the transgenic mouse lines looked normal and healthy, despite a significant amount of EGFP expression throughout the body. The expression of GFP therefore seemed to be non-

Table 1
GFP and its variants

Variant	Mutation	Max. excitation	Max. emission	EM (cm^{-1} M^{-1})	Characteristics	Reference
Wild-type						
GFP	–	395	509	21 000 (7150)	green emission	[6]
S65T	S65T	489	511	39 200	excited at 488 nm	[18]
GFPmut1 (EGFP)	F64L, S65T	488	507	250 000	35 times brighter	[17]
P4-3 (BFP)	Y66H, Y145P	380	440	37 000	blue emission	[9]
10C (YFP)	S65G, V68L, S72A, T203Y	513	527	36 500	greenish-yellow emission	[2]

The EM was measured at optimal excitation. The EM for 488 nm excitation of wt-GFP is presented in parentheses. There is an informative table listing GFP variants and many of them are commercially available: http://www.image1.com/.

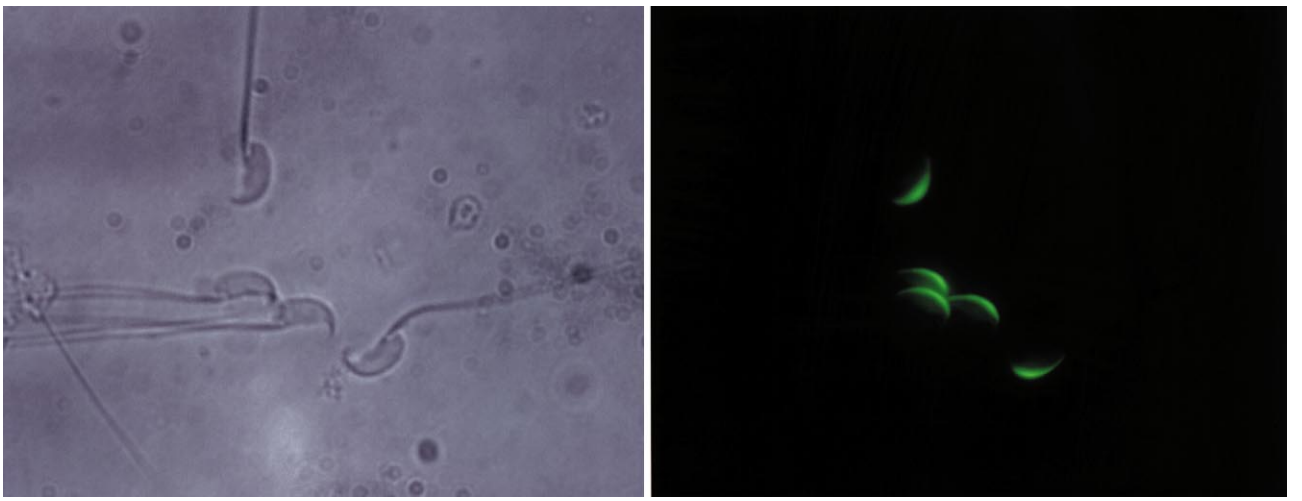

Fig. 2. 'Green sperm'. The sperm from the acrosin-GFP transgenic mouse lines had a green fluorescent acrosome. The sperm in the photo are not stained but naturally 'green fluorescent' under appropriate excitation light. Therefore we could observe 'green sperm' swimming under the microscope and interacting with eggs.

toxic. These results indicate that EGFP could be used as a novel reporter in almost the whole mouse body from pre-implantation stage to adulthood.

