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Internal ribosome entry site (IRES) from Encephalomyocarditis virus (EMCV) as a tool for shuttle expression plasmids

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ABSTRACT

In eukaryotes, IRES sequences aid the recruitment of factors needed for translation to occur, enabling protein production independent of 5′ capped mRNA. Many patents and commercially available plasmids exploit their properties for polycistronic expression of recombinant proteins. However, these applications have been restricted to eukaryotic organisms, since it was thought that elements of this origin were essential for their activity. Here, using two tricistronic vectors designed for expression in mammalian hosts, we present evidence of EMCV IRES activity in prokaryotes. This finding enables the development of new and more versatile plasmid vectors for the production of recombinant proteins in multiple hosts from a single construct. Additionally, it provides new hints for the elaboration of alternative models describing the molecular mechanism of EMCV IRES mediated translation, in the absence of eukaryotic elements that were considered indispensable for its function.

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1. Introduction

Initiation of translation in eukaryotes requires scanning and anchorage of the ribosomal subunits to the 5′ cap of the mRNA chain. Nevertheless, sequences that give an organism the ability to begin translation from an internal sequence, known as internal ribosome entry site (IRES), have been described extensively in the scientific literature [1–8]. One of the most broadly used and first described IRES was derived from the Encephalomyocarditis virus (EMCV) [9]. Its RNA harbors IRES elements responsible for recruiting ribosomes onto an upstream region of the coding sequences, forming a stem-loop structure, which secondary and tertiary assemblies are critical for initiation of translation [10–13]. Thus, the incorporation of these sequences in between the Open Reading Frame (ORF) of a polycistronic cassette enables the expression of multiple recombinant proteins in eukaryotic cells.

Variants of EMCV IRES have been obtained by deletions or insertions, showing a decreased efficiency during protein translation, when compared to the wild type version [10,14]. However, also the host and the vector structure are determinant factors for the efficiency of IRES mediated translation [15–20].

Recent findings have demonstrated that IRES are able to form interactions with bacterial ribosomes, that even being weaker and transient compared with those that occur in eukaryotes, are capable of successfully driving protein translation [21].

Even though not all IRES sequences are able to drive production in eukaryotes in an ubiquitous manner [22] and that EMCV IRES it not capable of driving translation in insect cells [23], they have shown to be able of translating protein in prokaryotes. The data here presented indicates functionality of EMCV IRES in different *Escherichia coli* strains.

From the findings here shown, we propose the use of EMCV IRES as a tool for new shuttle-expression vectors, as the propagation and selection of the DNA sequence of interest in more than one host [24] is particularly important for the production of complex or yet unknown proteins, which very often are impossible to express or require further purification steps using simple systems such as *E. coli*, due to intrinsic limitations of the host [25,26]. Thus, more complex platforms such as yeast, insect cells, plant based systems or mammalian cells are necessary, demanding additional cloning steps in the quest for a more suitable vector for the new host. In that scenario, it would be convenient for the gene of interest to be

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cloned in plasmids designed not only for its replication and selection in multiple organisms, but also for its efficient transcription and translation in more than one host. We believe that this finding will also contribute to the development of new theories and models for the understanding of the molecular mechanism by which this sequence is able to drive mRNA translation without needing any eukaryotic factor.

2. Materials and methods

2.1. Bacterial strains and plasmids

BL21 (DE3) (New England Biolabs; Massachusetts, US), JM103 (ATCC; US), Rosetta Gami (Millipore; Massachusetts, US), SHuffle Express (New England Biolabs), Top10 (Invitrogen; New York, US) and Top10F (Invitrogen) *E. coli* strains were transformed using pG3 and pAPIS vectors.

pG3 plasmid encodes for eCFP*, mCherry and eGFP, with two IRES sequences alternated in between the fluorescent proteins (Fig. 1a). pAPIS plasmid has the same structure, and encodes for three different human transcription factors commonly used for induced pluripotent stem cells generation, each of them harboring a 6xHis-tag used for their detection via Western Blotting by a single antibody (Fig. 1b). Both plasmids were synthesized by DNA 2.0 (California, US) with human codon optimization (Supplementary Data S1 and S2).

