Identification of a short domain within the non-structural protein NS2 of epizootic haemorrhagic disease virus that is important for single strand RNA-binding activity

J. Theron, H. Huismans and L. H. Nel*

Departments of 1Microbiology and Plant Pathology and 2Genetics, University of Pretoria, Pretoria 0002, South Africa

The role that a conserved amino acid motif, found in the non-structural protein NS2 of orbiviruses, plays in the interaction of this protein with single stranded (ss) RNA was investigated by mutation analysis of the NS2 of epizootic haemorrhagic disease virus. An NS2 mutant in which this motif (amino acids 75 to 83) was deleted was expressed in Spodoptera frugiperda cells by a recombinant baculovirus and found to be unable to bind to poly(U)-Sepharose. The deletion mutant also differed from wild-type NS2 in that it did not appear to be complexed with ssRNA in cells infected with the baculovirus recombinant. Furthermore, the deletion exerted an adverse effect on the ability of NS2 to form inclusion bodies in the cytoplasm of baculovirus-infected insect cells. To further characterize the role of this motif in RNA-binding, specific residues within the region were substituted by site-directed mutagenesis and the mutants were expressed in Escherichia coli as fusion proteins. Analysis of the different mutant proteins indicated that in each case ssRNA-binding was impaired relative to that of the wild-type NS2 control. The degree of impairment corresponded to the number of amino acid substitutions and the largest effects were associated with non-conserved substitutions. It is suggested that the conserved motif is an important structural determinant in the interaction of NS2 with ssRNA.

Introduction

Epizootic haemorrhagic disease, bluetongue and African horsesickness viruses (EHDV, BTV, AHSV) are three distinct serogroups of the genus Orbivirus in the family Reoviridae (Verwoerd et al., 1979). The genomes of these viruses consist of 10 segments of double-stranded RNA (dsRNA) (Bremer, 1976; Huismans et al., 1979; Kusari & Roy, 1986) that are surrounded by a double-layered protein capsid (Verwoerd et al., 1972). Each of the genome segments encodes at least one of the viral polypeptides, which include seven structural proteins (VP1 to VP7) and three non-structural proteins, NS1, NS2 and NS3 (Mecham & Dean, 1988; van Dijk & Huismans, 1988; Grubman & Lewis, 1992). The NS2 proteins of BTV (Huismans et al., 1987b; Thomas et al., 1990), AHSV (Uitenweerde et al., 1995) and EHDV (Theron et al., 1994) have been shown to bind to single-stranded RNA (ssRNA). This characteristic is shared by the non-structural protein βNS of reoviruses (Huismans & Joklik, 1976) as well as NSP3 (NS34) (Boyle & Holmes, 1986) and NS2 (NS35) (Kattoura et al., 1992) of rotaviruses. Therefore, such ssRNA-binding non-structural proteins appear to be common to different members of the family Reoviridae. It has been suggested that the ssRNA-binding activities of these non-structural proteins may play a role in the selection and condensation of the 10 viral ssRNA species into precursor subviral particles, prior to dsRNA synthesis (Huismans & Joklik, 1976; Stamatos & Gomatos, 1982; Huismans et al., 1987b; Mattion et al., 1992). This condensation probably takes place in the granular viral inclusion bodies (VIBs) observed in orbivirus-infected cells (Lecatsas, 1968; Murphy et al., 1971). It has been shown that the NS2 protein of BTV is a major component of the VIBs observed in BTV-infected cells (Eaton et al., 1987; Brookes et al., 1993). Recombinant baculovirus-expressed NS2 of BTV has also been shown to form inclusion bodies not unlike those observed in BTV-infected cells (Thomas et al., 1990). Similar results have been obtained for AHSV and EHDV (Uitenweerde et al., 1995). Little is known about the formation of these structures, although they are presumed to be involved in viral morphogenesis.

Although the corresponding ssRNA-binding proteins of members of the family Reoviridae, namely NS2 in the orbiviruses, βNS of reoviruses and NSP3 of rotaviruses show an extremely low level of sequence similarity, they
do share a nine amino acid consensus sequence [(I/L)-
XMM(I/L)(S/T)XXG] in which five positions have
identical or equivalent amino acids (Van Staden et al.,
1991). The functional importance of this consensus
sequence in ssRNA-binding activity is not known. We
investigated this by analysing a number of EHDV NS2
mutants in which the consensus sequence was either
deleted or modified by site-directed mutagenesis. The
results show that the conserved motif is important for
NS2 interaction with ssRNA. Deletion of the motif
prevented ssRNA-binding from taking place whereas
point mutations within this domain drastically dimin-
ished ssRNA-binding activity.

