Short Technical Reports

Green Fluorescent Protein Expressed in Living Mosquitoes—Without the Requirement of Transformation


ABSTRACT

Mosquitoes transmit viruses, protozoa and nematodes that are major causes of morbidity and mortality in humans. Details of arthropod anatomy and development, and the replication and development of pathogens in the arthropod vector, have relied upon examination of dissected or histologically processed material. We constructed a double-subgenomic Sindbis (dsSIN) virus expressing green fluorescent protein to demonstrate the potential of this protein for studying pathogen development in living arthropods. We were able to observe dissemination of virus, and furthermore, it was possible to observe components of the nervous system of mosquito larvae in extraordinary detail and record this on video tape. Although green fluorescent protein has been used as a reporter gene in a number of organisms, expression has relied upon transformation of cells or embryos. Transformation technology has limited applicability, thus we have described an alternative system that, due to the broad host range and viral tropisms of dsSIN viruses, may be useful to scientists in a range of disciplines. Green fluorescent protein may also provide a non-lethal selection method for use in transgenic arthropod research.

INTRODUCTION

An infectious expression system based upon the alphavirus Sindbis (7,9) has proven utility in the expression of genes in mosquitoes (12–14,23–25,27). An additional promoter and multiple cloning sites, immediately downstream of the structural genes, facilitate the expression of an introduced gene (9). Genomic RNA and two subgenomic RNAs are therefore produced, rather than genomic and a single-subgenomic RNA, as is typical for a wild-type Sindbis virus (12). This double-subgenomic Sindbis (dsSIN) system may be exploited to identify genes that have the potential to disrupt vector competence in transgenic mosquitoes (4,23,25). Recently, dsSIN viruses have been used to interfere with bunyavirus and flavivirus replication and transmission in vivo (23,25). In these studies, viral replication and gene expression could only be determined in killed and processed mosquitoes. Our knowledge of viral replication and tropisms in arthropod vectors is therefore primarily based upon techniques such as immunofluorescent or immunohistochemical staining of fixed tissues, which can destroy viral epitopes and tissue integrity. To determine if viral replication and protein expression could be visualized in living mosquitoes, the gene encoding green fluorescent protein (GFP) derived from the jellyfish Aequorea victoria (26), was cloned into the infectious SIN expression system (Figure 1).

GFP is a 27-kDa monomer of 238 amino acids and emits green light (λ\text{max} = 509) when excited with UV light (λ\text{max} = 395). Since GFP fluoresces without the necessity of cofactors, substrates or co-expressed genes, it can be visualized in situ in living organisms. GFP has been used as a reporter gene under the control of a neuron-specific promoter in transgenic Caenorhabditis elegans (5) to monitor the formation of neuronal processes in the developing nematode and to locate Exu protein during Drosophila melanogaster oogenesis (31). More recently, GFP has been used as a reporter of successful transformation in studies of zebra fish (1) and mice (22).

MATERIALS AND METHODS

Construction of Expression Vector and Production of Virus

Plasmid pTE3′2J/GFP was constructed from the pTE3′2J (9) using standard procedures (29). Unless otherwise stated, reagents were obtained from Promega (Madison, WI, USA) or Sigma Chemical (St. Louis, MO, USA). An XbaI/SpeI fragment from pGFP encoding the GFP protein (CLONTECH Laboratories, Palo Alto, 660 BioTechniques Vol. 21, No. 4 (1996)