The IRES sequences used in these constructs correspond to MPS1-ECAT and MPS1-ECAT374 as published by Jang et al. [10]. The fluorescent protein sequences are those published by Albagli-Curiel et al. [27].

Plasmids backbones are pTracer-CMV/Bsd (Invitrogen) and pJ609 (DNA2.0) for pAPIS and pG3 cassettes, respectively.

2.2. Cell growth and induction

Transformed bacteria were grown on Luria Bertani (LB) broth (Difco; Maryland, US) and LB agar (Difco) plates, containing 150 μg/mL of ampicillin. Liquid media cultures were incubated overnight at 37 °C with 250 RPM agitation. Solid media plates were incubated at the same temperature without agitation.

Unlike Top10 and Top10F, for JM103, BL21, Rosetta Gami and SHuffle strains, overnight liquid cultures were used as pre-inoculum for new cultures that were induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma–Aldrich; Missouri, US) when they reached an OD between 0.4 and 0.6. Induction was done for 16:30 h. In the case of solid media experiments, these strains were plated in LB agar plates with 150 μg/mL ampicillin and 1 mM IPTG.

After induction or just overnight growth (depending on the strain), liquid cultures were centrifuged for 20 min at 3200× *g* for pelleting. pG3 transformed bacteria grown in agar were incubated overnight at 4 °C to allow maturation of fluorescent proteins. Then, cells were collected using a spreader and resuspended in 600 μL of PBS, to be later centrifuged for 15 min at 13,000× *g* for protein extraction.

2.3. Protein extraction

Pellets were lysed using xTractor Buffer Kit (Clontech Laboratories; California, US) according to supplier instructions, and sonicated with 20 pulses of 5 s on level 7 using Model 100 sonicator (Fisher Scientific; Ontario, Canada), with 30 s ice incubation in between each pulse.

Cell lysates from pG3 were centrifuged at 17,000× *g* for 20 min at 4 °C. Supernatant was collected and used for the analysis.

pAPIS lysates were centrifuged for 3 min at 17000× *g* at 4 °C. Half of the supernatant was kept for total protein analysis (where total protein corresponds to the crude lysate) and the other half was treated to separate soluble and insoluble fractions. To obtain these two fractions, the total protein solution was centrifuged for 20 min at 17,000× *g* at 4 °C. Supernatant was kept as soluble fraction, and pellets (insoluble fraction) were washed twice with one volume PBS each time, to be later resuspended in 8 M Urea (Sigma–Aldrich). Total protein concentration of each sample was estimated using Nanodrop (Thermo Scientific; Massachusetts, US).

2.4. Fluorescence analysis

Fluorescent proteins expression was confirmed using H4 Synergy microplate reader (Biotek; Vermont, US). A sweep of excitation wavelengths from 420 to 560 nm was made, in order to include those used by Albagli-Curiel et al. [27]: 475 nm for eGFP, 546 nm for mCherry and for 436 nm eCFP, finding 434 nm the wavelength that enabled the visibility of the three peaks of interest, corresponding to each of the expected protein emission wavelengths when emission was read in between 440 nm and 660 nm. The emission wavelengths detected were 510 nm for eGFP, 610 nm for mCherry and for 470 nm eCFP [27].

Fluorescence microscopy analysis was performed on transformants grown in solid media, using an AXIO Scope.A1 microscope (Zeiss; Ontario, Canada). Filter set 69 (for eGFP) and filter set 43 (for mCherry) were used. Images were taken by Zen software, using a coupled AxioCam ICc 5 color camera.

2.5. SDS-PAGE

For lysates from pAPIS transformed bacteria, same amount of total protein samples, from untransformed and transformed strains, were ran in a 10% SDS-PAGE [28]. Running was done at 120 V for 1:40 h. Each well was loaded with 136.4 μg of the

Fig. 1. Expression cassettes. **a**. Expression cassette from G3 vector encoding eCFP*, eGFP and mCherry. IRES 1, MPS1-ECAT (wild type); IRES2, (MPS1-ECAT374) [10]. **b**. Expression cassette from pAPIS vector, encoding the transcription factors Oct4, Sox2 and Klf4. Each TF has a 6xHis-tag, allowing its detection via Western Blot, using the same antibody.

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