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Interactions between Sindbis virus RNAs and a 68 amino acid derivative of the viral capsid protein further defines the capsid binding site

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ABSTRACT

In previous studies of encapsidation of Sindbis virus RNA, we identified a 570nt fragment (nt 684 – 1253) from the 12 kb genome that binds to the viral capsid protein with specificity and is required for packaging of Sindbis virus defective interfering RNAs. We now show that the capsid binding activity resides in a highly structured 132nt fragment (nt 945 – 1076). We had also demonstrated that a 68 amino acid peptide derived from the capsid protein retained most of the binding activity of the original protein and have now developed an RNA mobility shift assay with this peptide fused to glutathione-S-transferase. We have used this assay in conjunction with the original assay in which the intact capsid protein was immobilized on nitrocellulose to analyze more extensive deletions in the 132-mer. All of the deletions led to a reduction in binding, but the binding of a 5′ 67-mer was enhanced by the addition of nonspecific flanking sequences. This result suggests that the stability of a particular structure within the 132nt sequence may be important for capsid recognition.

INTRODUCTION

Specific protein–nucleic acid interactions involve the recognition of discrete elements in both the nucleic acid and its cognate binding protein. For many DNA–protein interactions these sites have been well characterized (1). In contrast the sequences and structural relationships important in RNA–protein interactions are just beginning to be understood and appear to be both diverse and complex (2, 3, 4).

The formation of viral nucleocapsids during virion maturation is one example of a process that is initiated by a highly selective RNA–protein interaction. Viral RNA encapsidation signals have been identified for a number of plant, insect and animal viruses as well as for several bacterial viruses (5, 6). The corresponding RNA binding domain of viral capsid proteins has been examined in only a few cases (7, 8, 9, 10). For a number of these viruses the RNA binding domain lies within a highly basic amino terminal domain of the protein—a region that extends into the internal cavity of assembled particles (11).

In previous studies with the alphavirus, Sindbis virus, we identified a 570nt sequence near the 5′ terminus of the 49S genomic RNA that enhanced the binding of heterologous RNAs to the Sindbis virus capsid protein in an *in vitro* binding assay (12). This segment was also found to be important for the packaging of defective interfering RNAs in infected cells, suggesting that it played a role in determining the specificity of encapsidation of the genomic RNA.

The Sindbis virus capsid protein contains 264 amino acids and is divided into two domains based on the amino acid sequence (13) and on the three dimensional structure (14). The N-terminal domain of about 113 residues is very basic and X-ray diffraction analysis indicates that it is disordered (14). The structure of the C-terminal domain (amino acids 114 to 264) is similar to that of chymotrypsin-like serine proteases. The capsid protein exists in the crystal structure as a dimer. Geigenmüller-Gnirke *et al.* analyzed a variety of deletion mutants of the capsid protein to determine the region of the protein that was involved in the binding to the RNA packaging signal (8). They identified a 68 amino acid peptide that has almost the complete RNA binding activity of the intact Sindbis virus capsid protein.

We have continued to define these RNA–capsid interactions and describe here our studies which show that a 132nt sequence embedded within the 570nt packaging signal retained all of the binding activity of the larger segment. In addition, we developed an RNA mobility shift assay using the 68 amino acid peptide tethered to glutathione-S-transferase. We have used this assay in conjunction with the original assay in which the intact capsid protein was immobilized on nitrocellulose to analyze more extensive deletions in the 132-mer. Although all of the deleted RNAs showed reduced binding activity, binding was influenced by context suggesting that the structure of the RNA may be critical for capsid recognition.

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MATERIALS AND METHODS

Plasmid constructs

Plasmids for transcribing RNAs for binding studies. The plasmids used for obtaining the RNA transcripts presented in Fig. 1 were derived from pKSG1 which was described previously (8). pKSG1 contains a 582-bp region from nt 721 to 1306 of the cDNA of the Sindbis virus genome Toto1102 (15) inserted into a Bluescript vector (Stratagene, La Jolla, CA). This is essentially the region previously defined as the encapsidation signal for the Sindbis capsid protein (12). RNAs transcribed from pKSG1 and its deleted versions either have 55 nt (Fig 1) or 11 nt (Fig 5) derived from the polylinker at the 5′ terminus. The 3′ terminus of the RNA transcribed from these plasmids either had no extra 3′ sequences from the vector or additional vector sequences of various lengths depending on the restriction site used for linearizing the cDNA. (See Fig. 5).

