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Original Article

Analysis of the activity of virus internal ribosome entry site in silkworm *Bombyx mori*

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Internal ribosome entry site (IRES) has been widely used in genetic engineering; however, the application in silkworm (*Bombyx mori*) has hardly been reported. In this study, the biological activity of partial sequence of Encephalomyocarditis virus (EMCV) IRES, Rhopalosiphum padi virus (RhPV) IRES, and the hybrid of IRES of EMCV and RhPV were investigated in *Spodoptera frugiperda* (Sf9) cell line and silkworm tissues. The hybrid IRES of EMCV and RhPV showed more effective than EMCV IRES or RhPV IRES in promoting downstream gene expression in insect and silkworm. The activities of all IRESs in middle silk gland of silkworm were higher than those in the fat body and posterior silk gland. The hybrid IRES of EMCV and RhPV was integrated into silkworm genome by transgenic technology to test biological activity of IRES. Each of the positive transgenic individuals had significant expression of report gene *EGFP*. These results suggested that IRES has a potential to be used in the genetic engineering research of silkworm.

Keywords *Bombyx mori*; internal ribosome entry site; genetic engineering

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Introduction

The beginning of protein synthesis needs to assemble 80S ribosomes in the initiation codon of messenger RNA (mRNA) in eukaryotic cells. At present, there are two major mechanisms involved in recruiting ribosome. One is dependent on mRNA 5′-end cap structure, which is the main mechanism of protein synthesis in eukaryotes. The other relies on the internal ribosome entry site (IRES) to locate ribosome on the initiation codon AUG of mRNA or a nearby location to initiate protein translation, which does not need upstream non-translation sequences [1].

The exogenous proteins are often multimeric proteins that usually contain two or more different peptides, such as immunoglobulin [2] that consists of heavy and light chains. Therefore, multiple genes are often required to express in one cell to achieve their biological mission of expressing multimeric proteins. Although this can be done through co-infection of recombinant virus or plasmid vectors in a single gene, it is more efficient to load two expression frames to an individual vector than that in individual vectors with only one gene [3,4]. As a result, the IRES, independent of 5′-end cap structure translation mechanism, is widely used to construct polycistron expression vector in genetic engineering. To achieve the goal of translating multiple proteins from the same transcript simultaneously, it is important to ensure that their transcription levels are the same. This can be accomplished by multiple genes sharing the same promoter through the connection of IRES. In this way, the first exogenous gene is translated according to the classical capping way, yet the next exogenous genes are translated by non-add cap mechanism. Both mechanisms have been proved that their translations are synergetic rather than interfering with each other [5].

The IRES is originated from Encephalomyocarditis virus (EMCV) and functions well in vertebrate, such as mouse [6,7], and its mutant also has activity in COS cells that are derived from the cells of CV-1 (simian) in origin [8]. Similarly, the IRES derived from Rhopalosiphum padi virus (RhPV) works well in *Spodoptera frugiperda* (Sf9) cell lines (an insect cell line) [9] and has been used to construct the dual-expression gene vector systems of baculovirus [5].

To study the biological activity of IRES in silkworm (*Bombyx mori)* and develop its practical application in genetic engineering, we prepared three types of IRES by chemical synthesis and polymerase chain reaction (PCR) amplification. These IRESs were shown as: (i) a part of EMCV IRES, E-IRES; (ii) RhPV IRES, R-IRES; and (iii) the combination of E-IRES and R-IRES, RE-IRES. Then,

we compared their activities in promoting downstream gene expression in Sf9 cells and silkworm tissues. Our results may lay the foundation of its potential genetic application in silkworm.

Materials and Methods

Biological sample

Lan 10 (Chinese strain), the polyvoltine strain of silkworm, was preserved in our own laboratory. Cell line of Sf9 and dual-fluorescence plasmid pFastBacA3RLuc-FL650FLuc were kindly provided by Prof. Changde Lu (Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Shanghai, China). pB-4 and competent *Escherichia coli* DH10Bac [*EGT* gene had been knocked out from Autographa californica nuclear polyhedrosis virus (AcNPV) genome] were also preserved in Silkworm Genetic Laboratory [10,11].

