Reports

Construction of a bicistronic vector for the co-expression of two genes in Caenorhabditis elegans using a newly identified IRES

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Keywords: Caenorhabditis elegans; IRES; bicistronic; vector

Supplementary material for this article is available at www.BioTechniques.com/article/113821

The nematode Caenorhabditis elegans is an important model animal for biological research. Currently, transgenic C. elegans strains are mainly generated by injecting DNA encoding a gene of interest, in combination with a reporter gene, into the gonad. With this approach, the interpretation of negative results, such as the failure to observe reporter expression, is frequently required. Single, selectable vectors are urgently required. Internal ribosome entry site (IRES) elements are known to bind the eukaryotic ribosomal translation initiation complex and independently promote translation initiation. Bioinformatic analysis predicted an IRES motif upstream of the start codon of the C. elegans Hsp-3 gene. While this sequence has a Y-shaped double-hairpin secondary structure characteristic of IRES elements, it was unclear if it could function as an IRES. In the present study, this predicted Hsp-3 IRES was incorporated into a bicistronic vector driven by the myo-3 promoter, which allowed co-expression of RFP and GFP genes in the muscle tissue of C. elegans and thereby demonstrated that this IRES element is functional. This vector provides a novel, powerful tool for C. elegans research.

Caenorhabditis elegans is an important model organism in many areas of biology, including development, cell biology, and neurobiology. An essential aspect of C. elegans research is the generation of transgenic lines. The most commonly used method for transformation is microinjection of two vectors, one encoding a gene of interest and the other a reporter gene, such as rol-6 (roller phenotype), into the gonad of a C. elegans hermaphrodite (1). However, there are several disadvantages to this method of engineering transgenic worms. First, it is difficult to control the ratio of the two different vectors for injection. In addition, various events can lead to preferential silencing, such that expression of the reporter gene cannot be observed (2). Single selectable vectors are therefore urgently needed. A large number of single plasmid selectable vectors from the Fire Vector Kit are widely used. These vectors involve fusing a gene of interest with the GFP reporter gene. However, this approach may disrupt the topology and function of the target protein or inhibit GFP fluorescence.

Within the past decade, many studies have reported the use of internal ribosome entry site (IRES)-dependent vectors in viruses, yeast, bacteria, and mammalian cells (3–6) for bicistronic expression of genes. IRES elements were originally identified in virus RNA and were found to hijack translation initiation in the host cell. They bind the eukaryotic ribosomal translation initiation complex and independently promote translation initiation (7). They have also been discovered in the 5′- untranslated regions of some eukaryotic mRNAs (8). The structural features of IRES elements remain largely unknown (8,9); however, Le and Maizel predicted a common IRES motif that has a Y-shaped double-hairpin structure and is found upstream of the start codon of the C. elegans gene Hsp-3 (10). There was, however, no experimental evidence for the functional role of this putative IRES.

The standard method of defining the activity of an IRES sequence is to test its ability to initiate translation from the second open reading frame (ORF) of a bicistronic construct. According to this premise, we constructed a bicistronic vector containing the predicted Hsp-3 IRES sequence and driven by the myo-3 promoter. We then examined whether the construct was able to co-express RFP and GFP in the muscle tissue of C. elegans.

Materials and methods

Plasmid construction

The predicted Hsp-3 IRES (M26604) was amplified from C. elegans genomic DNA using HiFi DNA polymerase (Transgen, Beijing, China) and the primers IRES-F: 5′-TGCTCTCCTTT-CACCACCTCCATCG-3′ and IRES-R: 5′-GCCCAATAAGAA-TAGGTCTTCGATA-3′. This fragment contains 285 bp upstream of the Hsp-3 start codon and was ligated into the T1 vector using the pEasy-TI Cloning kit (Transgen) (Figure 1, step 2) to generate sufficient material for subsequent manipulations. The entire RFP ORF was amplified from pDsRed1-C1 using the primers RFP-F: 5′-GAGCTCATGCGGCTCCTCCGAGAAGCGTACTCA-3′ and RFP-R: 5′-GGATCCATCCACAAGGGTTGCTGGGGCCCC-3′, which contain SacI and BamHI sites, respectively (shown in italics). The entire GFP open reading frame was amplified from L3786 using the primers GFP-F: 5′-GATCATGAGTAAAGGA-3′ and GFP-R: 5′-TCTAGATTACT-3′.
Vol. 52 | No. 3 | 2012

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Worm culture, transformation, and microscopy

Wild-type (wt) C. elegans strain N2 was maintained at 22°C on nematode growth medium (NGM) according to standard methods (11). Five to six worms were moved to a new plate before microinjection. The worms were injected with the pMyo-3:RFP-IRES-GFP::let858.3 PCR product with mixtures of pRF4 (carrying a dominant rol-6 allele) at a ratio of 1:4. Control plasmid 1 [pMyo-3::RFP-GFP::let858.3] and control plasmid 2 [pRF4::let858.3] were each injected as mixtures with pRF4 at the same concentration. Roller phenotype transgenic worms, whether exhibiting both RFP and GFP fluorescence or not, were selected using an Olympus IX71 microscope coupled with a Digital Processing Center (DPC) controller (Olympus, Tokyo, Japan).