Plasmids that lacked the 55nt polylinker region were designated by the prefix pKG. They were constructed using a vector derived from KDI25 (16) which had been modified to contain a T7 promoter immediately upstream of an *EcoR*1 site. The Sindbis virus cDNA inserts, obtained either from pKSG1 or generated by PCR were ligated to an *EcoR*1/*Hind*III blunt ended vector fragment containing the T7 promoter sequence. All potentially correct cDNA clones were sequenced to confirm their predicted primary structure. RNAs transcribed from the Sindbis virus cDNAs inserted into the blunt ended *EcoR*1/*Hind*III site have 3 extra G residues at their 5′ end. The clone pKG4 contained nucleotides 945 to 1306 in Toto 1102. Transcription of this plasmid linearized at an NdeI site produced a 135nt RNA containing 132 nt from 945−1076 in Toto 1102 and 3 extra 5′ G residues. RNA transcribed from this plasmid linearized at an *Alu*I site contained 365 nt and was one of the RNAs used for chemical modification and primer extension (see below). Additional details about these constructions and the plasmids are available on request.

The plasmid pKDI25.34 is a derivative of KDI25 (16) and contains the sequence for a Sindbis DI RNA that can be transcribed into a biologically active DI RNA using the SP6 DNA dependent RNA polymerase promoter. This RNA contains one copy of the 132 nt sequence present in pKG4. (The original KDI25 contained two copies.)

Plasmids and bacterial strains for cloning of a 68 amino acid fragment of the capsid protein into the expression vector pGEX-2T. The 68 amino acid deletion derivative of the capsid protein has been described (8). This protein fragment was expressed as a glutathione-S-transferase (GST) fusion protein using the pGEX-2T vector. The plasmid vector was linearized within the polylinker region at a unique *EcoR*I site and was ligated to a fragment generated by PCR from the 68 amino acid capsid deletion clone Δ17 (8). The plasmid pGEX-2TΔ17 was transformed into MC1061 and selected clones were sequenced to establish that the inserted gene was correct.

Expression and partial purification of the GSTDΔ17 fusion protein and the thrombin released Δ17 deleted capsid protein

The *E.coli* B strain, BL21 (OmpT$^-$, lon$^-$) (17, 18) was transformed with plasmid DNA from a correct clone of pGEX-2TD17. The cells were grown at 37°C to late logarithmic phase and induced for 1.5 h with 0.4mM IPTG. The GSTΔ17 fusion protein was isolated from cells essentially as described by Bogerd *et al.* (19). The uncleaved fusion protein (approximately 33 kDa) had the size expected for a fusion protein between GST and the deleted Sindbis capsid protein. Thrombin cleavage generated two products, one the size of the GST protein and the other migrating at a position expected for the Δ17 polypeptide. This peptide contains 5 additional amino acids at the amino terminus and 3 extra amino acids at the carboxy terminus derived from the vector linker region.

In vitro transcriptions

For chemical modification experiments *Xho*I cut KDI25.34 was transcribed *in vitro* using the SP6 DNA dependent RNA polymerase and standard conditions with the addition of ^{3}H-UTP (16). pKG4 cut with *Alu*I was transcribed using the T7 RNA polymerase as previously described (8).

For binding assays cDNA clones were transcribed *in vitro* using either the T7 or the SP6 RNA polymerase and ^{35}S-CTP or ^{32}P-CTP under conditions previously described (12). Transcripts were purified from 6 or 8% urea−polyacrylamide gels by gel excision and elution (20).

Chemical modifications and primer extension analysis

Four modifying agents were used: dimethysulfate (DMS), diethylpyrocarbonate(DEP), kethoxal and 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMCT). DMS, CMCT and kethoxal modifications were carried out at 4°C for one h in 50 μl essentially as described by Moazed *et al.* (21). DEP reactions were carried out at 4°C in 80 mM Hepes, pH 7.8, 300 mM KCl, 10 mM $MgCl_2$ and 2.5−10μl DEP per 50μl reaction mixture (22). In all cases modified and unmodified RNAs were treated identically and were compared by primer extension analysis using a 5′ ^{32}P-end labeled oligonucleotide primer (nt 1167 to 1184 in the Sindbis virus genome). Primer and RNA were annealed at 70°C for two min and slow cooled to 30°C. Reverse transcription was performed at 50°C for 45 min using 7.5 units of AMV reverse transcriptase per 10 μl reaction. Extension products were separated on sequencing gels along with 4 dideoxy sequencing reactions obtained using the same primer and the cDNA clone pKG4.