Methods

Viruses and cells. Wild-type Autographa californica nuclear poly-
hedrosis virus (AcMNPV), a recombinant baculovirus AcEHDV-2.8
which expresses the full-length EHDV-2 NS2 gene (Theron et al.,
1994), and viruses expressing mutations of this gene were propagated
and assayed in suspension or monolayer cultures of Spodoptera
frugiperda (Sf9) cells at 27 °C in Grace's medium containing 10 % (v/v)
fetal calf serum according to the procedures described by Summers &

Deletion of specific residues in the N terminus of NS2. An NS2
deletion mutant, lacking sequences corresponding to amino acids 75 to
83 (nucleotides 242 to 268), was constructed by a PCR as described by
Imai et al. (1991). In this method oligonucleotide primers, designed to
amplify the target in inverted tail-to-tail direction, were used to amplify
the cloning vector together with the target NS2 sequence, thereby
generating the desired mutation, pNS2, a pUC18 plasmid containing a
dNA copy of the full-length EHDV-2 NS2 gene in the BamHI site, was
used as the template in all PCR amplifications. We have included an
unique XhoI restriction site in the design of the primers (Table 1). The
PCR mixture (100 µl) contained 10 µl of 10 × Taq polymerase buffer
(500 mM-KCl, 100 mM-Tris–HCl pH 8.4, 15 mM-MgCl2, 0.1 % gelatin),
1 µl of 25 mM-dNTP mix, 25 ng of template plasmid, 85 pmol of the
sense and antisense primers and 2 U of Taq DNA polymerase
(Promega). The sample was subjected to 30 cycles of amplification at
94 °C for 2 min, 50 °C for 1 min and 72 °C for 4.5 min. The amplicon
was gel purified, digested with XhoI, self-ligated and transformed into
competent Escherichia coli JM109 cells. A resultant NS2-specific clone
was characterized by XhoI digestion and enzymatic sequencing (Sanger et al.,
1977). The deletion mutant NS2 gene was recovered by BamHI
restriction and cloned into pGEX-1 (Smith & Johnson, 1989) and
pAcC129-1 (Livingstone & Jones, 1989) which were designated
pX1.DMNS2 and pAcE2.8DM, respectively. These recombinant
transfer vectors were used to express the mutant NS2 protein in
eukaryotic cells (pAcE2.8DM) and in E. coli (pX1.DMNS2).

Site-directed mutagenesis of NS2. Each of the NS2 site-specific
mutants was constructed by a method in which three primers and two
PCRs were used (Landt et al., 1990). Briefly, in the first round of PCR,
the reaction mixture consisted of 10 µl of 10 × Taq polymerase buffer,
1 µl of a 25 mM-dNTP mix, 25 ng of template plasmid (pNS2), 38 pmol
of a 5'-specific sense primer, 100 pmol of the antisense mutagenic
primer and 1.5 U of Taq polymerase. The thermal parameters used for
the PCR were 2 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C, for a
total of 30 cycles. The amplicons were gel purified and used as
'megaprimers' along with 100 pmol of a 3'-specific antisense primer in
the second round of PCR, as described above, except the cycling
conditions were as follows: 2 min at 94 °C, 2.5 min at 50 °C and 2 min
at 72 °C (40 cycles). The sequences of the mutagenic as well as 5'-
and 3'-specific primers, both of which included BamHI linker sequences,
are shown in Table 1. The amplicons were restricted with BamHI, cloned
into pUC18 and the point mutations were verified by dideoxynucleotide
sequencing. The mutant NS2 genes were recloned into pGEX-1 and
designated pX1.1MNS2, pX1.3MNS2, pX1.3KMNS2 and
pX1.4MNS2 (see Table 2). The construction of the recombinant
gene, was described (Theron et al., 1994).