Single Worm PCR

A single roller phenotype transgenic worm was picked into 10 µl worm lysis buffer and frozen in liquid nitrogen. The lysed worm was incubated at 65°C for 1 h and at 95°C for 10 min. PCR reactions were performed using RFP gene-specific primers RFP-F: ATGGCCTCCTCAGGG AGACCGTATCA and RFP-R: CTACAGGACAGGTGTTGACC GCCC and GFP gene-specific primers GFP-F: ATGAGTAAGAGAAGAACCTTTCCA and GFP-R: TTACCTGATGGCCCGTACCG to identify the specific genes in the worm.

Western blot analysis

One hundred roller phenotype worms were lysed in 1 × SDS-PAGE buffer. The worm lysates and Easysee Exposure Marker (Transgen) were separated by 12% SDS-PAGE and transferred to nitrocel-
lulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). After blocking with 5% (w/v) milk, membranes were incubated with a rabbit anti-GFP antibody (1:1000; Proteintech Group, Chicago, IL, USA) for 1 h at 37°C. After washing three times with PBS-Tween (PBS-T), membranes were incubated for 30 min with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000; MACGENE International, Beijing, China). After washing three times with PBS-T, membranes were incubated with ECL reagent (PerkinElmer, Waltham, MA, USA) for 1 min. Membranes were then exposed to X-ray film to develop the image. RFP was also detected using goat anti-RFP antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-goat antibody (1:2000; MACGENE International).

**Results and discussion**

In order to test if the predicted IRES sequence identified in the *Hsp-3* gene was functional, we constructed a bicistronic vector with an expression cassette consisting of the *myo-3* promoter, RFP gene, the putative IRES, GFP gene, and the let-858 3′ sequence. In transgenic worms carrying this *Pmyo-3::RFP::IRES::GFP::let858.3′* construct, RFP and GFP fluorescence was observed in muscle tissue (Figure 2 and Supplementary Movie S1, S2) in two independent transgenic lines. To verify the fluorescence seen in the transgenic worms was due to fluorescent protein (FP) expression and not autofluorescence, Western blot analysis was carried out and confirmed RFP and GFP expression in *Pmyo-3::RFP::IRES::GFP::let858.3′* transgenic worms, while no expression was evident in wt worms (Figure 3).
To confirm the IRES sequence is necessary for expression of the downstream GFP gene, we examined transgenic worms containing the control construct Pmyo-3:RFP-24ES(285bp)-GFP:let858.3′, in which the IRES was replaced with the *Hemodonchus contortus* 24ES sequence. In transgenic roller worms, RFP, but not GFP, fluorescence was observed (Figure 4A). Single worm PCR verified that both FP sequences were integrated in the Pmyo-3:RFP-24ES(285bp)-GFP:let858.3′ transgenic worms (data not shown). To confirm that the IRES sequence does not act as a cryptic promoter for the downstream GFP gene, we also injected the control construct RFP-IRES-GFP:let858.3′, which lacks the myo-3 promoter and failed to detect expression of either FP in the transgenic worms by fluorescence microscopy (Figure 4B) or Western blot analysis (data not shown). PCR verified that both FP sequences were integrated into the roller transgenic worms (data not shown). Two independent transgenic lines were analyzed for each of the two control constructs.

We did not closely examine the relative expression efficiency of the IRES-driven GFP compared with RFP in Pmyo-3:RFP-IRES-GFP:let858.3′ transgenic worms, making further studies necessary to determine how IRES affects relative expression efficiency.

In this study, Western blot analysis and microscopy observations demonstrated that the RFP and GFP genes, driven by the myo-3 promoter, can be co-expressed in muscle tissue from a bicistronic vector containing a predicted *C. elegans* IRES derived from *Hsp-3* (Bip) gene. The major advantage of the RFP-IRES-GFP plasmid is that it can solve the problem of gene silencing due to the interference between two plasmids. Furthermore, it avoids the use of multiple promoters in vectors of limited size. This vector can be used for the establishment of stable worm lines, and is applicable to biolistic bombardment protocols. Its ability to co-express genes can be used for specific applications, such as protein-protein interaction studies and immunization via co-expressed antigens and co-stimulatory proteins. Bicistronic vectors in *C. elegans*, based on the use of a spliced leader 2 sequence and trans-splicing, have also recently been described (12,13). However, IRES-dependent initiation of translation is potentially less complex than spliceosome-mediated RNA trans-splicing for bicistronic expression. Eukaryotic ribosomes have the intrinsic ability to bind mRNA in the absence of initiation factors and ATP (14). IRESs are descendants of splicesomal introns; therefore, IRES elements may be more efficient. In addition, RNA trans-splicing is site specific. An extrachromosomal array is formed following transformation in *C. elegans*, and the efficiency of trans-splicing may be different in each transformation because of the difference of the copy numbers and transmission rates among transgenic strains. Thus, our *C. elegans* IRES-dependent bicistronic vector offers a novel, powerful tool for *C. elegans* research.

Acknowledgments

We gratefully acknowledge support from the program for Chang Jiang Scholars, Innovative Research Teams in Chinese Universities (No.IRT0866) and the earmarked fund for Modern Agro-industry Technology Research System.

Competing interests

The authors declare no competing interests.

References


Received 16 July 2011; accepted 21 December 2011.

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