Main reagent

Pyrobest *Taq* enzymes, restriction enzymes, T4 ligases, and pMD19-T were purchased from TaKaRa (Dalian, China). Agarose gel recovery kit, plasmids extraction kit, and ampicillin sodium were bought from Sangon (Shanghai, China). Plasmid transfection reagent Lipofectamine 2000 was obtained from Invitrogen (Eugene, USA).

Synthesis of IRES

The sequences of RE-IRES and partial sequence of E-IRES were produced by chemical synthesis (**Fig. 1**). Using RE-IRES as a template, R-IRES sequence was amplified by PCR using these primers (forward, 5′-CTCGAGGATAAAAGAACCTA-3′ and reverse, 5′-CCATGGTTATAAATAGATAAA-3′). The underlined sequences were restriction enzyme sites of *Xho*I and *Nco*I, respectively, which were designed for plasmids construction.

Figure 1 Sequences of IRES (A) Sequence of RE-IRES. The shaded area was the sequence of R-IRES and boxed sequence was E-IRES. (B) Partial sequence of E-IRES. The underlined sequences were restriction enzyme sites of *Xho*I and *Nco*I. Sequences in green were initiation codons and the red ones were termination codons.

Construction and identification of recombinant baculovirus Bacmid

A pFastBac donor plasmid was conserved in Silkworm Genetic Laboratory, which contains A3 cytoplasmic actin gene promoter, *Renilla* luciferase gene (*RLuc*), Firefly luciferase gene (*FLuc*), and SV40 3′-untranslated sequences. Three types of IRES (E-IRES, R-IRES, and RE-IRES) were inserted, respectively, into plasmid between *RLuc* and *FLuc* genes. As a result, *RLuc*, IRES, and *FLuc* were driven by A3 promoter. Then the competent *E. coli* DH10Bac was transfected with three types of purified recombinant plasmids. Finally, recombinant virus DNA was constructed by means of Bac-to-Bac system.

In this study, we designed specific primers in both sides of inserted site and used PCR to amplify nearby sequences of transposition locus to identify recombinant virus DNA. Transposition was regarded as available if the PCR products were identical to anticipated size. Primer sequences and their location in recombinant virus were shown in **Table 1** and **Fig. 2**.

Table 1 Primer sequences used for recombinant virus identification

Primers name	Primer sequences	Expectancy fragments size
Gmr	5′-GACCAAGTCAAATCCATG-3′	978 bp
M13F	5′-GTTTTCCCAGTCACGAC-3′	
FLuc	5′-GGCGGAAAGTCCAAA-3′	1500 bp
M13R	5′-CAGGAAACAGCTATGAC-3′	

Gmr and M13F were used for amplifying left arm of transposon; FLuc and M13R were used for amplifying right arm of transposon.

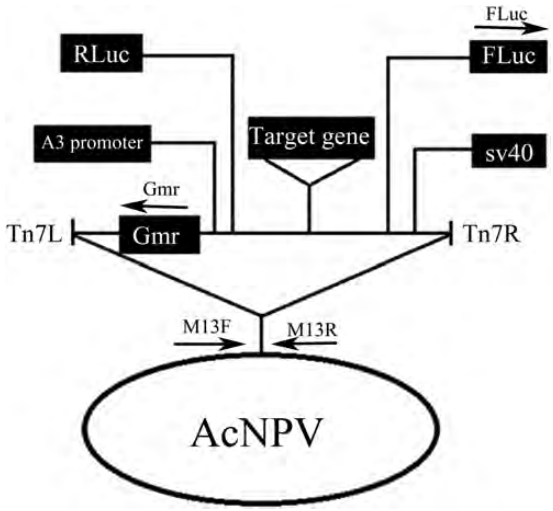

Figure 2 Structure chart of recombinant AcNPV Target gene: E-IRES, R-IRES, RE-IRES; Gmr, gentamicin antibiotic; Tn7L, left arm of pFastBac donor plasmid; Tn7R, right arm of pFastBac donor plasmid; M13F and M13R, primers for recombinant virus identify; arrows signaled position and direction of primers.

Cell transfection and fluorescence detection

One microliter of recombinant virus DNA was transfected in a six-well plate according to Lipofectamine 2000 transfection procedure. After being cultured for 3 days, cell supernatant including virus was collected, and then, a dual-luciferase detection kit (Promega, Madison, USA) was used to detect the activity of RLuc and FLuc.