Transfection and isolation of a recombinant baculovirus. A recombi-
nant baculovirus was obtained by co-transfection of Sf9 cells with
pAcE2.8DM and linearized AcRP23-lacZ DNA (Possee & Howard,
1987; Kitts et al., 1990). Progeny viruses with a white plaque phenotype
were selected and subjected to three successive rounds of plaque
purification. Sf9 cells were infected with these recombinant baculo-
viruses at an m.o.i of 10 p.f.u./cell and harvested at 48 to 72 h post-
infection. Protein samples were mixed with an equal volume of 2 ×
protein solvent buffer (PSB; 125 mM-Tris–HCl pH 6.8, 4 % SDS, 20 %
glycerol, 10 % 2-mercaptoethanol, 0.1 % bromophenol blue) and
analysed by electrophoresis on 12 % polyacrylamide gels containing
SDS (Laemmli, 1970) and by Western blotting.

Fractionation of infected Sf9 cells. Sf9 cells were infected with
recombinant AcEHDV-2.8 or AcEHDV-2.8DM, harvested and cyto-

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Polarity and type of primer</th>
<th>Oligonucleotide sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMNS2†</td>
<td>+ / Del</td>
<td>5'CAGCTCAGGGTAGAGGTAGCACG3'</td>
</tr>
<tr>
<td></td>
<td>- / Del</td>
<td>5'CAACTCGAGGACATGATTCATC3'</td>
</tr>
<tr>
<td>1MNS2</td>
<td>- / Sub</td>
<td>5'GCCCGAAATGATCATAACTA3'</td>
</tr>
<tr>
<td>3MNS2</td>
<td>/ Sub</td>
<td>5'CATTCCGCATAAGATCTACA3'</td>
</tr>
<tr>
<td>3KMNS2</td>
<td>/ Sub</td>
<td>5'CACCCGCGAATGACATCATTGC3'</td>
</tr>
<tr>
<td>4MNS2</td>
<td>/ Sub</td>
<td>5'TCCGAGATCGCTTAAATCTG3'</td>
</tr>
<tr>
<td>External†</td>
<td>+ / 3' end</td>
<td>5'CGAGGATCCGTTAAATCTG3'</td>
</tr>
<tr>
<td></td>
<td>- / 3' end</td>
<td>5'CGAGGATCCTGTTAAATCTG3'</td>
</tr>
</tbody>
</table>

* Mispaired bases are shown in bold italic lower-case letters.
† The unique XhoI sites included in the primers (see Methods) are underlined.
‡ NS2-gene-specific end primers used in site-directed mutagenesis procedures (see Methods). The
BamHI sites are underlined.

Table 1. Sequences of PCR primers used for constructing NS2 mutants
plasmic fractions (Huismans et al., 1987b) prepared in 0.01 M-STE-TX (10 mM-NaCl, 10 mM-Tris-HCl pH 7.4, 1 mM-EDTA, 0.5% Triton X-100). The particulate fraction (P100) was separated from the cytoplasmic fraction by centrifugation through a 1 ml layer of 40% sucrose (10 mM-NaCl, 10 mM-Tris-HCl pH 7.4, 1 mM-EDTA, 0.5% Triton X-100) with 10 mM-2-mercaptoethanol. The salt concentration of the 35S-labeled P100 fractions was adjusted to 0.15 M-NaCl and diluted to 0.15 M-NaCl just prior to being loaded onto the poly(U) columns. After extensive washing of the columns with equilibration buffer, the bound protein samples were eluted with 20 ml linear gradients of 0.15 to 1.15 M-NaCl in TE buffer (10 mM-Tris-HCl, 1 mM-EDTA, pH 7.4) containing 10 mM-2-mercaptoethanol as described by Huismans et al. (1987b). The protein content of each fraction was investigated by scintillation counting. Peak fractions were pooled, the proteins concentrated by KCl-SDS precipitation (Huismans et al., 1987a) and analysed by SDS-PAGE and autoradiography.

Sedimentation analysis. SF9 cells (1 x 10^7 cells) infected with the respective NS2 baculovirus recombinants were harvested and cytoplasmic extracts (300 µl) prepared as described above. Aliquots of each extract (100 µl) were left untreated or either treated with RNase A (20 µg for 10 min) or with high salt (0.5 M-NaCl). The NaCl concentration of each sample was adjusted to 0.15 M-NaCl just prior to analysis in 10 to 40% linear sucrose gradients (prepared in 0.15 M-STE-TX buffer). The gradients were centrifuged for 16 h at 40000 r.p.m. at 4 °C in a Beckman SW50.1 rotor and fractions were collected dropwise from the bottom of the gradients (approximately 400 µl/fraction). Each of the gradient fractions was analysed by SDS-PAGE and Western blotting.

