Chapter 9
Identification of Plant Virus IRES

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Abstrat Plant RNA viruses exploit nonorthodox strategies, such as the use of internal ribosomal entry sites (IRES), to express multiple genes from a single RNA species. IRES elements have been reported in tobacco etch virus (TEV), crucifer infecting tobamovirus (crTMV), hibiscus chlorotic ringspot virus (HCRSV), and many other animal and plant RNA viruses. In this chapter, the methodology used to identify and characterize a plant virus IRES element, including construction of a translation reporter vector for testing the IRES activity, testing the IRES activity in coupled in vitro transcription and translation systems and mammalian cells analysis of RNA stability, and sucrose gradient analysis and polysome profiling, is presented.

Keywords IRES; Plant virus; Translation; Gene expression

1 Introduction

In eukaryotes, translation initiation involves recruitment of 40S ribosomal subunits at either the 5′ m7G cap structure or internal ribosome entry sites (IRES). The ribosomal subunit together with other factors locates a start codon and protein synthesis begins following binding of the 60S ribosomal subunit, terminating at a stop codon (1, 2). To fully use their compact genomes, viruses have evolved various mechanisms either to redirect the translational machinery to favor viral transcripts or to regulate the expression of internal genes. Genome partitioning and the use of sgRNAs are common mechanisms used by many plant viruses to make their internal genes accessible for the ribosome (3). In addition, nonorthodox strategies such as the use of internal ribosomal entry sites (IRES) elements have been exploited by viruses to express multiple genes from a single RNA species. IRES elements are initially discovered in picornavirus RNAs to confer internal initiation independent of the 5' end (4, 5). Since then, functional IRES elements have been reported in various viral and cellular messenger RNAs (mRNAs). Cellular mRNAs that contain IRES elements encode a wide variety of

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proteins such as translation initiation factors, transcription factors, oncogenes, homoeotic genes, and growth factors (6). IRES elements have also been reported in plant viruses such as *Tobacco etch virus* (TEV) (7) and Crucifer infecting tobamovirus (crTMV) (8, 9).

In *Tobacco mosaic virus* (TMV) U1, only the 5' proximal gene of the gRNA is accessible to the ribosomes. An uncapped dicistronic sgRNA1 directs the expression of only the MP, while a capped monocistronic sgRNA2 is responsible for the expression of the CP (10). The gRNA of crTMV is able to direct the synthesis of CP in vitro. A 148-nt region preceding the CP of crTMV was tested in a bicistronic construct to contain an IRES element with relatively short and simple structure (8). IRES elements identified in crTMV have been reported to be active in rabbit reticulocyte lysate system (8) and have recently been demonstrated to function in yeast and HeLa cells (11).

IRES elements, most well studied in picornaviruses, share common features that are responsible for activity. Most of the known viral IRESs are located in 5' UTRs, highly structured and contain multiple conserved AUGs. Parts of the secondary structure of IRES elements associated with activity include sequences that form part of double-stranded regions (12, 13) or sequences located in apical or internal loops (14, 15). Disruptions of these regions have been associated with the modification of essential RNA–protein interactions (16). The GNRA tetraloop, an example of a conserved motif located at a distal loop in the central domain of *Foot and mouth disease virus* (FMDV) IRES, has been indicated to be involved in long range RNA interactions (14). Such RNA–RNA interactions, dependent on RNA concentration, ionic conditions, and temperature (17, 18), suggest dynamism in the tertiary structure of IRES that may play an important role in the IRES activity. Identification and characterization of a putative IRES element can be tested in wheat germ extract, rabbit reticulocyte lysate, and mammalian cells.

2 Materials

1. Acetic acid.
2. pBluescript®.
3. Coommasie blue R-250 (Sigma).
4. T7 RNA polymerase.
5. A bicistronic construct containing GFP gene as the 5' cistron and the envelope (E) protein gene of coronavirus infectious bronchitis virus (IBV) as the 3' cistron.
6. [α-^{32}P]UTP.
7. [^{35}S]methionine.
8. Phenol (pH 8).
9. 0.5 M NH_4OAc.
10. tRNA (10 mg mL^{-1}).

