Chapter 21
Probing Interactions Between Plant Virus Movement Proteins and Nucleic Acids

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Abstract Most plant viruses move between plant cells with the help of their movement proteins (MPs). MPs are multifunctional proteins, and one of their functions is almost invariably binding to nucleic acids. Presumably, the MP–nucleic acid interaction is directly involved in formation of nucleoprotein complexes that function as intermediates in the cell-to-cell transport of many plant viruses. Thus, when studying a viral MP, it is important to determine whether or not it binds nucleic acids, and to characterize the hallmark parameters of such binding, i.e., preference for single- or double-stranded nucleic acids and binding cooperativity and sequence specificity. Here, we present two major experimental approaches, native gel mobility shift assay and ultra violet (UV) light cross-linking, for detection and characterization of MP binding to DNA and RNA molecules. We also describe protocols for purification of recombinant viral MPs over-expressed in bacteria and production of different DNA and RNA probes for these binding assays.

Keywords Binding cooperativity; DNA; gel mobility shift; movement-protein–nucleic acid complexes; RNA; UV light cross-linking

1 Introduction

One of the general biochemical properties of many (and perhaps most) cell-to-cell movement proteins (MPs) of plant viruses is their ability to interact with nucleic acids. This protein activity makes biological sense because the main function of MPs is to transport the viral genome from the infected cell to the surrounding healthy cells, and the most direct way for MP to achieve this goal is simply to associate with the nucleic acid molecule and transport it through plasmodesmata. Since the ability to bind single-stranded (ss) RNA and DNA was originally demonstrated for MP of *tobacco mosaic virus* (TMV) (1), MPs from a large number of very diverse plant viruses, such as tobamoviruses, caulimoviruses, dianthoviruses, alfamoviruses, tospoviruses, umbraviruses, bromoviruses, cucumoviruses, fabaviruses, sobemoviruses, carmoviruses, necroviruses, tombusviruses, geminiviruses, hordeiviruses,

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potexviruses, pomoviruses, and luteoviruses, have been shown to exhibit nucleic acid-binding activities (reviewed in 2). Table 1 illustrates specific examples of viral MPs that bind nucleic acids and shows that most MPS exhibit the following four common characteristics of this binding: preference for single-stranded nucleic acids, comparable affinity toward ssRNA and ssDNA, cooperativity, and lack of sequence specificity. On the other hand, some viral MPs can also bind dsDNA and show preference for certain topological forms of the DNA molecules (Table 1). Furthermore, MPs of the viruses, such as *cowpea mosaic virus* (CPMV) (reviewed by 3), thought to move between cells exclusively as viral particles rather than as MP-viral genome complexes, have not been shown to possess nucleic acid-binding activities; instead, they may interact with the whole virions via MP-CP binding (4, 5).

Thus, when initiating a study of a plant virus MP, it is important to determine whether or not it interacts with nucleic acids and characterize the general parameters of this interaction. Here, we present protocols for biochemical assays that detect MP-nucleic acid-binding and define its hallmark features. It is important to note that, once the MP-nucleic acid-binding is demonstrated and initially characterized, additional studies can be performed that focus on detailed kinetic and structural aspects of this process. Although the methodology of such advanced studies is beyond the scope of this chapter, their experimental and conceptual approaches have been described and discussed in numerous papers, reviews, and monographs (e.g. 6, 7–11).

2 Materials

2.1 Equipment and Consumables

1. Environmentally controlled shaker (37 °C) for culturing *Escherichia coli*
2. Spectrophotometer for measuring optical density of bacterial cultures
3. Polymerase chain reaction (PCR) thermocycler for production of DNA probes
4. French press with a small (3 ml) chamber for breaking bacterial cells
5. 70 °C water bath
6. 56 °C water bath
7. Hot plate
8. Microfuge
9. 4 °C cold room or large refrigerator (cold box)
10. High speed centrifuge (e.g., Sorvall or Beckman)
11. Disposable 1–3 ml syringes with G26 needles
12. Dialysis tubing with 10 kDa molecular mass cut-off
13. Stir plates with stir bars
14. Vertical gel electrophoresis box, glass plates, and comb with 5-mm-wide teeth suitable for polyacrylamide gel electrophoresis (PAGE) (*see* note 1)
15. Horizontal gel electrophoresis box and comb with 5-mm wide teeth suitable for agarose gel electrophoresis
16. Power supply with leads

Table 1 Nucleic acid-binding properties of some plant viral MPs

Genus	Virus	MP	Binding to				Cooperativity	Sequence specificity	Reference
			ssRNA	ssDNA	dsRNA	dsDNA			
Alfamovirus	AMV	P3	++	++	–	–	+	No	(32–34)
Bromovirus	BMV	3a	++	++	–	–	++	No	(35, 36)
Caulimovirus	CaMV	P1	++	+	N.R.	–	++	No	(12, 37)
Carmovirus	TCV	p8	++	N.R.	N.R.	N.R.	++	No	(38, 39)
	CarMV	p7	++	N.R.	N.R.	N.R.	++	No	(40)
Cucumovirus	CMV	3a	+	+	–	–	+	No	(41–43)
Dianthovirus	RCNMV	35 kDa	++	++	N.R.	–	++	No	(44–46)
Fabavirus	BBWV-2	VP37	+	+	–	–	++	No	(47)
Geminivirus (bipartite)	SLCV	BV1/BC1	+/±	++/±	N.R.	–	N.R.	No	(48)
	BDMV	BV1/BC1	N.R.	++/–	N.R.	++/++	N.R.	2–9 kb open circles	(49)
Hordeivirus	PSLV	63 kDa TGBp1	++	N.R.	N.R.	N.R.	+	No	(50)
	BSMV	58 kDa TGBp1	++	–	++	–	N.R.	No	(51)
	BNYVV	42 kDa TGBp1	++	++	++	++	N.R.	No	(52)
Ilarvirus	PNRSV	32 kDa	++	–	±	–	++	No	(53)
Luteovirus	PLRV	17 kDa	++	++	N.R.	–	N.R.	No	(54)
Necrovirus	TNV	p7a	++	++.	N.R.	–	N.R.	N.R.	(55)
Pomovirus	PMTV	51 kDa TGBp1	++	N.R.	N.R.	N.R.	N.R.	No	(56)
		13 kDa TGBp2	++	N.R.	N.R.	N.R.	N.R.	No	(56)

(continued)

Table 1 (continued)

Genus	Virus	MP	Binding to ssRNA	Binding to ssDNA	Binding to dsRNA	Binding to dsDNA	Cooperativity	Sequence specificity	Reference
Potexvirus	PVX	25 kDa TGBp1	+	N.R.	N.R.	N.R.	+	No	(50, 57)
	BaMV		+	N.R.	N.R.	N.R.	N.R.	No	(58)
	FoMV	28 kDa TGBp1	++	N.R.	N.R.	N.R.	N.R.	No	(59)
	WClMV	26 kDa TGBp1 26 kDa TGBp1	++	N.R.	N.R.	N.R.	–	No	(60)
Sobemovirus	CoMV	P1	+	N.R.	N.R.	–	N.R.	No	(61)
Tobamovirus	TMV	30 kDa	++	++	N.R.	–	++	No	(1, 10)
	TVCV	29 kDa	++	N.R.	N.R.	N.R.	N.R.	No	(62)
Tombusvirus	TBSV	P22	++	N.R.	N.R.	N.R.	+	N.R.	(63)
Tospovirus	TSWV	NSm	++	N.R.	–	N.R.	N.R.	No	(64)
Umbravirus	GRV	ORF4	++	++	N.R.	–	–	No	(43)

