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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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'-TGCTCTCCCTT-CACCACTC-CCATCG-3' and IRES-R: 5'-GCCCA-ATAAGAA-TAAGGTCTTCATA-3'. This fragment contains 285 bp upstream of the *Hsp-3* start codon and was ligated into the T1 vector using the pEASY-T1 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'-GAGTCATGGCCTCCTC-CGAGAACGTCATCA-3' and RFP-R: 5'-GGATCCCTACAGGAACAGGTG-GTGGCGGCCC-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'-GATATCATGAGTAAAGGA-GAAGAACTTTTCA-3', which contains an *EcoRV* site, and GFP-R: 5'-TCTAGATTACT-

TGTATGGCCGGCTAGCGAAT-3', which contains *Xba*I and *Nhe*I sites to enable ligation into pPD118.25 (L3786). PCR was performed under the following reaction conditions: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and a final 7-min extension at 72°C. After digesting each of the purified products with the appropriate restriction enzymes, GFP and RFP sequences were ligated into pEasy-T1/IRES (Figure 1, step 3). pEasy-T1/RFP-IRES-GFP was then digested with *Kpn*I and *Nhe*I, and the RFP-IRES-GFP fragment was cloned into pPD118.25 (L3786), which contains an apparent internal enhancer from let-858, the upstream sequences of the let-858 translation start site, a multiple cloning site, a coding sequence for *gfp*, and the let-858 3' sequence (Figure 1, step 4). Since the GFP sequence in pPD118.25 contained an upstream reverse T7 promoter with a stop codon, it was replaced with the RFP-IRES-GFP cassette. The RFP-IRES-GFP-let858.3' fragment was amplified using primers sxRIGF: 5'-GAGCTCTCTA-GAATGGCCTCCTCCGAGAAC-3', which contains *Sac*I and *Xba*I sites to enable ligation of a *myo-3* tissue-specific promoter, and RIG3' R: 5'-TTAGATTTGGATT-GAATTAATTTT-3'. The PCR product was ligated into the pEasy T1 simple vector (Transgen). The *myo-3* promoter was cloned using the primers MyoF: 5'-GAGCTCTGT-GTGTGATTGCTTTTTCACAATC-3' and MyoR: 5'-TCTAGATGGATCTAGTG-GTCGTGGG-3', and the sequence was ligated into the T simple/RFP-IRES-GFP-let858.3' construct. A control plasmid was constructed where we replaced the IRES fragment with a filler sequence (285 bp) obtained by amplifying the *Hemonchus contortus* 24-kDa excretory/secretory protein (24ES; accession no. U64793.1, GenBank) sequence using primers 24ES-F: 5'-GGATCCTACGCTC-CTAAGGCAGCCAGGATGT-3' and 24ES-R: 5'-GATATCACTACACCA-CTCTACTGCGCATCCG-3' and then ligating the PCR fragment into T1 simple/Pmyo3::RFP-IRES-GFP:let858.3' using the *Bam*HI and *Eco*RV sites, which replaces the IRES fragment with 24ES. All cloning steps were verified by DNA sequencing.

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 Pmyo3::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 [Pmyo3::RFP-

Figure 1. Construction of plasmid Pmyo-3::RFP-IRES-GFP:let858.3'. (step 1) The *HSP-3* IRES was amplified from *C. elegans* genomic DNA. (step 2) The IRES sequence was ligated into pEasy T1 vector to create pEasy T1/IRES. (step 3) RFP and GFP genes were inserted into the pEasy T1/IRES vector. (step 4) RFP-IRES-GFP ligated into the L3786 expression vector pPD118.25. (step 5) RFP-IRES-GFP-let858.3' amplified and ligated into pEasy T1 simple vector to generate pEasy T1 simple/RFP-IRES-GFP-let858.3'. (step 6) *myo-3* promoter ligated into pEasy T1 simple/RFP-IRES-GFP-let858.3' to create pEasy T1 simple/Pmyo-3::RFP-IRES-GFP:let858.3'.

Figure 2. Transgenic worm expressing RFP and GFP from the Pmyo-3::RFP-IRES-GFP:let858.3' bicistronic vector. (A–D) Micrographs showing a transgenic worm in the same microscopic field under various conditions: (A) bright-field microscopy; (B) fluorescence microscopy with a filter to detect RFP; (C) fluorescence microscopy with a filter to detect GFP; and (D) merged view of panels B and C. Scale bar, 100 μm.

24ES(285 bp)-GFP::let858.3'] and control plasmid 2 (GFP-IRES-GFP::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: ATGGCCTCCTCCG AGA ACGTCATCA and RFP-R: CTACAG-GAACAGGTGGTGGCGGCCC and GFP gene-specific primers GFP-F: ATGAG-TAAAGGAGAAGA ACTTTTCA and GFP-R: TTA CT TGTATGGCCG-GCTAGCGAAT 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 lysate proteins and Easysee Exposure Marker (Transgen) were separated by SDS-PAGE on 12% gels and transferred to nitrocel-

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Figure 3. Western blot analysis of GFP and RFP expressed in transgenic worms from the Pmyo-3:RFP-IRES-GFP:let858.3' bicistronic vector. (A) Western blot analysis of GFP in transgenic worms. (B) Western blot analysis of RFP in transgenic worms. Lanes: 1, exposure marker (Transgen); 2, transgenic worms; 3, wild-type.

Figure 4. RFP and GFP expression in transgenic worms from the control vectors Pmyo:RFP-24ES(285bp)-GFP:let858.3' and RFP-IRES-GFP:let858.3'. (A) RFP is expressed in transgenic worms carrying Pmyo-3:RFP-24ES(285bp)-GFP:let858.3'. Note the absence of GFP expression. (B) No fluorescent protein expressed in transgenic worms carrying RFP-IRES-GFP:let858.3'. Scale bar, 200 μm.

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 *Hemonchus 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 spliceosomal 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.

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Competing interests

The authors declare no competing interests.

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