Table 2. Amino acid sequence of EHDV-2 NS2 and mutations constructed by mutagenesis

<table>
<thead>
<tr>
<th>NS2 proteins</th>
<th>Type of mutation</th>
<th>Amino acid sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTNS2</td>
<td>Non-mutant</td>
<td>I S L M I S A D G</td>
</tr>
<tr>
<td>DMNS2</td>
<td>Deletion</td>
<td>L E</td>
</tr>
<tr>
<td>1MNS2</td>
<td>Substitution</td>
<td>I S L I I S A D G</td>
</tr>
<tr>
<td>3MNS2</td>
<td>Substitution</td>
<td>I S L I V A A D G</td>
</tr>
<tr>
<td>3MNS2</td>
<td>Substitution</td>
<td>I S L K V A A D G</td>
</tr>
<tr>
<td>4MNS2</td>
<td>Substitution</td>
<td>I S L I V A A D E</td>
</tr>
</tbody>
</table>

*Bold amino acids indicate substituted amino acids in mutant proteins. Boxed amino acids indicate non-conserved substitutions. Numbering is based on the published EHDV-2 NS2 sequence (Van Staden et al., 1991).

Expression and purification of NS2 fusion proteins in E. coli. Wild-type NS2 and mutant NS2 were expressed as bacterial fusion proteins and purified as previously described (Theron et al., 1994). Briefly, cultures containing the recombinant pGEX plasmids were induced with IPTG for 5 h after which the cultures were harvested, lysed and the glutathione S-transferase (GST)–NS2 fusion proteins purified from the cleared supernatants by affinity chromatography with glutathione agarose (Sigma). Proteins were analysed by SDS–PAGE and Western blot analysis.

**RNA-binding analysis of bacterial-expressed mutant NS2 fusion proteins.** Equal volumes of the purified GST–NS2 fusion proteins were each incubated with 10 mg poly(U)-Sepharose in 500 µl TE-TX buffer at NaCl concentrations between 0.01 and 0.315 M-NaCl as indicated in Fig. 4. After allowing the proteins to bind to the poly(U)-Sepharose (30 min at room temperature with gentle agitation), the poly(U)-Sepharose was collected by centrifugation at 2000 r.p.m. for 1 min and washed twice with 500 µl of the corresponding binding buffer. The supernatant fractions were pooled and proteins precipitated with 4 vols of acetone at −70 °C for 1 h and pelleted by centrifugation at 18000 r.p.m. for 30 min. The bound protein [poly(U)-Sepharose pellets] and unbound protein (precipitated supernatants) were resuspended in PSB, separated by SDS–PAGE and the RNA-binding quantified by scanning of the Coomassie blue-stained protein bands representing the bound and unbound fractions. The bound protein fraction was expressed as a percentage of the total protein recovered, bound and unbound.

**Electron microscopy.** Monolayers of mock infected SF9 cells infected with either wild-type AcRP23-lacZ or recombinant baculoviruses were harvested 48 h post-infection and fixed for 30 min in 10 mM-sodium cacodylate buffer containing 3% formaldehyde and 0.1% gluteraldehyde. The cells were post-fixed in 0.25% osmium tetroxide for 1 h before being dehydrated in acetone and embedded in Quetol. Ultra-thin cell sections were collected on copper grids, stained in 5% uranyl acetate, counter-stained in 3% lead citrate and then viewed with a Hitachi H-600 electron microscope at 50 kV.