Computer analysis of RNA secondary structure

The energy minimization program of M.Zuker (23) was used to obtain optimal and suboptimal structures for the 132 nt capsid binding sequence. The program was run with and without constraining folding to take into account the nucleotides that were accessible to chemical modification.

Nitrocellulose dot blot binding assay

The nitrocellulose dot blot assay is identical to that described previously (12) except that the amount of capsid protein applied to each filter square was increased to 2.5 μg (equivalent to approximately 80 pmoles of capsid protein).

Gel shift assay

Solution binding was carried out at room temperature in a 10μl volume. Under standard conditions each reaction contained 0.2 pmoles of ^{35}S-cytosine labeled probe RNA, 10 mM Tris−HCl, pH 7.5, 50 mM NaCl, 1mM EDTA, RNasin, 10mM DTT, 800 μg BSA, 8% glycerol, 2.5 μg bacterial RNA and 20 pmoles (2μM) fusion protein. Reactions were incubated in the absence of probe RNA for 10 min. Probe RNA was then added and the

samples were incubated for 20 min. In experiments carried out in the absence of bacterial RNA, the concentration of both the fusion protein and the cleaved 68 amino acid fragment was lowered to 0.1μM. In the competition experiments, the probe and competitor RNAs were added at the same time and incubated for 20 min. Reaction mixtures were loaded directly onto a 6% (79:1) nondenaturing polyacrylamide gel containing 50 mM Tris−glycine (pH 8.8), and 3% glycerol (24). The gel was prerun for 1−1.5 h at 100 volts in a 50 mM Tris−glycine (pH8.8) running buffer. Electrophoresis was carried out at 120−140 volts for 3−3 1/2 h. Gels were dried, exposed to film and then analyzed using a betascope 603 blot analyzer(Betagen, Waltham, MA).

RESULTS AND DISCUSSION

A 132 nt sequence in Sindbis virus RNA specifically binds to the capsid protein

We had shown previously that in the Sindbis virus genome the region between nt 684 and 1253 conferred specificity in the binding of an RNA to the Sindbis virus capsid protein (12). The capsid protein was immobilized on nitrocellulose and binding was carried out in the presence of excess total *E.coli* bacterial RNA. To determine how much of this viral RNA was required for the binding activity, we constructed a series of cDNAs containing various deletions in this RNA (See Materials and Methods and Fig. 1). RNA transcripts derived by *in vitro* transcription from these cDNAs and two transcripts (catE and catS from the choramphenicol acetyltransferase cDNA), which did not contain any Sindbis virus genomic RNA sequences, were tested. The data show that a 132-mer (from nt 945 to 1076 in the Sindbis virus RNA) was more efficiently bound by the capsid protein than the larger RA (Fig. 1).

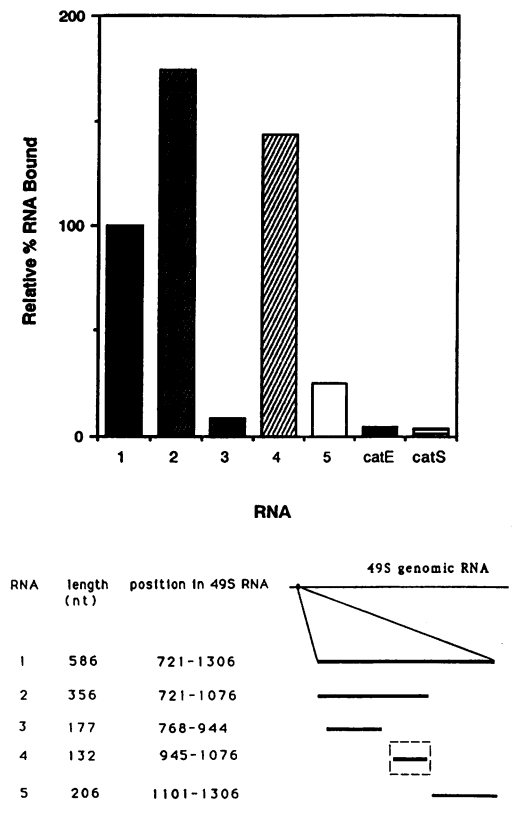

Figure 1. Identification of a 132-mer within the 586nt packaging signal that binds to the Sindbis virus capsid protein. The bar graph indicates the per cent of various ^{32}P-CMP labeled RNAs bound compared to the pmoles of the 586-mer bound which was set at 100%. The origin of each RNA is diagrammed below the graph and their location in 49S genomic RNA is indicated. Binding was carried out using the nitrocellulose binding assay and 80 pmoles of capsid protein derived from purified virions (12). The ^{32}P-labeled RNA probes contain 55 nt at their 5′ terminus derived from the bacterial vector. The catE and catS transcripts contain 277 nt and 589 nt, respectively, from the 5′ terminus of the CAT gene. The outlined segment indicates the 132nt region containing the capsid recognition sequence.

