Green Fluorescent Protein as a Transcriptional Reporter in Epithelial Cells: Real-Time Studies of the Human Involucrin Promoter

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Abstract

Green Fluorescent Protein (GFP) is a popular reporter for numerous uses in mammalian cells because its activity can be measured directly without cofactors or reagents. We show that an improved GFP variant, the cycle-three GFP gene in the pGlow-TOPO® vector is useful as a reporter of mammalian promoter activity. The 5´ regulatory region from the human involucrin gene was cloned upstream of the cycle-three GFP gene and the resultant constructs transfected into C33-A epithelial tumor cells and normal keratinocytes. Fluorescence analysis whole cells showed that cycle-three GFP in pGlow-TOPO® is reliable for reporting mammalian promoter function under different conditions. GFP activity was comparable to that of CAT, indicating that this method can be used in transcriptional studies for numerous mammalian promoters.

Introduction

Transcriptional regulation studies have been greatly simplified by the development of reporter genes coding for proteins that possess a unique activity, or produce a signal, that allows the protein to be easily discernable within a complex mixture of other proteins (1). Desirable characteristics in a reporter protein include detection with high sensitivity, wide dynamic range of response, and ease of use. The Green Fluorescent Protein (GFP) was originally isolated from the jellyfish *Aequorea victoria* (2,3). The protein has been extensively used as an *in vivo* marker and for monitoring dynamic processes inside living organisms (4,5,6). The fluorescence of GFP is the result of an internal chromophore formed by the autocatalytic post-translational cyclization of three amino acids, Ser65-Tyr66-Gly67 (7,8). GFP has several characteristics that make it an excellent reporter protein. Unlike other reporter proteins, GFP does not require substrates or cofactors to emit light (8). GFP retains its fluorescence capability upon exposure to mild denaturants, heat, detergents, and proteases because of the protected location of the chromophore inside the β-barrel of the protein (8). Most importantly, GFP mutants with different fluorescent properties, including increased fluorescence intensity and shifted wavelengths of excitation and emissions have been obtained. In the present study we used the pGlow-TOPO® vector, containing the cycle-three GFP mutant described by Crameri *et al.*, 1996 (9), to establish a novel reporter system for transcriptional analysis and promoter studies in eukaryotic cells. This novel system was used to monitor transcriptional activity driven by the human involucrin 5´ non-coding region in cultured epithelial cells. The system proved to be reliable in detecting activity changes under external signals affecting involucrin promoter activity. Cycle-three GFP fluorescence correlated with the transcriptional activity of the promoter and with the number of positively transfected cells. Finally, the GFP system was compared with the CAT reporter gene. Similar sensitivity and response range were noted, making cycle-three GFP and the pGlow-TOPO® vector a useful system for future transcriptional studies.

Materials and Methods

Plasmids. The pGlow-TOPO® vector (Invitrogen, Cat. no. K4830-01) contains the cycle-three GFP mutant obtained after optimization of codon usage in *E. coli* and three cycles of DNA shuffling. This GFP variant shows a significant improvement in whole cell fluorescence signal over wild-type GFP (9). Additionally, this allele can be easily detected with UV light in a wide range of assays (4,9). pGlow-TOPO® provides a highly efficient, one-step cloning strategy for the direct insertion of promoter sequences amplified by *Taq* polymerase upstream of the cycle-three GFP gene. *Taq* polymerase has a non-template dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. pGlow-TOPO® has a single, overhanging 3´ deoxythymidine (T) residue that allows efficient ligation of PCR products. pCAT-Basic (promoterless) and pCAT-Control vectors (Promega) were used as controls in the transfection and co-transfection assays. pCAT-Control contains the CAT gene cloned upstream of the SV40 promoter and enhancer. p220CAT contains the amplified fragment from nt -156 to +43 cloned in pCAT-Basic (10).

Promoter cloning. The 5´ non-coding region from the human involucrin gene (2456 nt), previously cloned into the