**Results**

**Expression of deletion mutant NS2 protein in infected SF9 cells.** To investigate whether the N-terminal amino acid residues 75 to 83 of EHDV-2 NS2 protein were required for ssRNA-binding or VIB formation, an NS2 deletion protein lacking these amino acids was prepared, as described in Methods, and expressed in SF9 cells by means of a recombinant baculovirus, AcEHDV-2.8DM. The proteins synthesized were analysed by SDS–PAGE (Fig. 1 a). In contrast to the β-galactosidase protein (M_r, 116000) synthesized by the wild-type baculovirus control (AcRP23-lacZ), two unique protein species with M_r values of approximately 46000 and 48000, respectively, were identified in the AcEHDV-2.8DM cell lysate. This separation of mutant NS2 into two distinct bands is in agreement with that observed in the case of the NS2 expressed by the wild-type NS2 recombinant (AcEHDV-2.8) and is typical for baculovirus-expressed NS2 of EHDV (Theron et al., 1994). Both bands reacted with specific antiserum in a Western blot (Fig. 1 b).
Binding of baculovirus-expressed NS2 proteins to poly(U)-Sepharose

The ability of the NS2 deletion protein to bind ssRNA was investigated by poly(U)-Sepharose affinity chromatography of the partially purified 35S-labelled NS2 deletion protein in comparison with a wild-type NS2 protein control. The proteins were obtained from the P100 fractions of AcEHDV-2.8DM- and AcEHDV-2.8-infected cells as described in Methods. The salt concentration of the P100 preparations was initially adjusted to 0.5 M-NaCl to dissociate any pre-existing protein-RNA complexes and then diluted to 0.15 M-NaCl before loading onto poly(U)-Sepharose columns. Unabsorbed proteins were removed by extensive washing in column equilibration buffer and the binding proteins eluted with a linear salt gradient (0.15 to 1.15 M-NaCl). A sample of each fraction was counted in a scintillation counter and the peak fractions, as indicated in Fig. 2(a), were analysed by SDS-PAGE and autoradiography, the results of which are shown in Fig. 2(b). Most of the wild-type NS2 was bound to the column and only eluted at a salt concentration of 0.35 M-NaCl. However, the NS2 deletion mutant did not bind and was eluted in the wash flow-through fraction of the column.

Sedimentation analysis of recombinant baculovirus-expressed NS2

The heterogeneous sedimentation patterns of BTV and EHDV NS2 expressed in Sf9 cells by baculovirus recombinants have been shown to be the result of the association of NS2 with ssRNA (Uitenweerde et al., 1995). To investigate if the NS2 deletion mutant was similarly associated with ssRNA, cytoplasmic fractions of cell lysates containing baculovirus-expressed mutant and wild-type NS2 proteins were centrifuged through 10 to 40% sucrose gradients which were fractionated and compared after SDS-PAGE and Western blot analysis (Fig. 3). The results showed that wild-type baculovirus-expressed NS2 sedimented as a large heterogeneous complex in the sucrose gradient (Fig. 3a). Treatment of the infected cell extract with RNase A prior to centrifugation resulted in the dissociation of the complexes and the NS2 sedimented as a discrete complex of about 7S (Fig. 3b), suggesting that NS2 is bound to ssRNA. Treatment of the wild-type NS2 with a high salt concentration (0.5 M-NaCl) had the same effect on the dissociation of the protein complex (not shown). The mutant protein, in contrast to the wild-type NS2, could only be recovered from the pellet fraction after gradient centrifugation (Fig. 3a). Treatment of the mutant NS2 complex with either RNase A or high salt concentration did not appear to have any effect on its sedimentation (Fig. 3b), in sharp contrast to what has been observed with the wild-type NS2. Thus, it is likely that the mutant protein is unable to bind ssRNA in vivo, which would confirm the results of the in vitro experiments (Fig. 2).

Prokaryotic expression of mutant forms of the NS2 protein

Bacterial overproduction of mutant nucleic acid-binding proteins, as fusion proteins, has been used extensively to facilitate the in vitro analysis of their RNA-binding capability (Bogerd et al., 1991; Hammes & Greene, 1993; Berkowitz et al., 1993; Lee et al., 1993). Thus, considering the simplicity of the bacterial system, the RNA-binding of the EHDV NS2 was further investigated by site-specific mutagenesis of the conserved domain followed by expression as fusion proteins in E. coli. Four substitution mutant gene vectors, pX1.1MNS2 (Met-78 changed to Ile), pX1.3MNS2 (Met-78, Ile-79 and Ser-80 changed to Ile, Val and Ala, respectively), pX1.3KMNS2 (Met-78 changed to Ile), pX1.4MNS2 (Met-78, Ile-79, Ser-80 and Gly-83 changed to Ile, Val, Ala and Glu, respectively) were prepared as described in Methods.