++, binding comparable to that of TMV MP; +, binding weaker than that of TMV MP; ±, very weak binding; –, no binding; N.R., not reported. Plant virus genera are according to (65). *AMV*, Alfalfa mosaic virus; *BMV*, Brome mosaic virus; *CaMV*, Cauliflower mosaic virus; *TCV*, Turnip crinkle virus; *CarMV*, Carnation mottle virus; *CMV*, Cucumber mosaic virus; *RCNMV*, Red clover necrotic mosaic virus; *BBWV-2*, Broad bean wilt virus 2; *SLCV*, Squash leaf curl virus; *BDMV*, Bean dwarf mosaic virus; *PSLV*, Poa semilatent virus; *BSMV*, Barley stripe mosaic virus; *BNYVV*, Beet necrotic yellow vein virus; *PLRV*, Potato leaf roll virus; *PNRSV*, Prunus necrotic ringspot virus; *TNV*, Tobacco necrosis virus; *PMTV*, Potato mop-top virus; *PVX*, Potato virus X; *BaMV*, Bamboo mosaic virus; *FoMV*, Foxtail mosaic virus; *WClMV*, White clover mosaic virus; *CoMV*, Cocksfoot mottle virus; *TMV*, Tobacco mosaic virus; *TVCV*, Turnip vein clearing virus; *TBSV*, Tomato bushy stunt virus; *TSWV*, Tomato spotted wilt virus; *GRV*, Groundnut rosette virus. Modified from ref. (*2*); Copyright 2004 from *The Ins and Outs of Nondestructive Cell-to-Cell and Systemic Movement of Plant Viruses* by Waigmann, Ueki, Trutnyeva, and Citovsky. Reproduced by permission of Taylor & Francis Group, LLC., http://www.taylorandfrancis.com

17. Vacuum gel dryer
18. Blotting paper (Whatman)
19. UV light cross-linker (e.g., Stratalinker 1,800 from Stratagene, Inc.) or a germicidal UV light lamp
20. X-ray film, film cassette, and intensifying screen for autoradiography. Alternatively, a PhosphorImager with its cassette and intensifying screen can be used to reduce exposure time and facilitate digital acquirement of the image
21. CCD gel documentation system or Polaroid camera with a UV light table
22. NucTrap probe purification columns (Stratagene)
23. Ice buckets
24. Work space for handling radioactive isotopes

2.2 Media, Antibiotics, Buffers, Enzymes, and Other Chemicals

1. Double-distilled water (ddH2O), autoclaved
2. Stock solution of 0.5 M isopropyl-beta-d-thiogalactopyranoside (IPTG) in ddH2O (see note 2). Aliquot and store at −20 °C for up to 30 days
3. 1,000× stock solutions of antibiotics: 20 mg ml^{-1} kanamycin or 100 mg ml^{-1} ampicillin in ddH2O (see note 2) and 25 mg ml^{-1} solution of chloramphenicol in ethanol. Aliquot and store at −20 °C for 30 days
4. Stock solution of 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0. Autoclave and store at room temperature
5. Stock solution of 1 M tris (hydroxymethyl) aminomethane hydrochloride (Tris/HCl), pH 8.0. Autoclave and store at room temperature
6. Stock solution of 1 M dithiothreitol (DTT) stock solution in ddH2O. Aliquot and store at −20 °C
7. Stock solution of 1 M phenylmethanesulphonyl fluoride (PMSF) stock solution in methanol or dimethylsulfoxide (DMSO). Prepare fresh before use
8. Buffer L: 10 mM Tris/HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 10% glycerol. Prepare freshly before use. Add DTT and PMSF to 1 mM each immediately before use
9. Buffer L with 1 M NaCl. Prepare fresh before use
10. Buffer L with 1 M NaCl and 4 M urea (see note 3). Prepare fresh before use
11. Luria broth (LB) liquid medium: 5 g yeast extract, 10 g tryptone, and 10 g NaCl in one liter of ddH2O. Autoclave and store at room temperature
12. LB solid medium: same as LB liquid medium, only add 15 g l^{-1} agar before autoclaving. Store at room temperature
13. 2× yeast/tryptone (YT) liquid medium: 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 1 l of ddH_2O. Autoclave and store at room temperature
14. Agarose-molecular biology grade (Fisher Scientific or any other brand).
15. Ethidium bromide 10 mg ml^{-1} in ddH_2O. Filter through a Whatman paper and store at room temperature in the dark (can wrap the container in aluminum foil). It is carcinogenic, so exercise caution

16. Tris/Borate/EDTA (TBE) buffer 10× stock solution: mix 108 g Tris base, 55 g boric acid, and 40 ml 0.5 M EDTA; check pH which should be around 8.0. Autoclave and store at room temperature.
17. Loading buffer 5× stock solution for native PAGE and agarose gel electrophoresis: 50% glycerol, 0.04% bromophenol blue (tracking dye) in TBE. Store at 4 °C.
18. 20% sodium dodecyl (lauryl) sulfate (SDS) in ddH_2O. Autoclave and store at room temperature.
19. SDS PAGE 5× sample buffer: 10% SDS, 25% beta-mercaptoethanol, 50% glycerol, 300 mM Tris pH 6.8, 0.04% bromophenol blue. Store at 4 °C.
20. SDS PAGE 4× stacking gel buffer: 0.5 M Tris-HCl pH 6.8, 0.4% SDS. Store at 4 °C.
21. SDS PAGE 4× resolving gel buffer: 1.5 M Tris-HCl pH 8.8, 0.4% SDS. Store at 4 °C.
22. SDS PAGE 10× running buffer: 1.92 M glycine, 250 mM Tris base, 1% SDS. Store at 4 °C.
23. Acrylamide, 30% stock solution (acrylamide 28.2%/bis acrylamide 0.8%) in ddH_2O. Store at 4 °C. It is a neurotoxin, so exercise caution.
24. Acrylamide polymerizing reagent: 10% ammonium persulfate (APS) in ddH2O. Can store at 4 °C up to 1 week.
25. Acrylamide polymerizing reagent: *N*,*N*,*N′*,*N′*-tetramethylethylenediamine (TEMED) (Pierce).
26. Protein staining solution: 0.2% Coomassie brilliant blue R-250 (Fisher) in 35% methanol, 10% acetic acid in ddH_2O. Filter through a Whatman paper and store at room temperature. Can be reused 2–3 times.
27. Protein destaining solution: 10% methanol, 10% acetic acid in ddH_2O. Store at room temperature.
28. Protein molecular weight markers such as BlueRanger Prestained Protein Molecular Weight Marker Mix (Pierce).
29. Coomassie (Bradford) Protein Assay Kit (Pierce).
30. Forward and reverse PCR primers specific for the selected DNA probe.
31. Deoxyribonucleotide triphosphate (dNTP) stocks for PCR (2 mM each of the four dNTPs as individual stocks).
32. A dNTP radioactively labeled with α-^{32}P; for example [α-^{32}P]dATP or [α-^{32}P]dCTP (400 Ci mmol^{-1}) (Amersham or Perkin-Elmer).
33. ATP radioactively labeled with γ-^{32}P, i.e., [γ-^{32}P]ATP (3,000 Ci mmol^{-1}) (Amersham or Perkin-Elmer)
34. UTP radioactively labeled with α-^{32}P, i.e., [α-^{32}P]UTP (3,000 Ci mmol^{-1}) (Amersham or Perkin-Elmer)
35. *Taq* DNA polymerase (see note 4)
36. GFX PCR purification kit (Amersham) or PCR Purification Kit (Qiagen)
37. Bacteriophage T4 DNA polymerase (Promega or New England Biolabs)
38. Bacteriophage T4 polynucleotide kinase (Promega or New England Biolabs).
39. Bacteriophage T7 RNA polymerase (Promega or New England Biolabs) and 5 mM stocks of each of the four nucleotide triphosphates (NTPs) for in vitro transcription, or an in vitro transcription kit (see note 5).

