INADVERTENT EXPRESSION OF DENGUE 2 VIRUS NS3-EGFP FUSION PROTEIN IN *ESCHERICHIA COLI* USING THE pEGFP-N1 MAMMALIAN EXPRESSION VECTOR

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ABSTRACT: Dengue 2 New Guinea C (NGC) virus NS3 protein, a potentially important virulence factor was cloned to the N-terminus of the *Aequorea victoria* enhanced green fluorescent protein (EGFP) using the pEGFP-N1 mammalian expression vector. During amplification of the recombinant plasmid in *E. coli*, transformants expressing the EGFP were detected *in vivo* when viewed using fluorescence microscopy. This inadvertent expression of the recombinant fusion protein was confirmed further by detection of the T7-Tag peptide cloned to the amino terminal of the fusion protein using T7-Tag specific monoclonal antibody. These findings represent perhaps the first reported expression of the T7-Tag-NS3-EGFP fusion protein using the pEGFP-N1 mammalian expression vector in *E. coli*. *(JUMMEC 1999; 1:41-46)*

KEYWORDS: Dengue, NS3, pEGFP-N1, fusion protein.

Introduction

The potential effects of specific virus genes on the host cells can be examined by cloning and expressing the genes within the host cells. The viral gene of interest can be cloned into a mammalian cell expression vector which normally contains inducible or constitutive viral promoter at the 5' end of the gene. Often times, to enable detection of the cloned gene expression, specific peptide sequence or protein is inserted either at the 3' end of the cloned viral gene or immediately after the promoter at the 5' end of the gene. These peptide sequences or tag proteins can be detected by using specific monoclonal antibodies. Most commercially available mammalian cell expression systems allow cloning and amplification of the recombinant plasmid to be performed in bacterial cells prior to transfection of the host cells. In most cases, no expression of the cloned gene is expected in the bacteria since in most mammalian cell expression vectors no bacterial promoter is included immediately upstream of the multiple cloning sites.

In an effort to investigate the potential effects of dengue 2 virus NS3 gene on the host cells, the virus gene was cloned into a mammalian expression vector, pEGFP-N1 (Clontech, CA, USA). This expression vector contains a strong constitutive CMV promoter for eucaryotic cell expression and no bacterial promoter immediately upstream of the multiple cloning sites. In addition, to facilitate detection of the cloned gene expression in transfected host cells, the dengue virus gene was cloned as a fusion partner to the EGFP protein which has been reported to fluoresce under ultraviolet light *in vivo* (1). It is reported here that during amplification of the recombinant T7-Tag-NS3-EGFP plasmid (pT7-Tag-NS3-EGFP), inadvertent expression of the cloned fusion protein in *E. coli* was detectable *in vivo* using fluorescence microscopy and by immunodetection method.

Materials and Methods

Virus infection and preparation of infected cells RNA

Dengue 2 New Guinea C (NGC) strain virus obtained from the American Type Culture Collection (ATCC, USA) was used in this investigation. Virus was propagated in the C6/36 mosquito cells as previously described (2). Briefly, semi-confluent cells (~ 70%) were infected with dengue 2 virus inoculum to give an estimated multiplicity of infection (MOI) of about 3 to 5 plaque forming unit (PFU) per cell. Approximately 7 to 8 days post-infection (PI) or when more than 90% of the infected cells have shown the cytopathic effects (CPE), cell cultures were frozen at -70°C. Crude virus inoculum was prepared by sequential centrifugation of the supernatant at 800 and 40,000 x g. The titer of the

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virus inoculum was determined by performing virus plaque assays using porcine spleen cells. Virus infection for RNA isolation was done as described above. Infected cells were harvested approximately 7 to 8 days PI and total RNA was isolated using the TRIzol™ Reagent (GIBCO BRL, Life Technologies Inc., USA) following protocols provided by the manufacturer.

Cloning of the NS3

Dengue 2 virus NS3 gene was amplified by the RT-PCR and cloned into the pGEM-T cloning vector (Promega Corp., Wisconsin, USA). It was noted, however, that the full length NS3 gene sequence was perhaps 'toxic' to the bacterial cells since no transformants were obtained after repeated attempts. To overcome this problem, the gene was initially cloned in two halves into the pET-23a(+) vector (Novagen, USA). The NS3 gene was amplified by the PCR as two separate segments using 2 sets of primers; NS3FXA (5'GGATCCATCGAAGGGCGCGCCGGAGTATTGT GGGATGTCCCTTCACCCCCACC) and NS3XR (5'CCATCTCTACTCGAGTTGAGATGTAT CCTCTAGC) which amplified the 5'-half of the NS3 fragment beginning at nucleotide number 4521 until 5440 (3), whereas, NS3NF (5'GCTAGCTAGAGGATA CATCTCAACTCGAGTAGAGATGG) and NS3NtR (5'GCGGCCGCCTTTCTTCCAGCTGCAAACTCC TTGAATTCC) which amplified the 3'-half of the NS3 beginning at nucleotide number 5409 until 6375. The two amplified fragments containing the NS3 *Xho* I restriction sites were then cloned separately into the pGEM-T cloning vector using the vector's T overhangs.