11. Chloroform.
12. 10–30% (w/v) linear sucrose gradient.
13. 25 mM Tris-HCl (pH 7.6).
14. 100 mM KCl.
15. 5 mM $MgCl_2$.
16. Ultrahigh speed centrifuge and rotors.
17. Absolute ethanol.
18. 1% formaldehyde agarose gel.
19. Cos-7 cells.
20. β-tubulin antibody.
21. Coupled in vitro transcription translation(TnT) kit (Promega).
22. 30 °C heat block.
23. Complete Dulbecco's modified Eagles medium (Gibco Life Technologies).
24. 10% bovine calf serum (Hyclone).
25. 1% penicillin/streptomycin (Gibco Life Technologies).
26. X-ray film and cassette.
27. X-ray film developer.
28. 0.1% SDS (v/v).
29. Recombinant vaccinia virus (vTF7–3).
30. DOTAP transfection reagent (Roche).
31. Humidified 5% CO_2 incubator.
32. GS-710 calibrated imaging densitometer (Bio-Rad).
33. Molecular Analyst computer software (Bio-Rad).
34. 1X Laemmli's sample buffer; 24 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 20% glycerol.
35. Protein gel running buffer: 2.88% glycine (w/v), 0.6% Tris-HCI, and 0.1% SDS (v/v).
36. Amplify™ (Amersham Pharmacia Biotech).
37. Nitrocellulose membrane (Stratagene).
38. Semidry transfer cell (Bio-Rad Trans-Blot SD).
39. Western blot blocking buffer: 5% skim milk powder in TBST including 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20.
40. IgG conjugated with horseradish peroxidase (Dako).
41. Chemiluminiscence detection kit (ECL, Amersham Pharmacia Biotech).

3 Methods (see notes 1–4)

3.1 Coupled In Vitro Transcription and Translation (see note 5)

1. Plasmid DNAs (5 μg) are linearized and extracted by phenol/chloroform and ethanol precipitated before adding to the 50 μL reaction mix containing the following components:

1. Linearized DNA 2 μg
2. 5X transcription buffer (Promega) 10 μL
3. 100 mM DTT 5 μL
4. NTPs (2.5 mM each of ATP, GTP, CTP, TTP) 10 μL
5. RNase inhibitor (40 U μL^{-1}) 0.5 μL
6. T7 RNA polymerase 1 μL
7. Sterile water to 50 μL

Incubate the reaction mix at 37 °C for 1 h.
Check the integrity of the transcripts by agarose gel electrophoresis.

2. In vitro translation reaction includes the following components:

8. 1 μg RNA template (after in vitro transcription) 2 μL
9. Wheat germ extracts 35 μL
10. Minus methionine amino acid mixture 1 μL
11. 10 mM methionine 1 μL
12. RNase inhibitor (40 U μL^{-1}) 1 μL
13. 50 μCi of [^{35}S] methionine 1 μL
14. Add sterile water to 50 μL

Incubate the reaction at 30 °C for 1 h.

3. Reaction products are separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

3.2 Transient Expression of Constructs in Cos-7 Cells

1. Culture Cos-7 cells (ATCC-CRL-1651) in complete Dulbecco's modified Eagles medium (Gibco Life Technologies) supplemented with 10% bovine calf serum (Hyclone) and 1% penicillin/streptomycin (Gibco Life Technologies) at 37 °C in humidified 5% CO_2 incubator.
2. Infect Cos-7 cells with 10 PFU/cell of recombinant vaccinia virus (vTF7–3) for 2 h at 37 °C prior to transfection.
3. Transfect the vTF7–3-infected cells with the plasmids (see note 6) using DOTAP transfection reagent (Roche).
4. After 20–24 h post-infection, harvest the cells.
5. Lyse transfected Cos-7 cells
6. Mix total proteins with 2X SDS loading buffer and subjected to SDS-PAGE.

3.3 Analyses of Protein Products using SDS-PAGE

1. Discontinuous polyacrylamide gel electrophoresis system (19) is used. Separating gels of different concentrations (12.5, 15, or 17.5%) and 3% stacking gels are cast between two glass plates.

2. Typically, an aliquot of the translation reaction is added to the 1X Laemmli's sample buffer and boiled at 100 °C for 2 min and cooled on ice before loading on a SDS-polyacrylamide gel.
3. In a reservoir of protein gel running buffer, gels are run vertically at 20 mA until the bromophenol blue dye reached the bottom of the gel.
4. After electrophoresis, gels containing [^{35}S]-methionine are fixed in 50% methanol and 10% acetic acid for 30 min.
5. The signal is enhanced with the use of Amplify™ (Amersham Pharmacia Biotech) for 15 min.
6. Gels are dried under vacuum at 80 °C for 1 h and later exposed to X-ray film (Biomax, Kodak) for autoradiography at −80 °C overnight.
7. Gels containing unlabeled polypeptides are stained in 50% methanol, 10% acetic acid and 0.05% coommasie blue R-250 (Sigma) for 30 min at RT°C and destained in 50% methanol, 10% acetic acid before drying.

3.4 Western Blotting

1. After SDS-PAGE, proteins are transferred to nitrocellulose membrane (Stratagene) by a semidry transfer cell (Bio-Rad Trans-Blot SD) and blocked overnight at 4 °C in blocking buffer.
2. The membrane is incubated for 2 h at room temperature in a dilution (1:1000) of a specific antiserum against the reporter gene in blocking buffer.
3. After three washes for 15 min each with TBST, the membrane was blocked for 20 min before it is incubated with either antirabbit or antimouse IgG conjugated with horseradish peroxidase (Dako) diluted 1:2500 in blocking buffer for 1 h at room temperature.
4. After three washes with TBST, the membrane was treated using a chemiluminiscence detection kit (ECL, Amersham Pharmacia Biotech) accordingly.