For bacterial expression, E. coli JM109 cells containing the wild-type NS2 or mutagenized NS2 genes under
control of the tac promotor in pGEX-1 vectors were grown in the presence of the tac operon inducer IPTG. The GST–NS2 fusion proteins were partially purified from bacterial extracts by glutathione agarose affinity chromatography and the yield was assessed by SDS–PAGE (not shown). Appropriately sized fusion proteins were in all cases found to be the major component in the cell lysate preparations and, after recovery from glutathione–agarose beads, the fusion proteins were purified to 90 to 95% homogeneity. There was no evidence of degradation of the full-length protein product and the sero-identity of the induced proteins was confirmed by Western blotting (not shown). Only highly purified and intact wild-type and mutant NS2 fusion proteins were used in subsequent experiments.

RNA-binding activity of mutant forms of the NS2 protein

The ability of the substitution mutant NS2 proteins to bind to ssRNA was investigated by a modified poly(U)–Sepharose affinity assay in which the purified proteins
Fig. 3. Sedimentation analysis of cytoplasmic extracts from AcEHDV-2.8- and AcEHDV-2.8DM-infected cells by sucrose density centrifugation. Cytoplasmic extracts were loaded onto linear 10 to 40% sucrose gradients and centrifuged at 40000 r.p.m. for 16 h at 4 °C in a Beckman SW50.1 rotor. Prior to centrifugation the respective extracts were either (a) untreated or (b) incubated with 20 μg RNase A for 10 min. Fractions were collected from the bottom (1) to the top (13) of each gradient and examined by Western blot analysis after resolution by SDS-PAGE. The pellet fractions are indicated by P in all cases.

Fig. 4. Analysis of the ssRNA-binding ability of bacterial-expressed wild-type and mutant NS2 fusion proteins. The fusion proteins were expressed, purified and assayed for poly(U)-Sepharose binding over a wide range of different concentrations of NaCl. The relative amount of NS2 proteins bound to poly(U)-Sepharose was determined by scanning densitometry of stained gels, as described in Methods. The data are the average of two separate experiments. ●, WTNS2; □, 1MNS2; ▲, 3MNS2; ○, 3KMNS2; ■, 4MNS2; △, DMNS2.

were allowed to interact with poly(U)-Sepharose in the presence of increasing concentrations of NaCl, as described in Methods. At higher salt concentrations only those protein-ssRNA complexes which interacted strongly remained as complexes. Thus the step-wise increase in salt concentration was used to measure changes in the degree of mutant NS2-ssRNA interaction (Siomi et al., 1994). The deletion mutant NS2 (pX1.DMNS2) and wild-type NS2 (pX1.WTNS2) served as internal controls. The results (Fig. 4) indicate that deletion of amino acids 75 to 83 (mutant DMNS2) has the most drastic effect on the ability of NS2 to bind to ssRNA. Only 5% of the deletion mutant still bound at a salt concentration of 0.045 M-NaCl while, under the same conditions, almost 80% of the wild-type NS2 remained bound. The effect of the mutation in which four amino acids were substituted (4MNS2) was also drastic with only about 12% of the protein still bound at 0.045 M-NaCl. In this mutant, three conserved residues were substituted with similar residues whereas the fourth was a non-conserved substitution of Gly-83 with the charged acidic residue, Glu (Table 2). The effect of this non-conserved substitution was evaluated by comparison with mutant 3MNS2 which differed from 4MNS2 only in that the Gly-83 residue was left intact. It is clear that this difference results in a marked increase in the binding efficiency of 3MNS2 with about 35% bound at 0.045 M-NaCl. The effect of non-conserved mutations was also investigated in the case of mutant 3KMNS2 which differed from 3MNS2 only in that the Met-78 was substituted with Lys, a charged basic residue. This change resulted in a reduced ssRNA-binding efficiency of 3KMNS when compared with 3MNS2. However, both the three-amino-acid substitution mutants were drastically impaired with respect to ssRNA-binding with a complete lack of binding at 0.09 M-NaCl as compared to more than 50% bound in the wild-type NS2 control. The smallest effect on RNA-binding was observed in the case of the Met-78 substitution with the similar residue Ile (1MNS2). However, this single substitution still impaired ssRNA-binding and resulted in a 25% reduction in the binding capacity relative to the wild-type NS2 control at 0.045 M-NaCl, but this reduction was less than 12% at the higher salt concentrations. The GST portion of the fusion protein expressed from the vector alone without the NS2-specific insert did not bind to poly(U)-Sepharose.