Construction of NS3-EGFP recombinant plasmids

Plasmids consisting of the 5'- and 3'-half of the NS3 were digested separately using the *BamH* I-*Xho* I and *Xho* I-*Not* I restriction endonucleases respectively. The resulting 3'-half (975 bp) and the 5'-half (919 bp) of the NS3 fragments were then ligated to the pET-23a(+) vector (Novagen, USA) which had been digested with *BamH* I and *Not* I, using three fragments ligation mixtures. The recombinant plasmids were then amplified, purified and the recombinant gene insert was excised using *Xba* I and *Not* I restriction enzymes. These restriction sites were chosen to enable ligation of the recombinant gene into the pEGFP-N1 vector using the compatible cohesive ends of the *Nhe* I and *Bsp* 120 I restriction sites of the expression vector. The excised fragment beginning at position 166 until position 276 of the pET-23a(+) vector consisted of a ribosome binding site (rbs) and a T7·Tag sequence at the 5' end which was intended to be used for detection of the fusion gene expression. The pT7·Tag-NS3-EGFP plasmid was then transformed into the Top10F' bacteria and cultured on LB/kanamycin plates for overnight at 37°C

Preparation of bacterial lysates and detection of the T7·Tag fusion protein

Detection of expressed T7·Tag-NS3-EGFP fusion protein was made by immunodetection using T7·Tag specific monoclonal antibody (Novagen, USA). Recombinant transformants were cultured overnight and then centrifuged at 10,000 x g for 3 min. The resulting pellets were resuspended in 1X sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue) and boiled for 7 min. Samples were then recentrifuged at 15,000 x g for 5 min, electrophoresed in 12.5% SDS-PAGE, and electrotransferred onto nitrocellulose membrane (MSI, Westborough, MA, USA). The membrane was blocked using blocking buffer (100 mM Tris base, pH 7.5; 150 mM NaCl) containing 5% skim milk and then developed using specific monoclonal antibody and conjugates as previously described (2).

Results and Discussion

Transformation of the Top10F' *E. coli* with the pT7·Tag-NS3-EGFP resulted in mixed transformants of large and small bacterial colonies (Figure 1). Since it was suggested earlier that the presence of the NS3 gene sequence was perhaps toxic to certain bacteria, it was possible

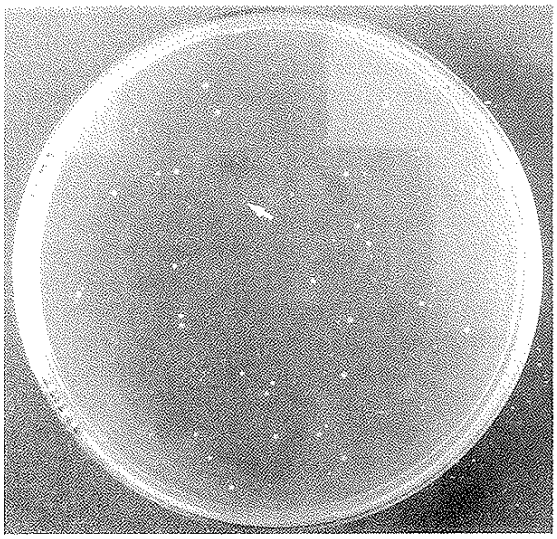

Figure 1. E. coli Top10F' transformed with pEGFP-N1 plasmid containing the T7·Tag-NS3 gene insert. Transformants consisted of a mixture of small (arrow) and big colonies.

that the resulting small colonies were also due to the toxic effects of the gene. To investigate this possibility, the presence of the gene insert was determined by screening the colonies with *BamH* I restriction enzyme which digests the 5' and 3' ends of the cloned NS3 gene

to yield a fragment of approximately 1960 bp. It was found that only the small colonies contained the expected gene insert (Figure 2). When the transformants were viewed under a fluorescence microscope (Zeiss Axiolab, Germany), only bacteria from the small colonies appeared to be fluorescent green (Figure 3a), suggesting that the EGFP protein was expressed. Transformants which formed big colonies did not have the insert or show the presence of fluorescing bacteria. *In vivo* expression of the EGFP was not expected since no bacterial promoter was cloned immediately upstream of the T7·Tag sequence of the pT7·Tag-NS3-EGFP. Nonetheless, this result suggested that the recombinant fusion protein was inadvertently expressed *in vivo*.