3.5 Densitometry

1. The intensities of the protein bands are measured by a GS-710 calibrated imaging densitometer (Bio-Rad) and analyzed using Molecular Analyst computer software (Bio-Rad).
2. The protein band of interest is normalized accordingly to the intensities of the corresponding p28 band or a consistent background band.

3.6 RNA Stability Test (see note 7)

1. Equal amounts of templates are linearized for in vitro transcription in the presence of [α-^{32}P]UTP.

2. Equal amounts of the labeled RNA (2 μL of the 25 μL reaction) are added directly into the in vitro translation reaction mixture. At specific time intervals, a portion (5 μL) is withdrawn from each translation reaction mixture.
3. Prior to adding 100 μL of phenol (pH 8), 94 μL 0.5 M NH_4OAc and 1 μL tRNA (10 mg mL^{-1}) are added to the reaction mixture. The sample is vortexed and centrifuged at 20,000 g for 5 min.
4. The supernatant is added to 100 μL chloroform, vortexed and centrifuged at 5,000 g for 5 min.
5. The RNA is precipitated by transferring the supernatant into 300 μL absolute ethanol and placed in −80 °C for 30 min before obtaining the pellet.
6. The extracted RNAs are resolved in a 1% agarose gel containing 0.1% SDS before autoradiography.

3.7 Sucrose Gradient Analysis and Polysome Profiling

The integrity of mRNA derived from a test construct is analyzed by sucrose gradient analysis as described by Pelletier and Sonenberg (5).

1. The DNA template is linearized for in vitro transcription in the presence of [α-^{32}P]UTP.
2. The in vitro synthesized RNA is extracted with phenol/chloroform, precipitated with ethanol, and incubated in a 50 μL translation reaction containing 35 μL of wheat germ extract at 30 °C for 10 min.
3. The translation mixture is cooled on ice and layered onto a 10–30% (w/v) linear sucrose gradient (2 mL) in buffer containing 25 mM Tris-HCl (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$. The gradient was then subjected to centrifugation at 259,000 g in an appropriate rotor at 4 °C for 60 min.
4. Fractions of 200 μL are collected from the top of the gradient and measured at absorbance 260 nm to obtain the polysome profile.
5. Based on the polysome profile, fractions are pooled and RNA extracted by phenol/chloroform and ethanol precipitation.
6. The transcripts are resolved on a 1% formaldehyde agarose gel and analyzed by autoradiography.

3.8 Functionality of the IRES Element in Mammalian Cells

To test if the plant virus IRES element is functional in mammalian cells, the GFP gene is cloned upstream of the IRES region in a bicistronic construct.

1. In a transient expression system (20), Cos-7 cells are infected with vaccinia virus, which possesses a T7 polymerase gene, and transfected with T7-promoter driven plasmid DNA.

2. The expression of GFP is detected in transfected cells by viewing under ultraviolet light.
3. The cells are harvested and western blot detection is performed using the expression of β-tubulin gene as a control to normalize the densitometry readings.
4. The expression of both GFP and reporter gene positioned after the IRES indicates that the IRES element s active in mammalian cells.
5. The IRES activity in Cos-7 cells is further analyzed by expression of deletion constructs.

4 Notes

1. Construction of a translation reporter vector. The vector can be a pBluescript® or any suitable plasmid that contains a T7-RNA polymerase promoter so that a full-length sequence containing a putative IRES element can be cloned into it for transcription. The transcribed RNA template can then be used for in vitro translation. A reporter gene (such as green fluorescent protein, GFP) at the 5' end is used as an internal control for the internal initiation event and the stability of RNA templates during translation.
2. The region containing the IRES element is inserted into a bicistronic construct containing GFP gene as the 5' cistron and the envelope (E) protein gene of coronavirus *Infectious bronchitis virus* (IBV) (21) as the 3' cistron, giving rise to construct pGFP-IRES-E.
3. A series of sequential deletions of the full-length sequence containing a putative IRES element is carried out to delineate the region responsible for internal initiation. When the expression of the reporter gene is observed to be reduced by several folds, it indicates that the IRES element has been deleted. Construct pGFP-E, containing the GFP gene and the E protein gene in different reading frames, are used as controls (22).
4. Expression of pGFP-E resulted in the detection of GFP. With the insertion of the putative IRES element inserted between the 5' and 3' cistrons, the GFP encoded by the 5' cistron and the E protein encoded by the 3' cistron were expressed.
5. To avoid repeated freezing and thawing of the coupled transcription and translation kit. It is advisable to aliquot the wheat germ extracts after the first usage.
6. Constructs containing deletions under the control of a T7 promoter are expressed transiently in semiconfluent monolayers of vTF7–3-infected Cos-7 cells.
7. To handle RNA with care so that it will not be degraded by RNase and affect the results.

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