Chemical modification of the 132-mer

The 132-mer can be folded into an extensively base paired structure using the energy minimization program of Zuker (Fig. 2). We carried out a series of chemical modifications of the RNA to identify accessible nucleotides which would provide some

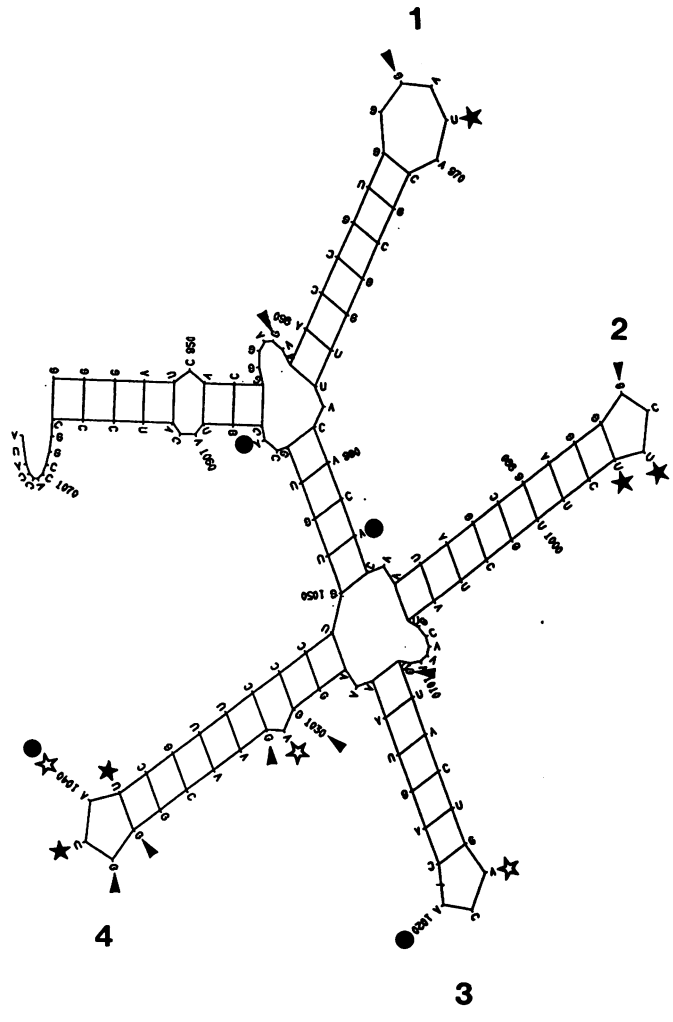

Figure 2. The optimal secondary structure of the 132nt capsid binding domain predicted using the Jaeger *et al.* (23) energy minimization program. The numbers indicate the 4 stem/loops present in this structure that are referred to in Fig. 4. The symbols indicate the 19 nucleotides that were strongly accessible to chemical modification by kethoxal (closed arrows), 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulfonate (closed stars), diethylpyrocarbonate (open stars) or dimethyl sulfate (closed circles).