40. Bacteriophage T4 gene 32 protein (United States Biochemical Corp. or New England Biolabs) and *E. coli* ssDNA-binding protein (United States Biochemical Corp.), two known ssDNA-binding proteins (6) for use as positive controls
41. Proteinase K (New England Biolabs)
42. Bacteriophage M13mp18 ssDNA and M13mp18 dsDNA (replicative form, RF) (New England Biolabs)
43. RNase A (Qiagen)

2.3 Bacterial Strains and Plasmids

1. *Escherichia coli* strains BL21(DE3), BL21(DE3)pLysS, or BL21(DE3)pLysE (see note 6). Can be purchased from Novagen.
2. A bacteriophage T7 RNA polymerase bacterial expression vector for expression of recombinant MP (e.g., pET series vectors, see note 7). Can be purchased from Novagen.
3. A vector for in vitro transcription of RNA probes from a bacteriophage T7 RNA polymerase promoter (e.g., pBluescript series vectors from Stratagene).

3 Methods

Studies of MP-nucleic acid interactions naturally begin with purification of MP, followed by production of DNA and RNA probes. As in many in vitro approaches, the preferred source of MP is a recombinant protein purified from bacteria. The major caveat in MP expression and purification is to develop a protocol that yields a reasonably soluble preparation. Here, we present such protocol developed for TMV MP; however, different viral MPs may require different approaches and specific modifications of the expression protocol. For probes, we use relatively short (100–800 nucleotides) radioactively-labeled ssDNA, dsDNA, and RNA as well as long (2,000–8,000 nucleotides) molecules. Because most MPs known to bind nucleic acids bind them without sequence specificity (see Table 1), the probes do not need to be of viral origin, whereas potential sequence specificity can be examined using competitor DNA and RNA derived from the viral genome.

Initial detection and characterization of MP-nucleic acid binding focuses on four goals: demonstration of the nucleic acid-binding activity, identification of the preferred binding substrate (i.e., DNA or RNA, single-stranded or double-stranded), determination whether the binding is cooperative or random, and investigation of sequence-specificity of the interaction. Technically, these objectives are most reliably addressed by two in vitro binding assays: native gel mobility shift (also called gel retardation and band shift) in which binding is detected by reduced electrophoretic mobility of protein–nucleic acid complexes, and ultra violet (UV) light cross-linking in which binding is detected as covalent attachment of protein to

cross-linked oligonucleotides (e.g., 1, 8, 10, 12). In addition, other techniques (not described here), such as nitrocellulose filter binding (13), coimmunoprecipitation (14), or electron microscopy visualization of the MP–nucleic acid complexes (10, 11), can occasionally be used.

3.1 Expression and Purification of Recombinant MP

1. Subclone the open reading frame of the MP to be tested into a bacteriophage T7 RNA polymerase bacterial expression vector (see note 7).
2. Freshly transform MP-expressing construct into BL21(DE3)pLysE cells (see note 8), plate on LB solid medium supplemented with the appropriate antibiotics (see notes 6, 7), and grow overnight at 37 °C.
3. Next day, inoculate a starter culture in 5 ml of LB liquid medium (see note 9) with the appropriate antibiotics. Grow for about 2 h at 37 °C with 250 rpm shaking.
4. Dilute the starter culture to optical density $A_{600} = 0.1$ into 20 ml of 2× YT liquid medium (see note 10) with the appropriate antibiotics. Grow for about 2 h at 37 °C with 250 rpm shaking until A_{600} reaches 0.7–1.0.
5. Add 20 µl 0.5 M IPTG (to final concentration of 0.5 mM) and let grow for another 3 h; centrifuge (10,000 g, 10 min, 4 °C) and save cell pellet. The pelleted cells can be processed immediately or stored at −70 °C for several months.
6. Resuspend cell pellet in 2 ml of buffer L, keep on ice. Break cells using French press in a 3-ml cell at 20,000 psi. Shear DNA by passing the lysate 4–5 times through a syringe with G26 needle. Transfer to 1.5-ml polypropylene microfuge tubes, centrifuge (12,000 g, 5 min, 4 °C), and save pellet (see note 11).
7. Resuspend the pellet in 1 ml of buffer L with 1 M NaCl by passing it 2–3 times through a syringe with G26 needle. Centrifuge as above. Save pellet (see note 11).
8. Resuspend as above in 0.5 ml of buffer L with 1 M NaCl and 4 M urea. Incubate for 10 min at 70 °C. Centrifuge as above. Save pellet (see note 12).
9. Resuspend as above in 1.5 ml buffer L with 1 M NaCl and 4 M urea and incubate for 15 min at 56 °C. Centrifuge as above. Save supernatant (see note 12).
10. Place the supernatant in a dialysis tube and dialyze for 2 h at 4 °C against 2 l of buffer L, then change buffer and dialyze for additional 2 h at 4 °C. Remove from the dialysis tube (see note 13), determine protein concentration (for example, using a Coomassie Protein Assay Kit), and aliquot. The purified MP can be assayed for the nucleic acid-binding activity immediately or stored at −70 °C for several months. This procedure yields 0.2–0.5 mg of purified MP. We also recommend to confirm the purity and the expected electrophoretic mobility of the isolated MP by SDS PAGE (15). Figure 1a illustrates such analysis of different stages of the TMV MP purification protocol, from total cell extract to the purified protein.

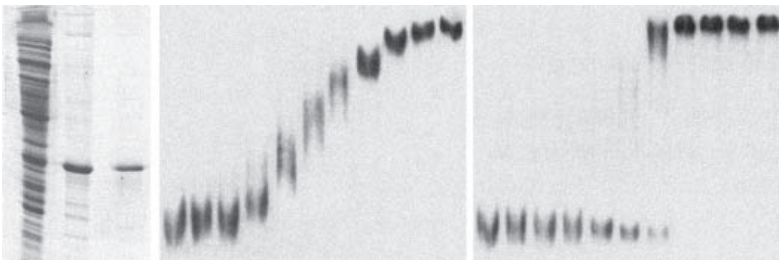

Fig. 1 SDS PAGE analysis of purified TMV MP and native polyacrylamide gel mobility shift analyses of low and high cooperativity of protein binding to ssDNA. (**a**) Purification of recombinant TMV MP. The protein was purified from inclusion bodies following over-expression from pPETP30 plasmid in *E. coli* (1) and analyzed on a 12.5% SDS polyacrylamide gel using Coomassie blue staining of protein bands. *Lane 1*, total bacterial cell lysate; *lane 2*, pellet fraction of the lysate containing TMV MP inclusion bodies; *lane 3*, purified TMV MP obtained by solubilization of protein aggregates. *Arrowhead* indicates the position of the 30 kDa TMV MP. (**b**) Low cooperativity binding of *E. coli* ssDNA binding protein to ssDNA. (**c**) High cooperativity binding of TMV MP to ssDNA. Radioactively labeled ssDNA probe (40 ng) was incubated with increasing amounts of protein followed by gel mobility shift assay on a 4% native polyacrylamide gel. *Lanes 1–11*, protein-to-ssDNA probe weight ratios 0:1, 0.5:1, 0.7:1, 1:1, 2.5:1, 5.0:1, 10:1, 20:1, 50:1, 75:1, and 100:1. *Arrowheads* indicate the positions of complete, fully saturated protein-ssDNA complexes and of the free ssDNA probe. All experimental conditions were as described in this chapter

3.2 Preparation and Labeling of Nucleic Acid Probes for Binding Assays

Interactions of MP with nucleic acids should be assayed using DNA and RNA probes. MP binding to nucleic acids can be assayed using shorter (70–1,000 nucleotides), radioactively end-labeled probes as well as long (2,000–7,000 nucleotides), unlabeled probes. The former are used in mobility shift assays on native polyacrylamide gels, while the latter are suitable for simple agarose gel electrophoresis (see note 14). Generally, DNA probes are prepared by PCR, but, because MP-DNA binding is often sequence nonspecific, it is possible to use commercially available ssDNA and dsDNA, such as genomic ssDNA and replicative dsDNA of bacteriophage M13mp18 (available, for example, from New England Biolabs), as probes for mobility shift assays on agarose gels. In the case of dsDNA preparations, they can be converted to ssDNA probes simply by separating the dsDNA strands following brief boiling and quick chill and storage on ice.