Figure 1. Map of pTE3′2J/GFP.
CA, USA) was cloned into the XbaI site of pTE3'2J. pTE3'2J was treated with calf alkaline phosphatase before ligation. pTE3'2J/GFP was linearized with XhoI to produce DNA templates for run-off transcription with SP6 polymerase. RNA was synthesized for 2 h at 37°C using a mMessage mMachinem kit (Ambion, Austin, TX, USA) without additional GTP. The resulting capped RNA was stored at -70°C in transcription buffer until needed. RNA was transfected by electroporation as previously described (16). Briefly, BHK-21 cells were trypsinized, washed once in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and twice in ice-cold phosphate-buffered saline (PBS), pH 7.4, and then resuspended in PBS to 1 × 10^7 cells/mL. For electroporation, 5 µg of each RNA, 2 µL of RNasin® (2.5 mM final concentration) were mixed with 500 µL of cell suspension and placed in 0.2-cm cuvettes. Cells were then immediately pulsed at room temperature in a Gene Pulser® apparatus (Bio-Rad, Hercules, CA, USA) with two consecutive pulses at 1.5 kV, 25 mF, with resistance set to infinity (time constant 0.7–1.0 ms). Cells were rested for 10 min at room temperature and were then diluted 1:20 in DMEM with 10% FBS and transferred to a 75-cm^2 flask (Falcon®, Becton Dickinson, Franklin Lakes, NJ, USA). Following incubation at 37°C for 24–48 h, TE/3'2J/GFP virus was harvested in tissue culture fluid from infected cells. Supernatant was removed, centrifuged (800× g, 5 min) and filtered through a 0.45-µm cellulose acetate filter. The production of GFP by TE/3'2J/GFP virus was confirmed by infecting BHK-21 cells grown to confluence on glass coverslips (8), and examination for fluores-

Figure 2. GFP expressed in stages of Ae. aegypti mosquitoes. (A) Comparison of control uninfected (upper) and infected (lower) larvae. (B) Ventral nerve chord and thoracic ganglia in larva. (C) Nerve fibres in larva. (D) Eye of pupa. (E) Comparison of control uninfected adult (center) with infected male (left) and female (right). (F) Common oviduct from dissected adult.
cence using a BH-2 epifluorescence microscope (Olympus, Lake Success, NY, USA) with fluorescein isothiocyanate (FITC) filters (data not shown).

Infection of Mosquitoes

For arthropod studies, Aedes aegypti mosquitoes (a white-eyed strain) were inoculated (28) with 0.5–1.0 μL of TE/3′2J/GFP virus (tissue culture infectious dose 50% endpoint [TCID₅₀] titer 7 log₁₀/mL) either as larvae or adults. Viral persistence and replication in vivo was further demonstrated by titration of triturated insects on mammalian cell culture. Within 3 days post-inoculation, TCID₅₀ titers increased to 5.5 log₁₀/mosquito.

Observation of GFP

Inoculated larvae, and adults emerging from these, were examined at various time points post-inoculation using an IMT-2 microscope (Olympus) with FITC filters for transmitted and epifluorescence. By attaching a VPC-920 1/2″ (1.27 cm) color CCD camera (5 Lux sensitivity), Panasonic AG-1960 video recorder and Sony PVM-1343 MD monitor (S & M Microscopes, Colorado Springs, CO, USA), tissues in which GFP was expressed could be recorded. This relatively inexpensive system allowed nerves to be traced in living, moving larvae and to be recorded in real-time (data not shown).

RESULTS AND DISCUSSION

Within 48 h post-inoculation, fluorescence was observed in tissues of live larvae (Figure 2). Nervous tissues (the ventral nerve chord, brain and fine nerve fibers) were clearly visible, with dissemination into fat body tissues occurring with time. The virus survived metamorphosis; fluorescence was apparent in the head of pupae and was seen through the eyes and in other tissues, including those of the nervous and occasionally reproductive systems of the emerged adults (Figure 2).

Since widespread fluorescence can hinder visualization of fine detail, we inoculated a noninfectious SIN replicon (14) expressing GFP. GFP was visible in the tissues (principally the nerve chord) of inoculated larvae but did not disseminate from those cells initially infected (data not shown). These results are consistent with previous observations (14). We have previously described the neurotropism of dsSIN viruses (27), which may be due to the E1 and E2 genes derivation from a neurovirulent strain of SIN (17).

The ability to observe viral gene expression without specimen processing represents a significant contribution to the study of viral infections in arthropod vectors. Since SIN viruses may replicate in a variety of insect species (15,30), and since the virus is able to survive metamorphosis, TE/3′2J/GFP may be suitable to study arthropod development. TE/3′2J/GFP replicates and expresses GFP in tissues of Culex pipiens, Anopheles gambiae (data not shown) and in D. melanogaster (A. Raymes-Keller, unpublished). Since SIN also replicates in vertebrates, TE/3′2J/GFP may also be useful for studies in higher organisms. With the development of improved GFP (6,11), the usefulness of GFP in a replicating expression system can be extended. As our ability develops to genetically engineer organisms such as Aedes densonucleosis virus (4), Trypanosoma cruzi (10), Rhodococcus rhodni (2), Ricketsia-like organisms (3) and other pathogens of or transmitted by arthropods, GFP can facilitate studies of these organisms and of their insect hosts in a manner never before possible.