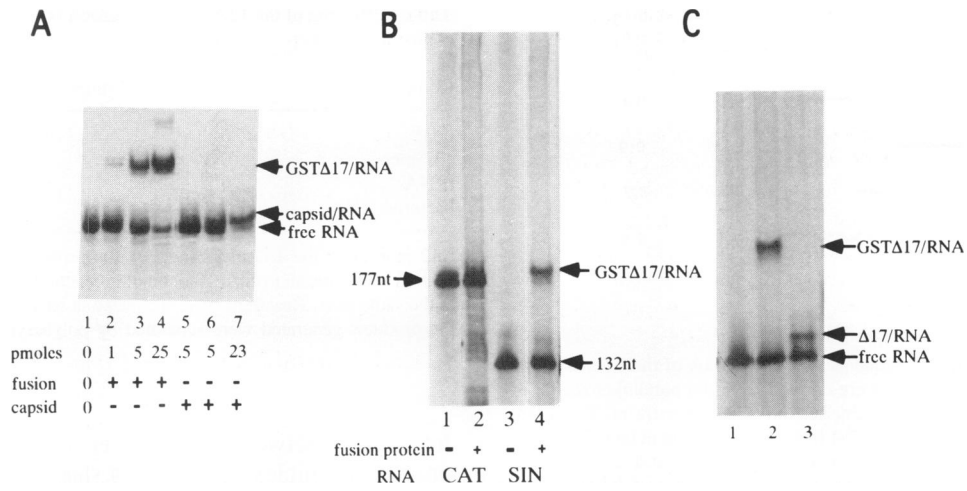

Figure 3. Polyacrylamide gel analysis of the complexes formed between the 132nt Sindbis virus capsid recognition sequence and different forms of the capsid protein. **A.** Complexes formed with the GST-capsid fusion protein or the intact capsid protein isolated from virions. A constant amount (0.02 μM) of the ^{35}S-cytosine labeled 132-mer was incubated with varying concentrations of either protein. **B.** Evidence that complex formation is specific. The autoradiogram compares complex formation between the fusion protein (1 μM) and either a 177nt CAT transcript (lanes 1 and 2) or the 132nt transcript (Lanes 3 and 4). **C.** Complexes formed with the fusion protein or the released 68 amino acid capsid moiety and the 132-mer (0.02 μM). Lane 1, no protein added; lane 2, 1 μM fusion protein; lane 3, 1 μM 68 amino acid capsid fragment.

experimental support for this type of structure. Two RNAs containing this region were chemically modified. One was a full length defective interfering (DI) RNA consisting of 2441nt that can be replicated in cells infected with Sindbis virus and that is packaged into extracellular particles by the Sindbis virus structural proteins (16). The other was an RNA transcript containing 362nt from position 945 to 1306 in the Sindbis virus genomic RNA. Modified positions within the 132-mer were determined by primer extension analysis. Strong and reproducible chemical modification was observed for both of these RNAs at 19 positions, indicated in Fig. 2. Computer assisted folding in which these 19nt were constrained to remain unpaired gave a structure nearly identical to the one shown in which no constraints were applied. All four stem-loop structures were conserved. The observation that the 132-mer was modified at the same positions when it was embedded in the DI RNA or in the much smaller 360 nt fragment indicates that the structure is the same in the two RNAs and was not affected by the greater complexity of the DI RNA.

Formation of a complex between the 132-mer and a 68 amino acid peptide derived from the capsid protein

Deletion analysis of the 264 amino acid capsid protein identified a 68 amino acid peptide that retained almost the complete binding activity of the intact protein (8). Amino acids 76 to 132 contain most of the essential binding domain of the capsid protein. Those results were obtained using an assay that detected the binding of *in vitro*-translated proteins to RNA based on their migration with the RNA during electrophoresis in an agarose gel. We have now prepared this peptide as a fusion protein linked to glutathione-S-transferase (GST) and have partially purified it from *E.coli* bacterial extracts. A complex between the fusion protein and the 132-mer could be detected by a distinct gel shift after electrophoresis in a polyacrylamide gel (Fig 3A, lanes 2 to 4). Under identical conditions, a complex between the 132-mer and the intact capsid protein purified from virions was only minimally retarded (Fig 3A, lane 7). The latter was difficult to resolve from

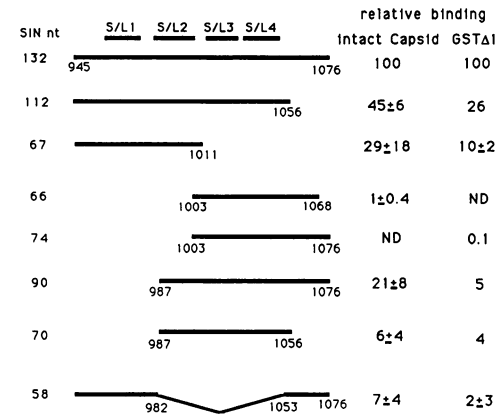

Figure 4. Deletion analysis of the 132-mer. Binding of various deleted RNAs to the intact capsid protein (80 pmoles) was determined by the nitrocellulose dot binding assay. Complex formation between these RNAs and the fusion protein (2 μM) was measured by gel shift. Binding is expressed as the per cent pmoles bound relative to the pmoles of 132-mer bound. In the nitrocellulose assay with intact capsid protein 22% of the 132-mer was bound. In the gel shift assay with the fusion protein 65% of the 132-mer was bound. These are the 100% values. S/L 1 through 4 indicate the approximate location of the 4 stem loops present in the computer generated structure for the 132-mer shown in Fig. 2. The transcripts which start at nt 987 contain one extra G residue at the 5′ end. The other transcripts contain 3 extra 5′ G residues and were derived from the pKG series of cDNAs described in Materials and Methods.