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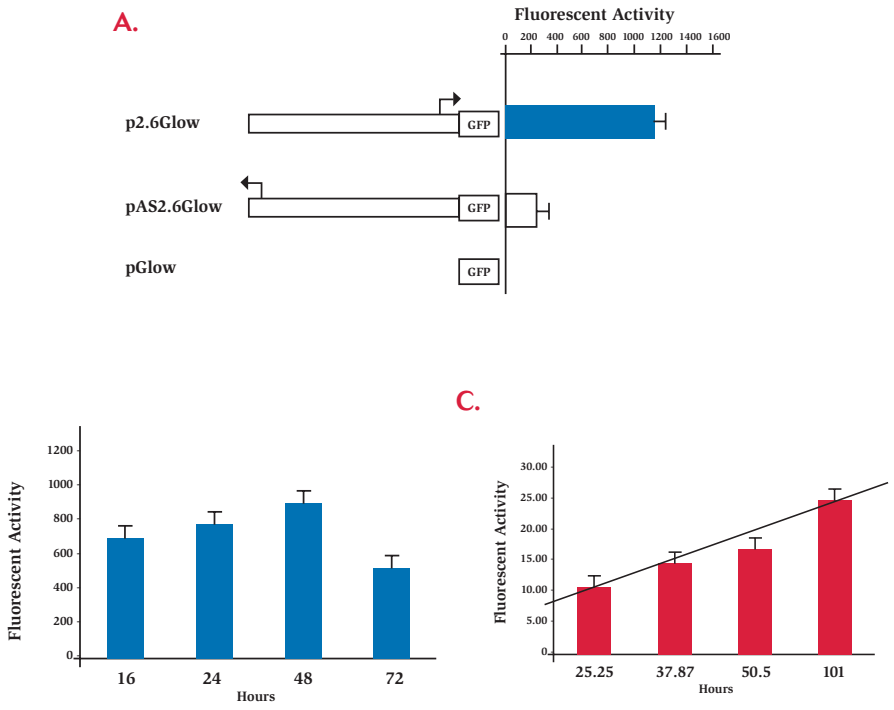
Figure 1 – Expression of cycle-three GFP from pGlow-TOPO® containing the human involucrin promoter.



A. C33-A cells were transfected with 3 µg of plasmid DNA from the sense and antisense constructs containing the involucrin promoter region (maps on the left) and harvested 48h post-transfection. B. Changes in fluorescence were recorded over time after C33-A cells were transfected with 2.5 µg of p2.6Glow plasmid and harvested at 16, 24, 48, and 72 hours. C. Correlation between the fluorescent signal and the number of GFP expressing-cells. 10^6 C33-A cells were transfected with 5 µg of p2.6Glow, harvested 48 hours post-transfection, counted, and divided in samples. In all cases, fluorescence was measured in a fluorimeter. Fluorescent activity was set by subtracting the fluorescence of the promoter less plasmid (pGlow) from the fluorescence in cells transfected with each construct and normalized by protein content. The fluorescent activity was obtained from four independent experiments.

p2.6CAT plasmid (10), was PCR amplified using the following oligonucleotides INVO2: 3´-GGG TCT AGA CAG ACT CAG AG-5´ and ENH1: 5´-CCC AAG CTT CTC CAT-3´. The oligonucleotides 220 linker 5´CCCAAGCTTCGTACGGGGCC TAAAGGGTTGC-3´ and INVO2 were used to amplify a 220 nt fragment (-156 to +43) containing the core promoter. Amplified fragments were ligated directly into the pGlow-TOPO® vector. The resulting constructs were transformed into chemically competent DH5α™ cells. Picked colonies were cultured overnight in LB medium containing ampicillin (100 µg/ml) followed by plasmid DNA isolation. Clones were analyzed for insertion and orientation by restriction analysis. Selected constructs were sequenced using the Sequenase system (USB Corp.).

Cell culture and transient transfection assays. The cervical carcinoma cell line C33-A was cultured in D-MEM/F12 (1:1) medium plus 10% fetal bovine serum and gentamicin, penicillin, and streptomycin (final concentration 100 µg/ml, each) at 37˚C in a 5% CO_2 atmosphere. Normal multiplying human keratinocytes (HK) from human foreskins were cultured in Gibco™ Keratinocyte-SFM medium (with 50 µg/l of bovine pituitary extract, 5 µg/l Epidermal Growth Factor (EGF), 100 µg/ml each penicillin, streptomycin, and gentamicin, in a 5% CO_2 atmosphere. Differentiation-induction in confluent cultures was performed with 2 mM $CaCl_2$ Keratinocyte-SFM lacking epidermal growth factor and bovine pituitary extract. C33-A cultures, seeded in 24-well plates and 80% confluent were transfected with different plasmid DNA concentrations and/or different plasmids using the calcium phosphate method described by Ausubel *et al.* (11). Normal human keratinocytes cultures, 70% confluent in 24-well plates were transfected with 6 µg of p2.6Glow or p2.6CAT using Lipofectamine™ (Invitrogen) according to the manufacturer's protocol (10). Transfection efficiency was determined in all experiments by fluorescent microscopy using a Nikon Eclipse E600 fluorescence microscope and filter with an excitation range between 480 to 510 nm and emission in 510 nm. Fluorometric and CAT determi-

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nations were done only when transfection efficiency did not vary more than 10%.