For very small probes, 70–100 nucleotide-long synthetic oligonucleotides can be used (10), completely eliminating the need for PCR-based preparation of the DNA probes. In this case, individual oligonucleotides serve as ssDNA probes, and dsDNA probes are made by annealing complementary oligonucleotides.

Similarly to binding to DNA, MP binding to RNA can be detected by native gel mobility shift assays. In addition, it can be easily analyzed by UV light cross-linking followed by SDS PAGE. Both assays utilize RNA probes generated by in vitro

transcription and labeled radioactively along their entire length by including a radioactive nucleotide in the transcription reaction.

3.2.1 Preparation of DNA Probes

1. Design15–17 nucleotide-long forward and reverse PCR primers specific for the selected DNA probe (e.g., viral genome for sequence-specific probes, or an unrelated plasmid for sequence nonspecific probes). Normally, the primers are designed to generate a 300–1,000-bp PCR product, but longer or shorter fragments are also suitable for use as probes.
2. Prepare a PCR cocktail with a total volume of 25 µl volume containing 100 ng template DNA, 0.2 mM of each of the four dNTPs, 0.2 µM of the forward primer, 0.2 µM of the reverse primer, and 2 U of *Taq* DNA polymerase with 2.5 µl of Taq 10× reaction buffer (see note 15).
3. Perform PCR with the following program of the thermocycler: 3-min denaturation at 94 °C, 30 cycles of 30 s at 94 °C and 2 min at 70 °C, and 2 min at 70 °C.
4. The PCR products are purified using the GFX PCR purification kit (Amersham) or PCR Purification Kit (Qiagen) to remove dNTPs, primers, and enzyme (see note 15).
5. Analyze a sample of the PCR products (1–2 µl) on an agarose gel, followed by ethidium bromide staining, to determine the yield of the amplification, verify the expected size of the PCR product, and estimate the amount of amplified DNA based. Calculate the latter based on the known amounts of DNA standards electrophoresed on the same gel.

3.2.2 Radioactive Labeling of DNA Probes

1. DsDNA probes produced by PCR are radioactively end-labeled using T4 DNA polymerase (see note 16) using standard molecular biology protocols (16, 17). Briefly, prepare a mixture with a total volume of 20 µl containing 1–2 µg DNA, 0.1 mM of each of the three dNTPs, 2 µCi of an aqueous solution of the fourth dNTP labeled with α-^{32}P, and 1 U μg^{-1} DNA of T4 DNA polymerase with 2 µl of T4 DNA polymerase 10× reaction buffer. Incubate for 10 min at 37 °C, and stop the reaction by heating for 5 min at 70–75 °C. This labeling reaction should yield probes with a specific activity of approximately 2×10^7 cpm μg^{-1} (see note 17). Purify the probe from unincorporated dNTPs using a NucTrap column.
2. Synthetic oligonucleotide probes are radioactively end-labeled using T4 polynucleotide kinase using standard molecular biology protocols (16, 17). Briefly, prepare a mixture with a total volume of 50 µl containing 1–2 µg oligonucleotide DNA, 150 µCi of an aqueous solution of [γ-^{32}P]ATP, and 20 Richardson units of T4 polynucleotide kinase with 5 µl of T4 polynucleotide kinase 10× reaction buffer. Incubate for 30 min at 37 °C, and stop the reaction by heating for 5–10 min at 70–75 °C. This labeling reaction should yield probes with a specific activity

of approximately 10^6 cpm μg^{-1} (see note 17). Purify the probe from unincorporated dNTPs using a NucTrap column.

3.2.3 Preparation of Radioactively-Labeled RNA Probes

1. Select a template for the RNA probe, which can be either a viral genome-specific fragment or an unrelated, nonspecific sequence, and subclone it under the bacteriophage T7 RNA polymerase promoter of an in vitro transcription vector (see note 18).
2. Linearize the template construct by cleavage with a restriction endonuclease at a site located immediately downstream of the probe template sequence.
3. To make RNA probes for gel mobility shift assay, prepare a mixture with a total volume of 20 µl containing 1–2 µg linearized template DNA, 0.5 mM of each of GTP, CTP, and ATP, 2.5 µM (15 µCi) of an aqueous solution of [α-^{32}P]UTP, 25 µM unlabeled UTP, and 20 U of T7 RNA polymerase with 2 µl of T7 RNA polymerase 10× reaction buffer (see note 19). Incubate for 1 h at 37 °C. This labeling reaction should yield probes with a specific activity of approximately 2–5 × 10^6 cpm μg^{-1} (see note 17). Alternatively, a complete in vitro transcription kit can be used according to the manufacturer's instructions (see note 5). Purify the probe from unincorporated NTPs using a NucTrap column per manufacturer's instructions (see note 20).
4. To make RNA probes for UV light cross-linking assay, prepare a mixture with a total volume of 20 µl containing 1–2 µg linearized template DNA, 0.5 mM of each of GTP, CTP, and ATP, 10 µM (60 µCi) of an aqueous solution of [α-^{32}P]UTP, and 20 U of T7 RNA polymerase with 2 µl of T7 RNA polymerase 10× reaction buffer (see note 19). Incubate for 1 h at 37 °C. This labeling reaction should yield probes with a specific activity of approximately 2–5 × 10^8 cpm μg^{-1} (see note 17). Alternatively, a complete in vitro transcription kit can be used according to the manufacturer's instructions (see note 5). Purify the probe from unincorporated NTPs using a NucTrap column per manufacturer's instructions (see note 20).

3.3 MP-Nucleic Acid-Binding Assays

3.3.1 Gel Mobility Shift Assays

3.3.1.1 Mobility Shift on Native Polyacrylamide Gels

1. Cast a 4% native polyacrylamide gel. For full-size/mini gels, combine: 2.7/1.7 ml 30% acrylamide and 17.3/10.8 ml 1× TBE, mix, add 20/12.4 µl TEMED and 200/125 µl 10% APS, mix, pour into the gel frame, and immediately insert comb (see note 21). Allow it to remain at room temperature until the acrylamide polymerizes completely (see note 22).

2. In a 1.5-ml polypropylene microfuge tube, combine 40 ng of radioactively-labeled DNA or RNA probe (see notes 23 and 24), 1 µg of purified MP (see note 25) in a total volume of 15 µl buffer L and incubate on ice for 10–30 min. See notes 26 and 27 for a recommended set of initial experimental and control-binding reactions.
3. Add 4 µl of 5× native gel loading buffer, mix, load into the gel well (see note 28), and electrophorese at the electric field strength of 8 V cm^{-1} (see note 29) at 4 °C (i.e., in a cold room or a cold box) until the tracking dye has migrated 1/2–2/3 way down the gel.
4. Remove the gel from the gel box, remove side spacers, and, using a spatula, slowly separate the glass plates. Place a sheet of blotting paper over the gel and carefully peel the gel off the glass plate. Cover the exposed side of the gel with saran wrap and dry in a gel dryer under vacuum (see note 30).
5. Expose the dried gel to X-ray autoradiography film for overnight at −70 °C with an intensifying screen. Alternatively, analyze the gel using PhosphorImager. See note 31 for general guidelines on how to interpret the results of this experiment.