free RNA and was only clearly discerned at the highest concentration of capsid protein (2.3 μM). The amount of free RNA and RNA−protein complex in each lane was quantitated using the Betascope blot analyzer. After incubation with the fusion protein, 100% of the input RNA was recovered in the free plus bound RNA. In contrast, with the intact capsid protein only 52% of the RNA was recovered (40% in the discrete free and bound RNA bands seen in lane 7 and 12% spread diffusely from the top of the gel). The rest of the RNA probably formed large

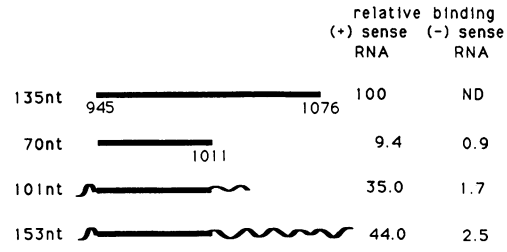

Figure 5. The effect of flanking sequences on the binding of the 67-mer to the fusion protein. Flanking sequences were derived from the polylinker region of the vector. The 5′ terminus of the chimeric RNAs has 11 extra nt. The 101nt RNA has an additional 23 nt and the 153nt RNA an extra 75 nt at their 3′ termini which were obtained by transcription of plasmids linearized at a *XhoI* site or a *BssHII* site respectively. The flanking sequences on (−) sense 67nt fragments are identical to those on the (+) sense fragments. The data are presented as per cent bound relative to the 132-mer which was 63%.

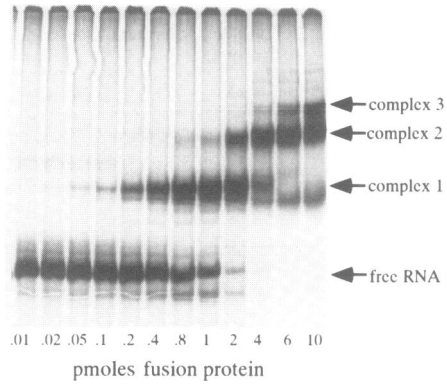

Figure 6. The effect of increasing concentrations of the fusion protein on complex formation in the absence of bacterial RNA. Assays were carried out at the indicated concentrations of fusion protein in the presence of 0.02 μM ^{35}S-cytosine labeled 132-mer.

aggregates (nucleocapsids?) with the intact capsid protein which did not enter the gel. Poor resolution of complexed and free RNA in a gel shift assay and the formation of large complexes that barely enter the gel have been observed with intact capsid proteins isolated from several other viruses (25, 26).

This gel shift assay retained the specificity previously demonstrated with the nitrocellulose assay. No complex was formed when a ^{35}S-cytosine labeled 177 nt transcript derived from the CAT gene was substituted for the 132-mer (Fig. 3B and see also Fig 4 and 5). The 68 amino acid fragment released from the GST moiety by treatment with thrombin formed a complex with the 132-mer which was retarded much less than the complex formed with the fusion protein (Fig. 3C, lanes 2 and 3). The GST moiety alone did not bind to the 132-mer (data not shown). The difference in mobility between the complexes was expected from the differences in the size of the peptides. In contrast, differences in size can not explain why the complex with the intact capsid protein was only minimally retarded compared to the complex with the fusion protein (Fig. 3A lanes 4 and 7). The size of the fusion protein monomer is not very different from that of the intact capsid monomer (30 kDa).

Table 1. Binding of the 132-mer to the fusion protein in the presence of various competitor RNAs[a]

Competitor RNA	Microgram Competitor	Relative % Bound
none	0	100
132-mer	0.3	9
tRNA	0.2	93
bacterial RNA	0.2	80

[a]0.2 pmoles of the 132-mer Sindbis virus probe RNA and 1 pmole of fusion protein were present in each 10 μl binding reaction. Competitor RNA was added at the same time. Reactions were incubated at room temperature for 20 min and the products generated were separated by polyacrylamide gel electrophoresis.