Virus injection

Ten microliters (1×10^6 copies/μl) of virus suspension was injected into the abdomen of the silkworms from internode segments by microinjector after rearing silkworms for 24 h at the fifth instar stage. After 120 h post-microinjection, the silkworms were dissected and middle silk glands, posterior silk glands, and fat bodies were collected to detect dual-fluorescent activity using dual-luciferase detection kit, each group with three biological replicates.

Microinjection and screening of transgenic silkworms

Briefly, the microinjection and screening of transgenic silkworms were carried out according to Zhong *et al*. [12]. Eggs of silkworms were collected and microinjected within 8 h after eggs being laid at the syncytial pre-blastoderm stage. The concentration ratio between transposition plasmids and helper plasmids was 1 : 0.5 and the injection volume for each egg was about 15–20 μl of mixed plasmids (total concentration of DNA was 0.3 μg/μl).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Transgenic molting silkworms at the third instar stage were selected as samples. Each sample was grinded for 5 min on the ice with a ratio of 10 μl of phosphate buffer saline (containing 65 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 400 mM NaCl, pH 7.6) to 1 mg of silkworm sample using a motor-driven plastic pestle. After being centrifuged at 25,000 *g* at 25°C for 10 min, 100 μl of supernatant was collected and isometric sodium dodecyl sulfate (SDS) was added. Then, the mixture was boiled for 3 min and centrifuged at 25,000 *g* for 10 min before being subjected to SDS–polyacrylamide gel electrophoresis (PAGE) with stacking gel (5%) and resolving gel (12.5%).

Finally, LAS-3000 scanner and Image Reader LAS-3000 (V2.2) software (Fijifilm, Tokyo, Japan) were used to visualize the gel and analyze the data. The band spectrum analysis of green fluorescent protein was performed by using Multi Gauge-Image (V3.0) software (Fijifilm).

Results

Construction of double-fluorescent plasmids and transgenic plasmids

When three types of IRES were inserted into the site between FLuc and RLuc of double-fluorescent plasmid pFastBacA3RLuc-FL650Fluc, respectively, sequences of FL650 promoter (650 bp length of Fibroin Light-chain promoter) were deleted. Then the pFastBacA3RLucIRESFLuc plasmid was constructed [**Fig. 3**(**A**)]. As described in our previous report [11], the plasmid pBTrans-RE-IRES-EGFP-3 × P3-RFP was constructed on the basis of plasmid structure of pB-4 transposon [**Fig. 3**(**B**)]. The 3 × P3 promoter controlled the expression of red fluorescent protein (*RFP*) gene, which acted as a marker gene for screening positive individual of transgenic silkworms. The expression frame of trans-promoter-IRES-EGFP-SV40 was used for detecting the biological activity of IRES in promoting gene expression in silkworm genome. Trans-promoter was an eukaryotic promoter that can make RE-IRES recruit ribosomes for the *EGFP* expression. Since our vectors were randomly inserted into genome and non-coding region (a major part of genome), it was hard to make sure that the RE-IRES was close to the promoters in a transgenic silkworm.

SDS–PAGE result showed that functionally targeted protein had the same molecular weight with standard protein (**Fig. 4**). If the expression of EGFP was caused by the trans-promoter, the gene may be translated from the first or second initiation codon in RE-IRES (**Fig. 1**). However, the first translation pathway would be terminated by one of the seven TAA termination codons (sequences in red color), nine TAG, and one TGA termination codons (unmarked).

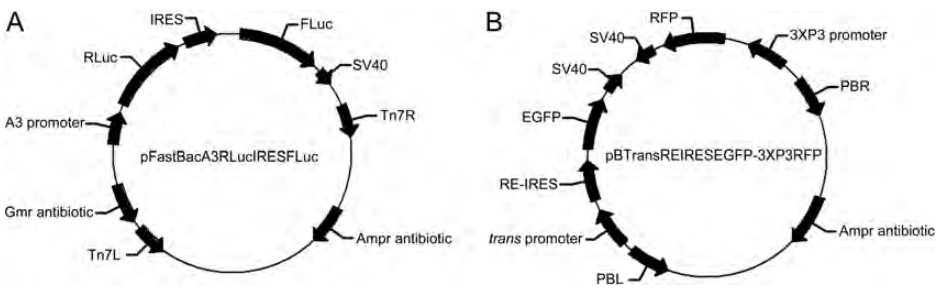

Figure 3 The plasmids structure chart of cell experiment and transgenic experiment (A) Plasmid for cell experiment. (B) Plasmid for silkworm transgenic experiment. RLuc, *Renilla* luciferase; FLuc, Firefly luciferase; A3 promoter, *B. mori* A3 cytoplasmic actin gene promoter; RFP, red fluorescent protein; SV40, 3′-untranslated sequences; PBL, *piggyBac* left arm; PBR, *piggyBac* right arm.