A common problem associated with the generation of transgenic mosquitoes (4), and we are currently working to optimize the expression of GFP in systems such as the Aedes densonucleosis virus (4) and retroviruses (18) that have the potential for DNA-based expression in mosquito cells and mosquitoes.

REFERENCES

Fluorometric Assay for DNA Polymerases and Reverse Transcriptase

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ABSTRACT

We report a quick, easy and inexpensive fluorometric assay that measures the activity of replication enzymes using PicoGreen™. The systems tested include replication of the natural template M13 Gori by E. coli DNA polymerase III holoenzyme and the replication of a synthetic homopolymer by human immunodeficiency virus reverse transcriptase. A direct comparison of the fluorometric assay with the conventional isotopic assay shows that the fluorometric assay accurately reflects the extent of replication. By performing the assay reactions directly in 96-well plates and using a fluorescence plate reader to determine the extent of reaction, the time required to measure replication activities is significantly shortened.

INTRODUCTION

Conventional assays of polymerase activity involve measuring the extent of incorporation of radiolabeled nucleotides into acid-insoluble poly nucleotides (5,6,8). Such procedures are cumbersome and time-consuming because of the need to separate incorporated and free nucleotides by a series of filtration and washing steps.

Several spectroscopic assays have been introduced recently. These include a reverse transcriptase (RT) assay that uses 4',6-diamidino-2-phenylindole, the fluorescence of which is enhanced by binding to poly(A)-poly(dT) during the course of the reaction (1). The fluorescence of this dye is unchanged in the presence of double-stranded DNA (dsDNA) and, therefore, cannot be used to assay conventional DNA-dependent DNA polymerases (1).

To detect transient intermediates in the reactions catalyzed by E. coli DNA polymerase I and T4 DNA polymerase, Frey et al. (3) have used changes in the fluorescence of the base analog 2-aminopurine upon incorporation into DNA. In a different approach, Griep (4) has recently described a fluorometric assay for E. coli DNA polymerase III holoenzyme based on changes in the fluorescence of single-stranded (ss) DNA-binding protein (SSB) during DNA replication. However, this assay is limited to systems requiring SSB.

The fluorescence of the recently introduced dye PicoGreen™ is enhanced specifically upon binding to dsDNA, and it is relatively nonfluorescent in the presence of ssDNA. Here, we report a quick, easy, sensitive and inexpensive fluorometric assay, utilizing PicoGreen, that can be used to monitor the synthesis of both dsDNA and DNA-RNA hybrids and is, therefore, applicable to a wide range of DNA polymerases and reverse transcriptases.

MATERIALS AND METHODS

E. coli DNA polymerase III holoenzyme (pol III holoenzyme), human immunodeficiency virus (HIV) RT, dnaG primase, SSB and M13 Gori DNA are commercially available from Enzyco (Denver, CO, USA). PicoGreen was obtained from Molecular Probes (Eugene, OR, USA). Synthetic poly nucleotides were obtained from Pharmacia Biotech (Piscataway, NJ, USA).

Fluorescence Instrumentation

Fluorescence measurements were made using either an SLM Model 48000 fluorometer (SLM Instruments, Urbana, IL, USA) or an SLT FluoroStar microplate fluorometer (SLT Instruments, Research Triangle Park, NC, USA). The SLM fluorometer was configured with excitation and emission wavelengths of 500 and 526 nm, respectively. The slit widths were varied between 4 and 16 nm. All measurements were performed at room temperature except where noted. The SLT microplate fluorometer was equipped with 485 and 538 nm interference filters on excitation and emission, respectively.

Solutions

Pol III holoenzyme primer-template solution: 60 mM HEPEs, pH 7.5, 14 mM magnesium acetate, 2.8 mM ATP, GTP, CTP and UTP, 14%