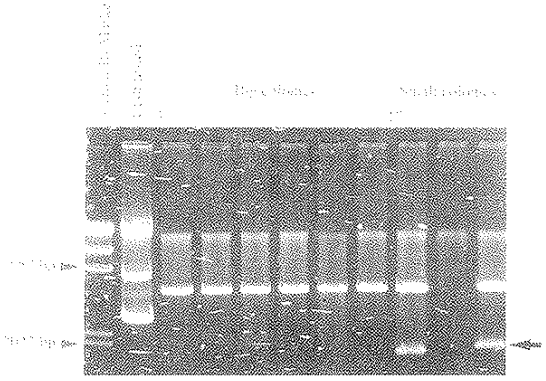

Figure 2. Restriction endonuclease digestion of plasmids from the small and big transformant colonies. Screening of the transformants were performed using *BamH* I enzyme. Arrow indicates the resulting NS3 fragments of about 1960 bp.

To confirm this possibility further, immunodetection was performed using monoclonal antibody specific against the T7·Tag peptide sequence, which was fused to the N-terminus of the NS3-EGFP. The monoclonal antibody was noted to recognize a polypeptide band of approximately 80 kD (Figure 4, Lanes 2-7) which was the predicted size of the T7·Tag-NS3 fusion protein. This polypeptide was present only in samples derived from the small colonies, suggesting that expression of the recombinant fusion protein somehow affects bacterial growth. A possible explanation for detection of only the 80 kD but not the expected full length T7·Tag-NS3-EGFP of about 102 kD is perhaps due to an intracellular cleavage of the fusion protein at a protease cleavage site (4) located at the carboxy end of the NS3. Nonetheless, since expression of the T7·Tag was detectable in the bacterial cell lysates and the bacteria also fluoresced when shown under ultraviolet light, it was apparent that the dengue 2 NS3 fusion protein cloned into the pEGFP-N1 eucaryotic expression vector was inadvertently expressed in *E. coli*.

The mechanisms leading to the expression of the fusion protein is not known. It is perhaps possible that the

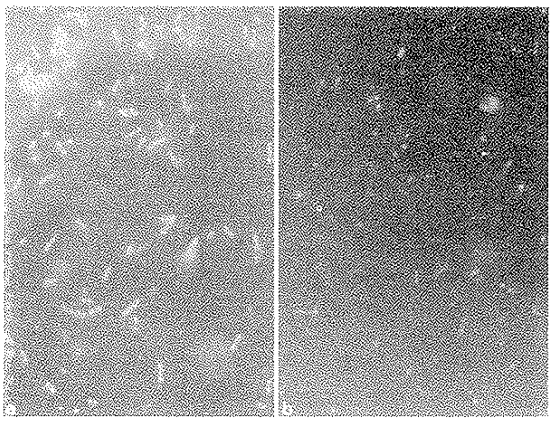

Figure 3. Expression of the pEGFP-N1 plasmid in *E. coli*. Bacterial cells transformed with the pEGFP-N1 plasmid carrying the T7·Tag-NS3 insert (a) and untransformed cells (b) were viewed with a Zeiss Axiolab fluorescence microscope using a single-pass filter set for FITC.

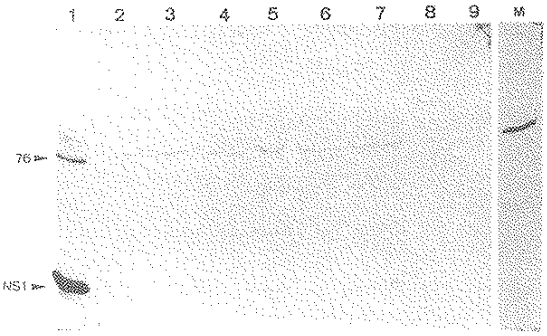

Figure 4. Detection of T7·Tag-NS3 by Western blot analysis using T7·Tag specific monoclonal antibody. T7·Tag-NS3 positive samples showed a band representing the expressed protein. Biotinylated protein marker (lane M), positive control for T7·Tag (lane 1) using T7·Tag-NS1 fusion protein which was cloned and expressed in the pET-23a(+). Positive clones (lanes 2-7), *E. coli* Top10F' transformed with pEGFP-N1 alone (lane 8), *E. coli* Top10F' (lane 9).

expression is mediated through the P_{amp} promoter which was the only bacterial promoter noted in the pEGFP-N1 plasmid. The presence of a ribosome binding site immediately upstream to the cloned fusion gene perhaps facilitates translation of the fusion protein. A possible immediate application of this finding is that it enables rapid screening of successful bacterial transformants during amplification of the recombinant plasmid. This will ensure that the cloned gene of interest has been cloned in frame to the EGFP and no stop codons were introduced prior to transfecting the recombinant plasmids into the mammalian host cells.

In summary, results presented here demonstrate inadvertent expression of genes cloned into a eucaryotic expression vector in *E. coli*.

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