3.3.1.2 Mobility Shift on Agarose Gels

1. Make a 0.3% solution of agarose in 1× TBE, boil until agarose dissolves fully, cool to 55 °C, and pour into a horizontal gel tray, insert comb. Allow it to remain at room temperature until agarose solidifies completely (see note 22).
2. In a 1.5-ml polypropylene microfuge tube, combine 1 µg of unlabeled probe (see notes 23 and 32), 25 µg of purified MP (see note 25) in a total volume of 15 µl buffer L, and incubate on ice for 10–30 min. See note 26 for a recommended set of initial experimental and control-binding reactions.
3. Add 4 µl of 5× native gel loading buffer, mix, load into the gel well, and electrophorese at the electric field strength of 5–7 V cm^{-1} (see note 29) at room temperature until the tracking dye has migrated to the end of the gel.
4. Stain the gel (see note 33) for 10 min at 4 °C in 2 µg ml^{-1} of ethidium bromide in 1× TBE, and destain for 1–3 h at 4 °C in 1× TBE supplemented with 1.5 M NaCl (see note 34).
5. Visualize the stained probe and record the image using a CCD gel documentation system or a Polaroid camera with a UV light table. See note 31 for general guidelines on how to interpret the results of this experiment.

3.3.2 UV Light Cross-Linking Assay

1. Cast a 12.5% resolving SDS polyacrylamide gel. For full-size/mini gels, combine: 6.7/2.1 ml 30% acrylamide, 4.0/1.25 ml 4× resolving gel buffer, and 5.3/1.65 ml ddH_2O, mix, add 15/2.5 µl TEMED and 60/25 µl 10% APS, mix, pour into the gel frame, and immediately overlay with ddH_2O (see note 35). Leave at room temperature until the acrylamide polymerizes completely (see note 22).
2. Aspirate ddH_2O from the polymerized resolving gel and cast a stacking polyacrylamide gel. For full-size/mini gels, combine: 0.75/0.375 ml of 30% acrylamide,

1.25/0.625 ml of 4× stacking gel buffer, and 3/1.5 ml ddH_2O, mix, add 5/2.5 µl TEMED and 50/25 µl of 10% APS, mix, pour into the gel frame, and immediately insert comb (see note 21). Leave at room temperature until the acrylamide polymerizes completely (see note 22).

3. In a 1.5-ml polypropylene microfuge tube, combine 10 ng of RNA probe (see note 23), 0.2 µg of purified MP (see note 24) in a total volume of 15 µl buffer L, and incubate on ice for 10–30 min. See notes 25 and 27 for a recommended set of initial experimental and control-binding reactions.
4. Open the reaction tubes, place them in a UV light cross-linker, and irradiate with 1.8 J of UV light. Alternatively, incubate the samples for 30 min on ice at a distance of 6 cm under a germicidal UV light lamp.
5. Add 1 µl of 0.5 mg ml^{-1} RNase A in ddH_2O and incubate for 30 min at 37 °C to digest RNA probe unprotected by the bound protein.
6. Add 4 µl of 5× SDS gel sample buffer, mix, load into the gel well (see notes 28 and 36), and electrophorese at the electric field strength of 10 V cm^{-1} (see note 29) until the tracking dye has migrated to approximately 0.5 cm from the bottom of the gel.
7. Remove the gel from the gel box, remove side spacers, and, using a spatula, slowly separate the glass plates. With a razor blade, remove and discard the bottom 0.5–1 cm of the resolving gel, which contains radioactive nucleotides derived from the digested unbound probe. Also, the stacking gel – which is sticky and difficult to handle and which should not contain the protein bands of interest – can be removed and discarded.
8. Place the resolving gel in a glass or plastic box with the protein staining solution, stain for 30 min at room temperature with gentle shaking (see note 37). Remove staining solution, add destaining solution and destain at room temperature with gentle shaking until molecular weight marker bands are visible (see note 38).
9. Remove the gel from the destaining solution, place it on a sheet of blotting paper, cover the other side of the gel with saran wrap, and dry in a gel dryer under vacuum (see note 30).
10. Expose the dried gel to X-ray autoradiography film for overnight at −70 °C with an intensifying screen. Alternatively, analyze the gel using PhosphorImager. See notes 39 and 40 for general guidelines on how to interpret the results of this experiment.

3.4 Characterization of MP-Nucleic Acid-Binding Cooperativity and Sequence Specificity

3.4.1 Binding Cooperativity

For proteins that bind nucleic acids without sequence specificity – such as most viral MPs (*see* Table 1) – nucleic acid molecules present a continuous lattice of potential binding sites, rather than individual discrete and isolated binding sites as in sequence-specific binding. Sequence nonspecific binding of proteins to nucleic

acid lattices occurs in two major modes: random and cooperative. In the random-binding mode, every nucleotide initiates attachment of protein with the same probability such that protein molecules that bind independently of each other are randomly distributed on the nucleic acid lattice (18). Thus, at subsaturating concentrations of protein, all protein molecules associate with all probe molecules with comparable affinity. In native gel mobility shift assays, which represent the major and simplest tool for determination of the protein–nucleic acid-binding mode, random protein binding is detected as a ladder of discrete protein-probe complexes, the size of which increases with the increase in protein concentration (13, 19) (illustrated in Fig. 1b).

In the cooperative-binding mode, protein association with the nucleic acid is not random; instead, protein molecules tend to bind in long clusters such that some nucleic acid molecules become fully coated with the protein, while others are still protein-free. Thus, cooperatively binding proteins exhibit an "all-or-none" behavior in native gel mobility shift assays, i.e., at subsaturating concentrations of protein, only two species of probe exist, free probe and maximally shifted complete protein-probe complexes, and no intermediate bands representing partly coated probe are detected (e.g., 1, 13) (illustrated in Fig. 1c). On the basis of this rationale, potential cooperativity (or the lack thereof) of MP binding to nucleic acids can be easily determined from the dose response to protein concentration in gel mobility shift assays.

1. Prepare a 4% native polyacrylamide gel or a 0.3% agarose gel as described above.
2. In 1.5-ml polypropylene microfuge tubes, set up a series of reactions using the constant amount of probe as described earlier for mobility shift assays on native polyacrylamide or agarose gels. In each tube, vary only the amount of added protein. We recommend using the following protein-to-probe weight-to-weight ratios: 0:1, 0.2:1, 0.5:1, 0.7:1, 1:1, 2.5:1, 5.0:1, 10:1, 20:1, 50:1, 75:1, and 100:1 (see notes 41 and 42). For suggested positive controls that generate reference, gel mobility shift patterns characteristic for high and low cooperativity-binding modes, see note 43.
3. Incubate on ice for 10–30 min.
4. Perform the gel mobility shift assay and detect formation of protein-probe complexes as described earlier. See note 44 for general guidelines on how to interpret the results of this experiment, and note 45 on how to use these data to estimate the minimal size of the nucleic acid-binding site of the tested protein.

3.4.2 Sequence Specificity of Binding

Most viral MPs bind nucleic acids irrespective of their sequence (see Table 1). It is possible, however, that, in addition to its general nucleic acid-binding ability, an MP may exhibit preferential binding toward the viral genome. Relative affinity of MP to various RNA and DNA sequences can be accurately assessed from binding competition experiments (e.g., 1) using unlabeled specific and nonspecific competitors.