Interactions between the 132-mer and the fusion protein may produce a complex of a different shape or different electrostatic properties than that produced with the intact capsid protein and this could explain the differences in mobilities. The mobility differences might also reflect differences in the ratio of RNA to protein.

Deletion analysis of the 132-mer

Seven different deletions of the 132-mer were tested for their ability to bind the fusion protein in the gel shift assay and for their ability to bind the intact capsid protein in the nitrocellulose assay (Fig. 4). The boundaries of the deletions and the four stem-loops in the 132-mer identified in the computer generated model (Fig. 2) are indicated. Both assays gave similar results, with perhaps the most significant conclusion being that all of the deletions affected binding. Loss of the 5′ 58 nt (nucleotides 945 to 1002, containing stem-loops 1 and 2) had the strongest effect on binding. Binding activity of RNA transcripts that retained these 5′ nt was higher than that of transcripts which initiated at nt 1003 or the transcript which had an internal deletion starting at nt 983 (Fig. 4).

We considered the possibility that sequences downstream of nt 1011 might stabilize an upstream binding site and tested two chimeras of the 5′ 67-mer which had 5′ and 3′ flanking sequences derived from the plasmid. Both showed enhanced binding (Fig. 5). The flanking sequences themselves did not bind to the fusion protein as indicated by the poor binding of RNA transcripts in which the flanking sequences were essentially identical, but the Sindbis virus nucleotides were complementary [(−) sense] to the 67-mer. These results suggest that the loss of binding activity that occurred when deletions were made in the 132-mer was due to an effect on the overall stability of the structure of the RNA. This proposal is supported by the data presented in Fig.7.

Binding at limiting protein concentrations: competition experiments

The evidence for specificity in the gel shift assays was based on the low level of binding of the capsid fusion protein to the CAT transcript (Fig.3B lane 2), to several of the deleted forms of the 132-mer (Fig. 4) and to several minus strand transcripts (Fig. 5). Those experiments were carried out in the presence of excess bacterial RNA. We also analyzed specificity by competition experiments and as a first step examined the binding of the 132-mer to the fusion capsid protein as a function of its concentration in the absence of bacterial RNA (Fig. 6). At the lower protein concentrations, the complex formed (Fig. 6, complex 1) had the same mobility as that previously described