The difference was not simply due to greater sensitivity of EGFP, allowing detection in tissues with lower expression. For example, while blood vessels were always negative in transgenic mice expressing wt-GFP, this tissue was one of the brightest parts of the body in more than 50 lines of all the transgenic animals expressing EGFP. Since the difference between EGFP structure and others is only a few amino acid substitutions, codon optimization must be responsible for the ubiquitous expression. However, as indicated earlier, with a combination of human CD4 cDNA, the CAG promoter did not serve as a ubiquitous promoter. The real cause of the

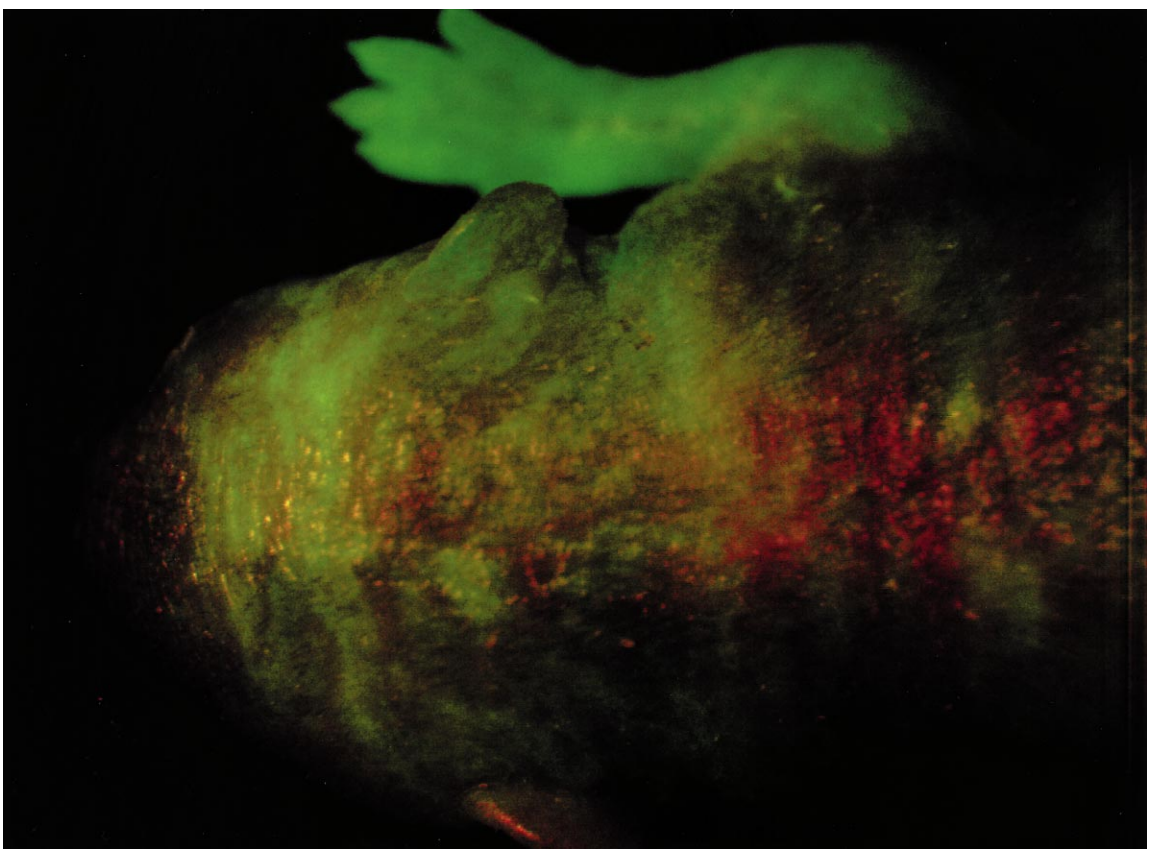

Fig. 3. A female pup from one of our X-linked transgenic 'green mice'. Although the GFP transgene should be distributed all over the body as hemizygous transgenic mouse, only half of the body surface glows 'green', probably because half of the X chromosome carrying the transgene has become silent by X inactivation. The photo was taken under a Leica stereotype fluorescein microscope under normal lighting together with GFP excitation lighting. As shown in the photo, the green fluorescence was strong enough to observe in a bright environment.

different expression pattern with different coding sequence remains to be solved.