1. Prepare a 4% native polyacrylamide gel or a 12.5% SDS polyacrylamide gel as described earlier (see note 46).
2. In 1.5-ml polypropylene microfuge tubes, set up a series of reactions as described earlier for mobility shift assays on native polyacrylamide gels or for UV light cross-linking. Each tube should contain constant amounts of protein and probe and increasing amounts of unlabeled nucleic acid competitor (see notes 47 and 48). We recommend using the following competitor-to-probe weight-to-weight ratios: 0:1, 0.5:1, 1:1, 5:1, 10:1, 50:1, 100:1, 500:1, and 1,000:1 (see note 41). At least two different competitors should be used: specific (e.g., viral genomic sequences) and nonspecific (e.g., M13mp18 ssDNA or even the unlabeled probe itself).
3. Incubate on ice for 10–30 min.
4. Perform the gel mobility shift assay or UV light cross-linking assay, dry the gel, and detect formation of protein-probe complexes by autoradiography as described earlier.
5. Align the autoradiogram with the dried gel and mark the location of the bands corresponding to the complete protein-probe complexes. Excise the corresponding gel regions with sharp scissors or a razor blade, and determine their radioactivity by counting Cerenkov radiation (no scintillation fluid is required).
6. Calculate the amount of the bound probe as percent of maximal binding (i.e., in the absence of competitor). Then, plot these data as a function of probe-to-competitor weight ratio. From the resulting competition curves, calculate IC50 (inhibitory concentration 50%) values for each unlabeled competitor using the probe-to-competitor weight ratio required to reduce binding by 50%. See note 49 for general guidelines on how to interpret the results of this experiment.

4 Notes

1. Boxes for either full-size gels ($20 \times 20\,cm^2 \times 1\,mm$) or minigels ($10 \times 7\,cm^2 \times 1\,mm$) can be used. These apparati can be purchased from BioRad, BRL, Hoefer, or any other manufacturer, or made in-house.
2. Filter sterilize IPTG, ampicillin, and kanamycin before aliquoting and storage. The choice of antibiotics depends on the bacterial strain and expression vector used to produce MP (see notes 6 and 7).
3. To facilitate preparation of this solution, add all liquid ingredients from stock solutions, i.e., 1 M Tris/HCl pH 8.0, 5 M NaCl, 0.5 M EDTA, and 100% glycerol, add solid urea, and ddH_2O to about 1/2 of the final desired volume, dissolve urea by heating the mixture at 70 °C, bring up to desired volume with ddH_2O and keep on ice until use.
4. Because PCR is used to produce relatively short DNA fragments for use as binding probes, there is no need for expensive high fidelity DNA polymerases, such as *ExTaq* or *Pfu*.
5. In vitro transcription can be performed using the reaction mixture described here. Note that all reagents, such as NTP stocks, used in preparation and handling of RNA should be RNase-free and prepared using RNase-free water (for standard protocols of preparation of RNase-free reagents, see, for example, 16, 17). Alternatively, one can use a complete kit, such as the Riboprobe system (Promega), which is designed for in vitro preparation of high specific activity single-stranded RNA probes.

6. Normally, the bacterial strain BL21(DE3) is sufficient for IPTG-induced over-expression from the bacteriophage T7 TNA polymerase expression vectors. However, viral MPs, potentially due to their strong single-stranded nucleic acid binding activity, are often toxic to bacterial cells; thus, even background levels of MP expression due to "leaky" production of the T7 RNA polymerase from its IPTG-inducible promoter may significantly impair bacterial growth. To suppress the MP expression until the cell cultures are ready for IPTG induction, the BL21(DE3) cells must carry a pLysS or pLysE plasmid. These plasmids, which carry the T7 lysozyme gene under the tet promoter from the pACYC184 plasmid (20), produce T7 bacteriophage lysozyme, which adsorbs and sequesters T7 RNA polymerase (21–23), preventing background expression of MP. Both pLysS and pLysE carry resistance to chloramphenicol, and they are maintained in the BL21(DE3) cells by culturing in the presence of 20 μg ml^{-1} of chloramphenicol. pLysE produces more lysozyme than pLysS, resulting in a tighter control of background expression, but both of them allow good over-expression after IPTG induction.
7. Our protocols utilize recombinant MP over-expressed in *E. coli* using the T7 RNA polymerase system, but any source of purified MP, such expression in baculovirus (24) or in yeast (25) is suitable for these experiments. For T7 RNA polymerase expression, we use pET-based vectors (23, 26) ranging from the early pET3 series to the more recent pET21 and pET28 series to the latest pET53 vectors, which allow fusion of different epitope tags to the over-expressed protein. We prefer to express either an untagged protein from a pET3 vector or a protein tagged at its C-terminus with hexahistidine from pET21 or pET28 vectors; the latter constructs allow further purification of the expressed protein on Ni columns.
8. We noticed that BL21(DE3)pLysE cells harboring the MP expression construct do not express well if used from a frozen glycerol or DMSO stock; thus, we freshly transform bacteria with the expression construct before each experiment. In addition, because BL21(DE3)pLysE cells produce lysozyme (see note 6) and are relatively fragile, we prefer to use the standard heat shock transformation method (e.g., 16, 17) rather than electroporation.
9. Normally, inoculation with a single colony further ensures the genetic homogeneity of the culture. However, since all colonies on the transformation plate derive from the same clone of the expression construct, we use multiple colonies to increase the inoculum and decrease growth time for the starter culture.
10. LB liquid medium can be used instead of 2× YT, but we consistently observe better expression in a richer 2× YT medium.
11. This pellet contains inclusion bodies of the over-expressed MP. With TMV MP, we observe virtually no over-expressed protein in the soluble fraction (supernatant). The insolubility of the inclusion bodies can be advantageous because it allows easy removal of contaminating bacterial proteins by multiple washes with high salt (see Fig. 1a).
12. The first wash with urea solubilizes about one half of the protein and further removes contaminants that have not been washed away by high salt. Importantly, incubation at 70 °C inactivates ssDNA-specific nucleases, which are present in the bacterial lysate, and, if not inactivated, preclude the use of the MP preparation in ssDNA-binding assays. Although half of the produced protein is lost during the first urea solubilization, we recommend this step for optimal purification and better refolding of the remained MP, which is achieved by the second urea solubilization performed at 56 °C (see Fig. 1a).
13. Normally, the MP solution remains clear after dialysis, but if some of the protein precipitates (looks like a fine white dust in the dialysis tube), it can be returned into solution by 2–3 passes through a G26 needle. It is important to note that this MP preparation is not completely bona fide soluble as the protein will precipitate when centrifuged at high speeds; this reduced solubility may be an intrinsic biological property of MP molecules that tend to interact with each other for cooperative binding to single-stranded nucleic acids (1). However, MP prepared using this protocol binds ssDNA and RNA (1), and it enhances plasmodesmal permeability when microinjected into plant tissues (27).
14. Although somewhat more laborious, native PAGE provides better resolution between different protein–nucleic acid complexes.