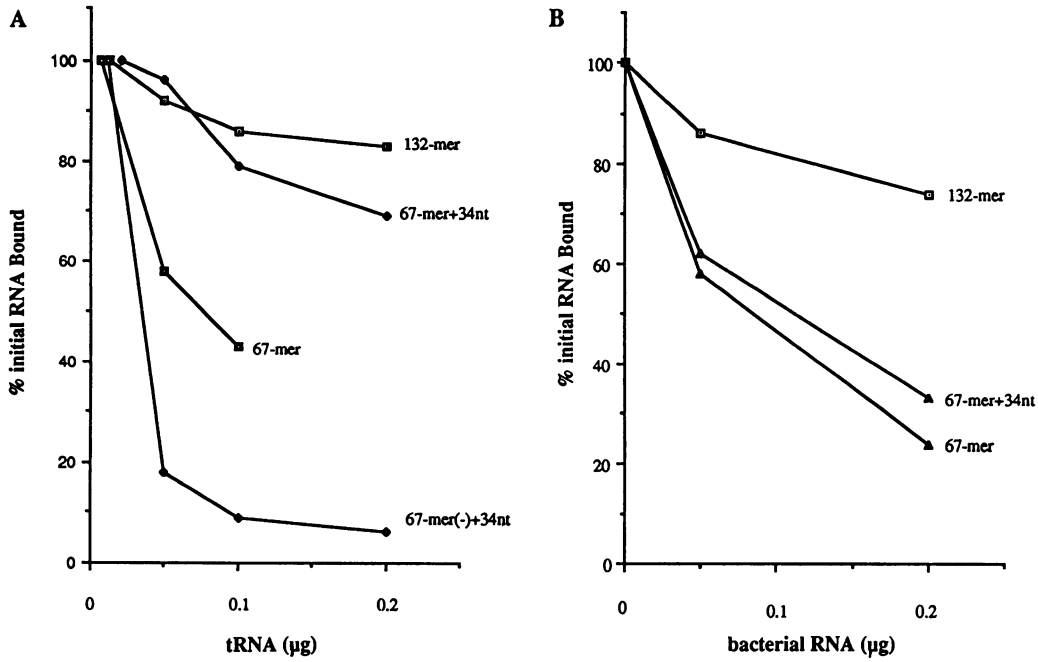

Figure 7. The effect of increasing concentrations of nonspecific RNAs on complex formation. All samples contained 0.1μM fusion protein and 0.02 μM RNA. **A.** Binding of the 132-mer and the 67-mer with and without flanking sequences in the absence and presence of tRNA. The normalized 100% value was displaced because each probe RNA contained a small amount of tRNA introduced during gel purification. **B.** Binding of the 132-mer and the 67-mer with and without flanking sequences in the absence and presence of bacterial RNA. The values for the binding of the probe RNAs in the absence of added competitor were: 132-mer, 71% bound; 67-mer, 81% bound; 67-mer with flanking sequences, 52% bound and the antisense 67-mer with flanking sequences, 26% bound. These are the 100% values.

(Fig. 3). With increasing concentrations of protein this complex was replaced by two discrete higher order complexes, complex 2 and 3 (Fig. 6, last 4 lanes). A similar shift into two higher order complexes was also seen when the thrombin-cleaved 68 amino acid capsid polypeptide was used at the higher concentrations (data not shown). These higher order complexes may reflect the presence of lower affinity binding sites on the RNA. Alternatively, they may be the result of additional protein−protein interactions. The 68 amino acid capsid peptide lacks the amino acids involved in dimer formation (8,14), but other regions of the peptide may be involved in protein−protein interactions which led to the formation of discrete structures.

Competition experiments were carried out with 0.1μM fusion protein, a concentration at which complex 1 was the major gel-shifted product. Table 1 shows the results obtained when the 132-mer, yeast tRNA or total bacterial RNA was used as competitor; only the 132-mer showed significant competition. We also examined the ability of nonspecific RNAs to compete with the binding of the 5′ 67-mer. tRNA competed effectively with the unadorned 67-mer but not with the longer chimeric transcript (Fig. 7A). Total bacterial RNA competed effectively with the 67-mer both in the absence and the presence of flanking sequences (Fig.7B). Differences between this result and that shown in Fig. 5 may be a consequence of the 20-fold higher molar ratio of protein to RNA used in the early experiments.

CONCLUSIONS

In these experiments we attempted to identify the smallest oligoribonucleotide that would retain specificity in its ability to form a complex with a 68 amino acid peptide derived from the Sindbis virus capsid protein. The addition of nonspecific bacterial RNA was required to demonstrate the type of specificity seen in Fig. 4. Those results were obtained using a molar ratio of protein to RNA of 100:1. Some discrimination between the different RNA fragments could be seen even in the absence of bacterial RNA, but only when the protein to RNA ratio was reduced to 5:1. Under conditions in which 52% of the 67-mer with flanking sequences was detected as a complex (see the legend to Fig.7), 26% of the (−) sense counterpart and 9% of the 3′ 74-mer (described in Fig. 4) formed complexes. Only the 132-mer and the 67-mer with flanking sequences, however, had a high enough affinity for the fusion protein to prevent them from being displaced by tRNA and even the chimeric 67-mer was competed by bacterial RNA. Thus, the 132-mer represents the smallest oligoribonucleotide that retained a high affinity for binding to the fusion protein or to the intact capsid protein.

The boundaries of the 132nt capsid binding region were originally arbitrary and had been based on convenient restriction sites in the viral cDNA. For that reason, at first, it seemed surprising that disruptions of this region had considerable effect on binding. The observation that binding of several of the deleted RNA fragments was enhanced by the addition of nonspecific flanking sequences suggests that at least some of the deletions may be perturbing an overall structure rather than eliminating a binding site. This type of disruption has been proposed previously, for example, in studies of the packaging signals in Moloney murine leukemia virus (27) and human immunodeficiency virus RNAs (28).

Encapsidation signals have now been identified for a wide spectrum of different RNA viruses (5, 6). They range in size from about 30 nt for the bacteriophage R17 and Qβ RNAs (29) and the insect nodavirus RNA (30) to 90−100 nt for human hepatitis B virus RNA (31, 32, 33) and up to a 1200−1400

discontinuous stretch of nt in the duck hepatitis B virus RNA (34). Some of these and several others have been identified as a stem-loop or a bulged stem-loop structure (28, 31, 32, 33, 35, 36, 37). Our results show that the context of a 67nt fragment can affect its ability to interact with the 68 amino acid RNA binding domain of the Sindbis capsid protein. They suggest that the stem-loop structure of this region may play a role in this interaction.

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