Fluorometric assays. For GFP assays, cells were harvested at different times post-transfection in PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4). Whole cell pellets were re-suspended in 50 µl PBS buffer and transferred to a 96-well plate containing black wells (Labsystems). Fluorescence was measured using the Fluoroskan Ascent 374 (Labsystems) and the following filter settings: excitation at 380 nm and emission at 510 nm. Fluorescence was corrected for background activity shown by cells transfected with the pGlow-TOPO® vector alone and for the cell number between individual experiments by cell protein content determined using the Bio-Rad protein assay system.

CAT assays. CAT assays were carried out as described in López-Bayghen, *et al.* (10). Cells were harvested 48 hours post-transfection in TEN buffer (40 mM Tris-HCl pH 8.0, 1 mM EDTA, 15 mM NaCl). Protein lysates were obtained by freeze/thaw methods followed by precipitation in 0.25 M Tris buffer. Standardized amounts of protein lysates were incubated with 0.25 µCi of [^{14}C]-Chloramphenicol (50 mCi/mmol, USB corp.) and 0.8 mM Acetyl-Co-A at 37˚C. Acetylated forms were separated by thin-layer chromatography and quantitated using an AMBIS 4000 radioactive image analyzer (Scanalytics). CAT activities were expressed as the acetylated fraction corrected for activity in pCAT-Basic.

Results

To characterize the level of fluorescence produced by the cycle-three GFP/in pGlow-TOPO® reporter, we selected the previously characterized involucrin promoter (10). The cervical carcinoma cell line C33-A was transfected with the following constructs containing the regulatory region from the human involucrin gene upstream of cycle-three GFP: p2.6Glow (5´-3´, sense), pAS2.6Glow (3´-5´, antisense), and the pGlow-TOPO® empty vector (without promoter). Initially, cells were transfected with 3 µg of plasmid DNA and harvested 48 hours post-transfection. Total fluorescence activity was determined by subtracting the background fluorescence produced by cells transfected with the promoterless plasmid (pGlow-TOPO®) from that produced by cells transfected with a promoter-containing construct. Only the p2.6Glow construct, with the promoter inserted in the sense orientation, demonstrated significant transcriptional activity (Figure 1A, page 17). Activity from antisense pAS2.6Glow was clearly lower, indicating that the fluorescent activity is dependent on the functionality of the promoter. A time-course study was conducted to determine when fluorescence activity peaked. C33-A cells were transfected with 3 µg of p2.6Glow plasmid (Figure 1B, page 17). Fluorescence peaked 48 hours post-transfection and decreased after 72 hours, as is usual in transient experiments. In order to determine the minimal number of cells needed to yield a measurable fluorescent signal, cell cultures in a 60 mm dish format were transfected with 5 µg of p2.6Glow, counted with a hemocytometer (Neubauer), and resuspended in PBS buffer prior to loading into a 96-well plates to measure fluorescence (Figure 1C, page 17). The lower detection limit was determined to be about 252,500 cells. The fluorescent signal was plotted against the cell number and it proved to be linear. Under the same transfection efficiency (determined by fluorescence microscopy), an increase in GFP expressing cells correlated with fluorescent measurements.

To determine the optimal DNA concentration needed to detect fluorescent activity and to make a comparison between the CAT reporter and this novel reporter system, we co-transfected C33-A cells with different concentrations of the p2.6Glow and pCAT-Control vectors. Cells were harvested 48 hours post-transfection and assayed for fluorescence and CAT activity. The results (Figure 2A, page 19) show that DNA amounts between 3 and 5 µg of DNA are sufficient to produce fluorescence. Both reporters demonstrated increased activity as DNA concentration increased. We also tested the same promoter directing both reporters under the same conditions (Figure 2B, page 19). For this purpose we transfected normal human keratinocytes with p2.6Glow, p2.6CAT, p220Glow, and p220CAT constructs under multiplying and calcium-induced differentiation conditions. Only plasmids containing the complete involucrin regulatory region (2.6 kb) displayed similar 2-fold activation once the cells were induced to