15. If thermocycler without a heated lid is used, overlay the reaction mixture with 50 µl of mineral oil to prevent evaporation during amplification; thermocyclers with heated lid do not require mineral oil. If mineral oil is used, it should be removed three extractions with one volume of chloroform each, followed by phenol precipitation, before use.
16. This approach is based on the terminal transferase activity of Taq polymerase, used to produce the probe (see above), which adds a single A overhang to each 3′-end of the PCR product and allows such DNA molecules with protruding 3′ ends to be easily end-labeled with T4 DNA polymerase. T4 DNA polymerase has 3′-to-5′ exonuclease and 5′-to-3′ polymerase activities, which remove the 3′ overhang and exchange the base of the dsDNA with the corresponding radioactively-labeled nucleotide, resulting in a blunt-ended probe with radioactive label incorporated at or very near to the termini of the DNA molecule (16).
17. To determine specific activity of the DNA or RNA probes, combine 1 µl of 1:10 dilution of the probe with 0.5 ml ice-cold 10% trichloracetic acid (TCA), place on ice for 10 min, collect the precipitate by vacuum filtration on a Whatmann GF/C glass fiber filter (before adding sample, prewet the filter with a small amount of 10% TCA), and read the Cerenkov radiation (do not use scintillation fluid). Calculate the specific activity of DNA probes as follows: total incorporated counts per minute (cpm)/total DNA input into the labeling reaction, where total incorporated cpm is TCA-precipitated cpm × 10 (i.e., dilution of probe sample) × total volume of probe preparation (i.e., 20 µl for dsDNA labeled using T4 DNA polymerase, and 50 µl for oligonucleotides labeled using T4 polynucleotide kinase). Calculate the specific activity of RNA probes as follows: total incorporated cpm/total RNA synthesized, where total incorporated cpm is TCA-precipitated cpm × 200 (i.e., dilution of probe sample × total volume of probe preparation), and total RNA synthesized is percent incorporation (i.e., TCA-precipitated cpm/cpm in the same sample before TCA precipitation × 100) × maximum theoretical RNA yield (i.e., total nmol of labeled + unlabeled limiting NTP × 4 × average molecular mass of a nucleotide within RNA, or for the reaction described here, total nmol of UTP × 4 × ~320 ng $nmol^{-1}$).
18. For technical simplicity and because most MPs do not interact efficiently with dsRNA (see Table 1), we focus only on ssRNA probes. If necessary, MP binding to dsRNA can be tested as described elsewhere (see references in Table 1).
19. Factors to be considered for maximal efficiency of the in vitro transcription reaction: Salt concentrations exceeding 50 mM may inhibit the T7 RNA polymerase activity; DTT, which is required for the T7 RNA polymerase activity, is unstable, even when frozen; thus, supplementing the reactions with the final concentration of 10 mM of freshly-made DTT may increase the reaction yield. Higher yields may also be obtained by raising NTP concentration to 4 mM each, adding Mg^{2+} to 4 mM above the total concentration of NTPs. Also, supplementing the reaction with inorganic pyrophosphatase (e.g., from New England Biolabs) to a final concentration of 4 U ml^{-1} will solubilize pyrophosphate precipitate that sequesters Mg^{2+}.
20. Alternatively, RNA probes can be purified on denaturing polyacrylamide gels as described elsewhere (e.g., 28).
21. Completely fill the gel frame with the acrylamide solution. The gel should be cast and comb inserted quickly because, once APS is added, the polymerization process begins. When inserting the comb, slightly tilt the gel to allow release of air bubbles and avoid their trapping under the comb teeth, which should be completely inserted into the gel solution. Do not disturb the gel until it has polymerized completely; polymerization can be easily detected as slight shrinking of the gel in the areas between the comb teeth and appearance of a thin layer of water (released following polymerization) above these areas. If gel does not polymerize, use freshly made 10% APS. Although degassing the acrylamide solution is often recommended, we find that this step can be safely omitted from our protocol.
22. Usually, this step takes about 30 min.
23. Because most viral MPs bind both ssDNA and RNA without sequence specificity (see Table 1), and because DNA probes are technically simpler to produce and handle than RNA probes, we recommend to begin studies of MP-nucleic acid interactions with sequence nonspecific DNA probes.

24. This experimental design is suitable for both DNA and RNA probes (e.g., 1). Probe amounts are calculated based on their specific activities (see note 17). For dsDNA probes, PCR fragments are used directly, and complementary oligonucleotides should be annealed by mixing them at 1:1 molar ratio, incubating for 5 min at 70 °C, 30 min at room temperature, and keeping on ice until use. For ssDNA probes, oligonucleotides are used directly, and PCR fragments should be denatured by boiling for 5 min and immediately placed on ice until use. RNA probes are used directly.
25. It is difficult to predict the optimal probe-to-MP ratio at which the binding is detected; thus, for initial experiments, we suggest to use a clear excess of MP.
26. Suggested reactions for initial testing of MP binding to DNA using gel mobility shift assays. Experimental reactions: MP + dsDNA probe, MP + ssDNA probe. Negative controls: dsDNA probe alone, ssDNA probe alone, MP + dsDNA probe treated for 30 min at 37 °C with 1 mg ml^{-1} of Proteinase K (New England Biolabs), MP + ssDNA probe treated for 30 min at 37 °C with 1 mg ml^{-1} of Proteinase K, bacteriophage T4 gene 32 protein + dsDNA probe. Positive control: bacteriophage T4 gene 32 protein + ssDNA probe.
27. Suggested reactions for initial testing of MP binding to RNA using gel mobility shift assays. Experimental reaction: MP + RNA probe. Negative controls: RNA probe alone, MP + RNA probe treated for 30 min at 37 °C with 1 mg ml^{-1} of Proteinase K. Positive control: bacteriophage T4 gene 32 protein + RNA probe. Note that, although the T4 gene 32 protein exhibits a much lower affinity to ssRNA than to ssDNA (6, 29), it is still suitable as a positive control.
28. Slowly remove the comb, taking care not to disturb the wells in the polymerized gel, place the gel into the gel box, rinse the wells by filling them with running buffer (1× TBE for native gels or 1× SDS gel running buffer) and removing the buffer with a vacuum aspirator or a Hamilton glass syringe, and, using a micropipette, slowly load the sample into the empty well, allowing it to slide along one side of the well to avoid trapping bubbles. Slowly overlay the loaded sample with running buffer to completely fill the wells. Then, fill both chambers of the gel box with running buffer. Fill the top chamber slowly, and never add running buffer to the top chamber before the samples in the wells have been completely covered with running buffer; this avoids mixing the loaded sample with the running buffer and helps to obtain sharper bands.
29. For vertical gel electrophoresis, total applied voltage is calculated based on the height of the gel, which corresponds to the entire native gel height for gel mobility shift assays and to the height of the resolving gel for UV light cross-linking assays. Alternatively, the vertical gels can be electrophoresed at maximal voltage and constant current of 25 mA; the ability to maintain constant current is found in more expensive power supplies, but electrophoresis at constant current usually yields better resolution of protein bands, especially with SDS polyacrylamide gels. For horizontal gel electrophoresis, total applied voltage is calculated based on the distance between the electrodes.
30. Usually, the drying process is finished after 1–1.5 h at 80 °C. Make sure the gel is completely dry before removing it from the dryer; if vacuum is broken while the gel is still wet, the gel will crack.
31. The results of gel mobility shift assays should be interpreted using the following general guidelines. On polyacrylamide gels, free dsDNA migrates faster than ssDNA, and on agarose gels, free dsDNA migrates slower than ssDNA. Protein-probe complexes always migrate much slower than the corresponding free probe, with large protein–nucleic acid complexes (i.e., multiple protein molecules bound to a molecule of probe) remaining very close to the loading well of the gel (*see* Fig. 1b). Proteinase K treatment should abolish retardation of the probe. The bacteriophage T4 gene 32 protein, known to bind ssDNA and ssRNA (6, 29), should cause reduced mobility of ssDNA and RNA probes, but not of dsDNA probe.
32. Agarose gel mobility shift assays are best suited for DNA probes detected by ethidium bromide staining (e.g., 19, 30) [although detection by Southern blot hybridization is also possible (31)]. Thus, higher amounts of probe are recommended. Also, for nonspecific probes (see note 23), we recommend commercially available preparations of M13mp18 ssDNA and dsDNA.