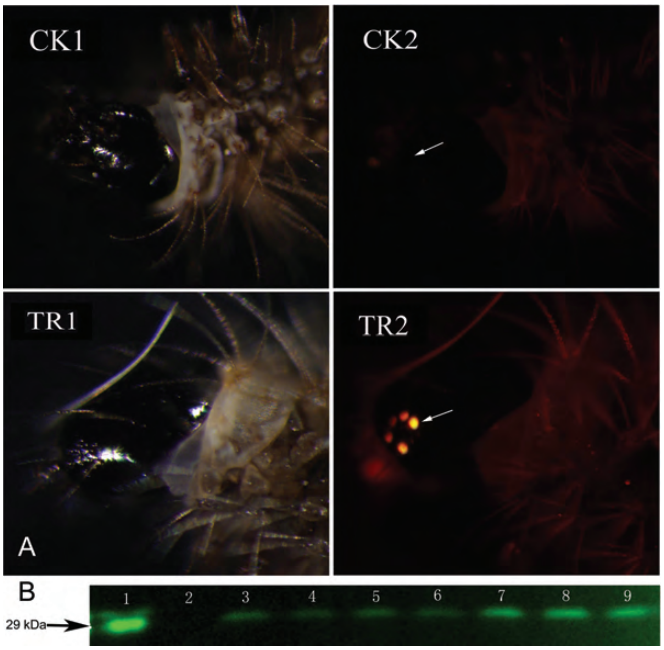

Figure 4 Silkworm transformation and SDS–PAGE identification (A) Red fluorescence detection results of transgenic silkworm. CK, the larva head of control silkworm; TR, the larva head of transgenic silkworm; CK1 and TR1, detection under white light; CK2 and TR2, detection under red fluorescence. (B) Results of SDS–PAGE and green fluorescence detection. 1: standard protein of EGFP (29 kDa); 2: non-transgenic silkworms (control); 3–9: positive individual of silkworms.

Moreover, the second translation pathway was similar to the first one. Therefore, it can be inferred that the EGFP protein could not be expressed in either the first or second translation pathway. Even if these translation pathways have not been terminated by those termination codons, 545 or 443 bp length sequence of RE-IRES (about 181 or 147 amino acids) would be translated with EGFP to a fusion protein, leading to a larger molecular weight of final product compared with standard protein. In addition, frame-shift mutation would deprive fusion proteins of green fluorescent activity. Therefore, the expression of *EGFP* protein may rely on RE-IRES, which recruits ribosomes to mRNA initiation codon or a nearby location to initiate protein translation. Results suggested that RE-IRES plays an important role in protein translation in silkworm.

Detection of IRES biological activities in cells

Recombinant virus was obtained by means of Bac-to-Bac system to detect the activity of double-fluorescent enzymes after transfecting sf9 cells for 72 h.

As shown in **Fig. 5**, E-IRES could not trigger translation of Fluc in insect cells but R-IRES worked well. Both fluorescent enzymes were successfully expressed without mutual interference. In addition, the new recombinant RE-IRES had a higher activity than that of single one (**Fig. 5**).

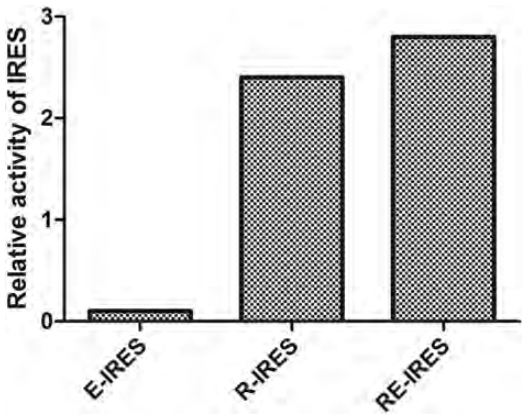

Figure 5 Relative activity of IRES in Sf9 cells (IRES-FLuc/A3-RLuc)