5. Green sperm and others

In contrast to ubiquitous expression, cell or organ specific expression of GFP is also possible if we use an appropriate promoter to drive GFP in transgenic mice and the fusion with a signal peptide leads GFP to the target organelle. The use of the acrosin promoter enabled male germ cell specific expression. When the amino-terminus of EGFP was fused at the carboxy-terminus of the acrosin signal peptide, the fluorescence localized inside the sperm acrosome. Sperm need to cause vesiculation of the acrosome before acquiring fertilizing ability. This phenomenon is known as the acrosome reaction, which occurs in the female reproductive tract and it has been extensively studied in mice. In order to detect the change in acrosome, antibodies, fluorescent dye, and agglutinins have been utilized. The green sperm we made would serve as a convenient tool to monitor the acrosomal status non-invasively Fig. 2.

The visualization of astrocytes was achieved by astrocyte specific glial fibrillary acidic protein (GFAP) promoter. According to their comparison, GFP served better than lacZ in terms of mimicking the GFAP expression pattern, suggesting a possibility of interference of gene expression by the lacZ sequence [24].

6. GFP as a transgenic marker

The main methods used in detection of transgenes are PCR analysis or Southern blotting following extraction of DNA from the tails of 3–4 week old pups [25]. However, the procedures require skill and consume time if applied to a large number of transgenic animals. Since the 'green mice' are distinguishable by their green fluorescence when they are born, the pCX-GFPs is useful as a transgenic marker when co-injected with a desired transgene. When the GFP gene was injected with a target gene (molar ratio; 1:1), more than 80% of transgenic founder mice carried both genes, while the remaining 20% carried one (Okabe et al., unpublished data).

GFP expressing constructs can be used for the efficient production of transgenic animals. To date, the low efficiency of transgenic animal production by microinjection has been a serious problem, especially in large mammals. Using GFP as a reporter gene, it was shown that eggs injected at the pronuclear stage become fluorescent until the blastocyst stage and transgenic eggs can be selected in a considerably high ratio before implantation [26].

After the production of the founder transgenic animals, it would be necessary to propagate the transgenic animals. However, animals such as bovine need a long gestation period and the number of offspring is limited. If the preimplantation stage transgenic embryos could be selected after in vitro fertilization using sperm from a transgenic founder male with eggs from a wild-type female, the efficiency of transgenic animal expansion would be greatly improved. As shown in the experiment using transgenic mice, it was shown to be easy to separate transgenic eggs before the implantation stage according to the green fluorescence from transgenic eggs. Many mixed transgenic and non-transgenic embryos were separated under fluorescent microscope and subjected to PCR analysis and the separation was confirmed to be accurate. When separated embryos were transferred into the uterus of pseudopregnant females (day 2.5), all the mice born from green eggs proved to be carrying the GFP transgene while the non-green eggs did not carry the transgene at all [14]. These data imply that an efficient production and propagation of transgenic livestock could be achieved by GFP co-injection.

Recently, embryo sexing by PCR analysis from a part of the embryo has been performed in a variety of species such as human, bovine and mouse [27,28]. However, if we could produce 'green mice' that have the GFP transgene on the sex chromosome, the 'green eggs' represent male (or female) embryos depending on the incorporated sex chromosome. We have so far produced 1–5 transgenic mouse lines for each of the chromosomes 1–19, X and Y with a genetic background of B6C3F1 mice. The photo in Fig. 3 shows a 'green mouse' with the GFP transgene integrated in the X chromosome. Half of the skin was green and the rest was not green due to X-inactivation (Okabe, in preparation).

Since the first report of GFP as a marker protein, this new probe has been providing us with numerous new experimental methods. The unprecedented nature of this GFP challenges us to find brand new uses for the protein. GFP fusion proteins, 'chameleons', which measure Ca^{2+} concentration, are one of the examples for new GFP usage. In the future, we need to keep our eyes not only on brighter mutant GFPs, but also on innovative uses for this vital marker.

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