33. Low percent agarose gels are very fragile. To reduce handling and risk of breakage, we recommend leaving the gel in the gel tray throughout the staining/destaining procedure. Also, if the gel tray is UV transparent, keep the gel in the tray during visualization of DNA bands.
34. High concentration of NaCl in the destaining buffer helps to dissociate protein from the DNA while ethidium is still present in the gel and is able to intercalate (e.g., stain), allowing to visualize the DNA probe, which had been covered by protein and, thus, more difficult to stain.
35. Fill about two thirds of the gel frame so that enough space is left to fit the teeth of the comb and allow 1–2 cm of stacking gel between the comb and the resolving gel. The gel should be cast and overlaid with ddH_2O quickly because, once APS is added, the polymerization process begins. Overlay is required to protect the acrylamide solution in the gel frame from molecular oxygen, which inhibits acrylamide polymerization and causes formation of jagged, uneven gel edge. Some protocols overlay resolving gels with isobutanol, which is light and does not easily mix with acrylamide, but isobutanol should be extensively washed out before casting the stacking gel. In contrast, ddH_2O slowly and carefully overlaid to about 0.5–1 cm height above the acrylamide solution requires no wash, and it can be simply aspirated before pouring the stacking gel. After overlaying, do not disturb the gel until it has polymerized completely; polymerization can be easily detected as a clear and sharp interface between ddH_2O and the gel. If gel does not polymerize, use freshly made 10% APS. Although degassing the acrylamide solution is often recommended, we find that this step can be safely omitted from our protocol.
36. Remember to load molecular weight markers in one of the wells of the gel.
37. We recommend to perform the staining procedure to fix the gel and visualize molecular weight markers and MP. However, this step is not absolutely necessary, especially if prestained molecular weight markers are used. Instead, the unstained gel can be directly dried and autoradiographed.
38. Use small volumes of staining or destaining solutions, which are just sufficient to cover the gel. The gel is destained faster if the destaining solution is frequently changed or if a small piece of sponge, which absorbs Coomassie blue and removes it from the destaining solution, is placed in the box with the gel.
39. The results of the UV light cross-linking assay should be interpreted using the following general guidelines. Protein–RNA complexes are detected as radioactively-labeled protein bands that represent protein covalently cross-linked to the probe. Note that, because RNase A treatment removes all probe sequences that are not in close contact with the bound protein, very short oligonucleotides remain cross-linked to the protein causing only slight reduction in its electrophoretic mobility. In the absence of MP or following Proteinase K treatment, no radioactively-labeled protein bands should be observed. The bacteriophage T4 gene 32 protein, known to bind ssRNA (6, 29), should produce a radioactively-labeled band.
40. One unique and important advantage of the UV light cross-linking assay, when compared with most other assays for protein–nucleic acid binding, is that it directly identifies the protein species that interacts with the probe, allowing to rule out potential artifacts due to contaminants in the tested protein preparations.
41. We recommend using weight, rather than molar, ratios because, as described in this section, sequence nonspecific binding occurs along the entire nucleic acid lattice and, thus, depends on the total amount of polynucleotides in the reaction, which is better reflected by the weight of the probe than by the number of its molecules.
42. For some MPs, it may be impossible to obtain preparations concentrated enough to allow testing of very high protein-to-probe ratios; however, most MP preparations should be suitable for testing 10:1 to 20:1 ratios, which are normally sufficient to detect cooperative binding (e.g., 1, 13).
43. As positive controls and reference gel mobility shift patterns typical for high and low cooperativity binding, we recommend using commercially available preparations of the T4 gene 32 protein and *E. coli* ssDNA-binding protein. The former represents a paradigm for a protein with high cooperativity of binding whereas the latter exhibits a lower-binding cooperativity,

which allows detection of partly coated probe molecules with intermediate degree of mobility shift (6). Note that, low-binding cooperativity of the *E. coli* ssDNA-binding protein is better expressed at higher salt concentrations, i.e., 200–300 mM NaCl, while at lower salt concentrations, i.e., 20–50 mM NaCl, this protein exhibits higher binding cooperativity (13, 19).

44. Most plant viral MPs that bind ssDNA and ssRNA without sequence specificity exhibit various degrees of binding cooperativity (see Table 1). Thus, one would expect to detect at least some degree of cooperative binding that is manifested as a sharp transition from free, non-retarded probe to protein bound, strongly retarded probe upon increasing the protein-to-probe weight ratio. The higher the binding cooperativity is, the more narrow becomes the range of protein concentrations over which the change from zero to essentially complete protein-probe binding occurs (compare Fig. 1b and c).
45. The gel mobility shift experiments described in this section also define the minimum protein-to-probe weight ratio needed for complete binding. Knowing this ratio, the size of the probe, and the molecular mass of the tested protein allows one to calculate the size of the nucleic acid binding site, i.e., a number of nucleotides associated with one protein molecule. Obviously, this calculation is based on the assumption that all molecules in the tested protein preparation are equally active and, thus, represents the minimal value for the size of the binding site. Although falling short of by far more complicated spectroscopy experiments traditionally used to determine the precise size of nucleic acid-binding sites (e.g., 19), this approach still represents a valuable tool for initial characterization of MP–nucleic acid interactions.
46. Because estimating binding competition requires the use of a labeled probe and unlabeled competitor, gel mobility shift on native polyacrylamide gels and UV light cross-linking are the most suitable assays for these experiments.
47. We suggest using the lowest protein-to-probe weight ratio that yields the complete shift of all probes in the reaction mixture. This ratio minimizes the presence of free, excess protein or probe, and it can be determined from the dose response experiments described for studies of binding cooperativity.
48. To determine true binding competition, rather than displacement, both the competitor and the probe must be present in the reaction mixture before the protein is added.
49. Preferential binding to a specific competitor results in a more efficient competition, which manifests as a shift of the corresponding competition curve toward the lower probe-to-competitor weight ratios and the proportionately reduced value of IC50 when compared with those of a nonspecific competitor. Conversely, sequence nonspecific binding results in virtually identical competition curves and IC50 values for specific and nonspecific competitors.

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From Viral Sequence to Protein Function

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Preface

Following the considerable success of the Plant Virology Protocols in the Methods in Molecular Biology volume, Humana Press invited us to produce a second edition of this volume.

The first book *Plant Virology Protocols: From Virus Isolation to Transgenic Resistance* had a trend running through it, which people liked, which was – methods to isolate a virus, clone it, express it, and transform it into plants, and evaluate those plants for transgenic resistance.

For the second edition, we have decided on a different trend running through the book that is – *From Viral Sequence to Protein Function*, which will cover the many new techniques that we now can apply to analyze and understand plant viruses.

This book has been divided into five major parts, containing 44 chapters in total.

Part 1 provides a general introduction to some typical plant viral proteins, and their role in infection and interactions with other viral proteins, with the host, vectors, etc.

Part 2 provides a range of techniques for investigating viral nucleic acid sequence as well functional analysis, with Part 3 covering protein analysis and investigation of protein function.

Part 4 has a wide-ranging remit but centered on techniques for microscopy/GFP visualization and analysis/protein tagging/generation of infectious clones and other such tools.

Part 5 covers the emerging area of genomics, interactions with host factors, and plant-based studies, a theme that will probably expand over the coming years to require an entire book dedicated to this theme alone, perhaps *Plant Virology Protocols Vol 3*!

Plant Virology Protocols is the product of the hard work and major efforts of a large number of individuals who have been supportive and patient during the editing process. The editors would like to thank them all; we hope they and others will find the book useful and informative.

Gary Foster would like to thank, or should it be apologize to, his family (Diana, James, and Kirsty) for agreeing to take on another book, yes I know I promised I would not take another book, but this one was too nice an idea.

Yiguo Hong would like to thank Gary Foster for inviting him to become involved in this project. Thanks also go to Po Tien, Bryan Harrison, John Stanley, and

Michael Wilson who have consistently inspired him to the tiny but extremely exciting world of plant viruses. Yiguo Hong would also like to thank his family (Mei, Elizabeth, and Lucy) for their support.

Elisabeth Johansen would like to thank Gary Foster for the invitation to participate in the challenging process leading to the publication of this book. Thank you for your guidance and encouragement.

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