Detection of IRES biological activities in different silkworm tissues

To further assess the biological activities of IRES in different tissues of silkworms, virus suspension was injected into silkworm abdomen from internode segments. Middle silk glands and posterior silk glands as well as fat bodies were collected to examine double-fluorescent activity separately after silkworms were dissected. Results showed that E-IRES did not work in silkworm tissues, but RhPV-IRES worked well, especially RE-IRES, which showed the strongest activity (**Fig. 6**). Obviously, these results were further confirmed by cell experiments, which the activity of IRES in middle silk gland was the strongest, followed by fat body, and posterior silk gland, respectively.

Detection of IRES biological activity in transgenic silkworm

To detect the biological activity of IRES in silkworm genome, transgenic plasmid pBTrans-RE-IRES-EGFP-3 × P3RFP and helper plasmid were simultaneously injected into eggs of *Lan 10* strain. The survivors of G0 generation were hybridized with wild type of *Lan 10* moths. Positive silkworms from G1 generation were picked out by distinguishing red eyes [**Fig. 4**(**A**)], which account for 9.25%. This indicated that exogenous gene *RFP* had been integrated into silkworm genome and it could be stably inherited to the next generation. As trans-RE-IRES-EGFP-SV40 was closely linked with *RFP* gene, we speculated that RE-IRES-EGFP was also integrated into silkworm genome and steadily transferred to the offspring. Subsequently, we extracted soluble protein from seven RFP positive molting silkworms at the third instar stage for SDS–PAGE electrophoresis and green fluorescent protein examination. The results also revealed that each RFP positive silkworm had a same protein band of 29 kDa, which was exactly identical to the molecular weight of green fluorescent protein [**Fig. 4**(**B**)]. All those results collectively confirmed the above conclusions and testified that

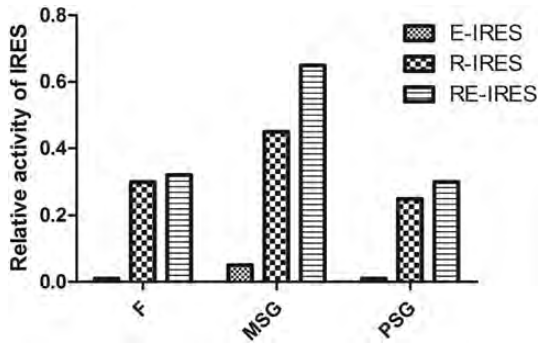

Figure 6 Relative activity of IRES in silkworm tissues (IRES-FLuc/A3-RLuc) F, fat body; MSG, middle silk gland; PSG, posterior silk gland.

RE-IRES function as a ribosomes recruiter to promote protein translation in silkworm genome.

Discussion

In the present study, we investigated biological activities of E-IRES, R-IRES, and RE-IRES in Sf9 cells and tissues of the silkworm. The results showed that IRES from different sources has different activities in diverse cell lines or organisms. This can be explained that the specific types and the number of trans-acting factors of IRES were diverse in cells; however, they are needed to activate translation activity in IRES-depended translation mechanism [13–15]. Also, the significant difference in the activity of IRES in silkworm tissues is likely due to the different susceptibility of those tissues to the baculovirus carrying IRES.

Polivirus and rhinovirus are hardly active in rabbit reticulocyte lysate, but their activities could be obviously improved when the salt-washed ribosomes of HeLa cells are added [16]. Likewise, cell-derived IRES shared the same characteristic. To date, it is revealed that the activity of c-Myc IRES sequence in HeLa cells is 20-fold higher than that in MCf7 cells, which is associated with different trans-acting factors in these two cell lines. However, the mechanism of trans-acting factors has still not been completely understood so far. They may act as RNA molecular partners changing or stabilizing the structure of IRES, or protein conjugate to combine proteins, such as ribosome subunits. Anyway, our results suggested that cytokines in insect cells and silkworms might suit for R-IRES rather E-IRES sequence. This is because cytokines might bind to IRES sequence and recruit ribosomes for protein translation by transforming IRES's spatial structure. However, it still needs further study.