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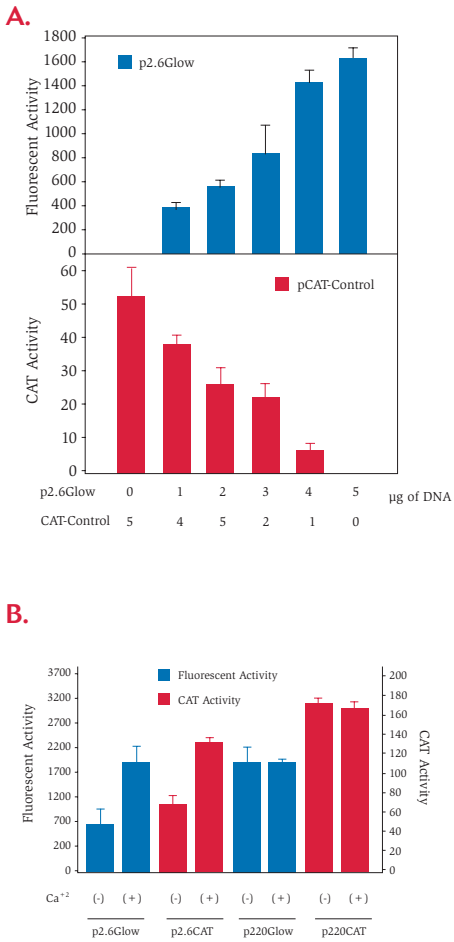
Figure 2 – Comparative activity of GFP and CAT reporters in transfection assays



A. C33-A cells were co-transfected with p2.6Glow and pCAT-control plasmids. Cells were harvested 48 hours post transfection. Average fluorescence and CAT activity were obtained from three independent experiments.
B. The activity of GFP and CAT genes under the control of the same promoter (the involucrin promoter) is shown. Normal human keratinocytes cultures were transfected with 6 µg of p2.6Glow, p220Glow, p2.6CAT or p220CAT plasmids. Calcium-induced differentiation in keratinocytes was done switching to 2 mM $CaCl_2$ medium 12 hours after transfection. Cells were harvested 48 hours post-transfection and fluorometric and CAT assays were carried out. Average fluorescence was obtained from four independent experiments and for CAT activity from six independent experiments.

differentiate by switching to 2 mM $CaCl_2$. This condition has been reported before as a stimulus for involucrin transcription (10), proving that the cycle-three GFP in pGlow-TOPO® reported here is equally sensitive as CAT for registering transcriptional changes in a defined promoter.

Discussion

We established a novel transcriptional reporter system using the cycle-three GFP gene as a reporter of relative promoter activity in quantitative assays. Through a number of experiments we have proven that cycle-three GFP displays sensitivity and dynamic range equivalent to CAT. The fluorescence assays developed here are rapid, simple, and sensitive. They allow quantitation of relative promoter strength over an activity range comparable to that of CAT for assessing promoter activity. Fluorometric detection of GFP does not require the use of substrates or cofactors to emit light. Furthermore, GFP can be detected in a non-destructive manner. We show here that it is not necessary to prepare cell extracts, making GFP useful for the study of tissue-specific gene expression in intact tissues or in organotropic cultures. A simple fluorometric assay is used to measure GFP fluorescence in whole cells. Since mammalian cells do not possess an activity similar to that of GFP, the sensitivity of the system is only limited by the "background noise" of the fluorometer. This assay permits the use of a small number of cells because of the increased sensitivity, being less expensive and less time-consuming than other systems and allowing data acquisition in a matter of minutes rather than hours or days. One drawback of this reporter gene system is that a fluorometer is a relatively expensive and uncommon laboratory instrument. Fortunately, fluorescence can be visualized by confocal and fluorescence microscopy, providing a useful method for determining transfection efficiency as well.

The cycle-three GFP in the pGlow-TOPO® reporter system is also useful for determining the level of promoter response to external stimulus in the same range shown in a traditional CAT assay. We think all of the advantages of this novel system, as described in this article, will improve transcriptional regulation studies. n

Acknowledgments

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References

1. Alam, J. and Cook, J.L. (1990) *Anal. Biochem.* **188**: 245.
2. Bronstein, I., Fortin, J., Stanley, P.E., Stewart, G.S., and Kricka, L.J. (1994) *Anal Biochem.* **219**: 169.
3. Prasher, D.C. (1995) *Trends Genet.* **11**: 320.
4. Bennett, R.P., Cox, C.A., and Hoeffler, J.A. (1998) *BioTechniques* **24**: 478.
5. Chalfie, M., Tu,Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994) *Science* **263**: 802.
6. Tannahill, D., Bray, S. and Harris, W.A.. (1995) *Dev. Biol.* **168**: 694.
7. Heim, R., Prasher, D.C., and Tsien, R.Y. (1994) *Proc. Natl. Acad. Sci.* USA **91**: 12501.
8. Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J. (1992) *Gene* **111**: 229.
9. Crameri, A., Whitehorn, E.A., Tate, E., and Stemmer, W.P.C. (1996) *Nat. Biotechnol.* **14**: 315.
10. López-Bayghen, E., Vega, A., Cadena, A., Granados, S.E., Jave, L.F., Gariglio, P., and Alvarez-Salas, L.M. (1996) *J. Biol. Chem.* **271**: 512.
11. Ausubel, F.M. ed. (1999). *Current Protocols in Molecular Biology*. John Wiley & Sons.