During transgenic vector construction, the target gene is often connected with selective marker gene, which not only reduces the size of transgenic vector and improves transgenic efficiency, but also enhances the efficiency of transgenic screening [6]. It is also in this process that the different expression levels of exogenous gene in positive individuals are probably owing to the distinction of its insertion sites and copy numbers. However, this could be avoided by the use of IRES [17]. Notably, our data provide evidence that RE-IRES could be applied in silkworm transgenic bioreactors construction due to its high transgenic activity.

In addition, our results indicated that the coding frame mediated by R-IRES or RE-IRES could recruit ribosomes to initiate downstream protein translation and both the coding frames connected by IRES expressed smoothly after transcription of upstream promoter. The RE-IRES is more powerful in promoting downstream gene expression, and thus is greatly beneficial for future application in silkworm genetic engineering.

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References

1 Pelletier J and Sonenberg N. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 1988, 334: 320–325.
2 Hsu TA and Betenbaugh MJ. Coexpression of molecular chaperone BiP improves immunoglobulin solubility and IgG secretion from *Trichoplusia ni* insect cells. Biotechnol Prog 1997, 13: 96–104.
3 Bertolotti-ciarlet A, Ciarlet M, Crawford SE, Conner ME and Estes MK. Immunogenicity and protective efficacy of rotavirus 2/6-virus-like particles produced by a dual baculovirus expression vector and administered intramuscularly, intranasally, or orally to mice. Vaccine 2003, 21: 3885–3900.
4 Roy P, Mikhailov M and Bishop DH. Baculovirus multigene expression vectors and their use for understanding the assembly process of architecturally complex virus particles. Gene 1997, 190: 119–129.
5 Chen YJ, Chen WS and Wu TY. Development of a bi-cistronic baculovirus expression vector by the Rhopalosiphum padi virus 5′ internal ribosome entry site. Biochem Biophys Res Commun 2005, 335: 616–623.
6 Ghattas IR, Sanes JR and Majors JE. The encephalomyocarditis virus internal ribosome entry site allows efficient coexpression of two genes from a recombinant provirus in cultured cells and in embryos. Mol Cell Biol 1991, 11: 5848–5859.
7 Mountford P, Zevnik B, Duwel A, Nichols J, Li M, Dani C and Robertson M, *et al*. Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc Natl Acad Sci USA 1994, 91: 4303–4307.
8 Lindeberg J and Ebendal T. Use of an internal ribosome entry site for bicistronic expression of Cre recombinase or rtTA transactivator. Nucleic Acids Res 1999, 27: 1552–1554.
9 Domier LL and Mccoppin NK. *In vivo* activity of Rhopalosiphum padi virus internal ribosome entry sites. J Gen Virol 2003, 84: 415–419.
10 Guo TQ, Wang JY, Guo XY, Wang SP and Lu CD. Transient *in vivo* gene delivery to the silkworm *Bombyx mori* by EGT-null recombinant AcNPV using EGFP as a reporter. Arch Virol 2005, 150: 93–105.
11 Zhuang LF, Wei H, Lu CD and Zhong BX. The relationship between internal domain sequences of piggyBac and its transposition efficiency in BmN cells and *Bombyx mori*. Acta Biochim Biophys Sin 2010, 42: 426–431.

12 Zhong BX, Li J, Chen J, Ye J and Yu SD. Comparison of transformation efficiency of *piggy*Bac transposon among three different silkworm *Bombyx mori* strains. Acta Biochim Biophys Sin 2007, 39: 117–122.
13 Jang SK and Wimmer E. Cap-independent translation of encephalomyocarditis virus RNA: structural elements of the internal ribosomal entry site and involvement of a cellular 57-kDa RNA-binding protein. Genes Dev 1990, 4: 1560–1572.
14 King HA, Cobbold LC and Willis AE. The role of IRES trans-acting factors in regulating translation initiation. Biochem Soc Trans 2010, 38: 1581–1586.
15 Semler BL and Waterman ML. IRES-mediated pathways to polysomes: nuclear versus cytoplasmic routes. Trends Microbiol 2008, 16: 1–5.
16 Bushell M, Wood W, Carpenter G, Pain VM, Mmorley SJ and Clemens MJ. Disruption of the interaction of mammalian protein synthesis eukaryotic initiation factor 4B with the poly(A)-binding protein by caspase- and viral protease-mediated cleavages. J Biol Chem 2001, 276: 23922–23928.
17 Mountford PS and Smith AG. Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. Trends Genet 1995, 11: 179–184.