To exclude the possibility that aggregation and insolubility of mutant fusion proteins accounts for their binding behaviour, purified fusion proteins were centri-
fuged at high speed in a microfuge, and the presence of
the NS2-related products in the soluble supernatant or
insoluble pellet fractions was investigated. All of
the proteins were recovered in the supernatant (not shown).
The altered RNA-binding abilities of these mutant
proteins are therefore unlikely to result from non-specific
aggregation of the proteins but rather from specific
structural changes, as demonstrated by sucrose centri-
fugation of deletion mutant NS2. It is noteworthy that,
with the exception of 1MNS2, the ability of the mutant
NS2 proteins to interact with the poly(U) oligo-
nucleotides was completely abolished at salt concen-
trations that were lower than the physiological salt
concentration of 0.15 M-NaCl. This would imply that
these mutant proteins are not capable of forming
NS2–ssRNA interactions in vivo.

Electron microscopy of baculovirus-infected Sf9 cells
The lack of ssRNA-binding by the deletion mutant NS2
protein at physiological salt concentration could in-
fluence the ability of this protein to form VIBs. To
investigate Sf9 cells in which wild-type NS2 or the NS2
deletion mutant was expressed were prepared for electron
microscopic analysis as described in Methods. Granular
structures were observed in the cytoplasm of cells infected
with the respective recombinant baculoviruses (Fig. 5)
and no similar structures were observed in control mock
infected or AcRP23-lacZ virus-infected cells (not shown).
However, in comparison with cells expressing the wild-
type NS2 in which the VIBs were large and dense (Fig.
5a), the VIB-like structures formed by the deletion
mutant NS2 protein (Fig. 5b), expressed to the same high
level, were always dispersed and less defined. Thus,
although the deletion did not affect the subcellular
targetting of the protein, it clearly did have a deleterious
effect on the assembly of NS2 into VIB-like structures.

Discussion
A central unresolved question regarding the morpho-
genesis of viruses with a segmented dsRNA genome
concerns the mechanism whereby individual segments
are recognized, sorted and assembled into mature virus
particles. It has been assumed that the ssRNA species are
encapsidated prior to being used as templates for
negative strand RNA synthesis in the maturing virions.
An important issue in the process of RNA encapsidation
is thus the identification of the portions of the protein
that are required for binding of the ssRNA species. It has
been shown for BTV NS2 that deletion of the first 92
amino acids of the N-terminal end abolished the RNA-
binding ability of the protein (Bremer et al., 1992). In
addition, we have previously shown that the sequence
(I/L)XXM(I/L)(S/T)XXG is present in the N-terminal
region of NS2 or its equivalent in various different
dsRNA viruses (Van Staden et al., 1991). In this paper
we report on the importance of this domain with respect
to ssRNA-binding.

Firstly, a baculovirus-expressed NS2 protein from
which the consensus amino acid motif was deleted lost its
ability to bind to ssRNA on a poly(U)–Sepharose

Fig. 5. Electron micrographs of thin sections prepared from recombinant baculovirus-infected Sf9 cells. The cells were infected with
recombinant baculoviruses AcEHDV-2.8 (a) or AcEHDV-2.8DM (b), harvested at 48 h post-infection, and then processed for electron
microscopy as described in Methods. Sections were stained with uranyl acetate and destained with lead citrate. The bar marker
represents 1.25 μm. N, nucleus; IB, inclusion body.
column. It was also shown that wild-type NS2 existed as a heterogeneous complex in association with ssRNA, whereas deletion mutant NS2 appear to be uncomplexed with ssRNA within infected cells. These results were consistent with the data obtained from the in vitro assays and it was concluded that the amino acid motif plays an important role in nucleic acid-binding.

NS2 forms perinuclear cytoplasmic VIBs which are thought to be the sites at which virus synthesis and assembly occurs in infected cells (Hyatt et al., 1992; Brookes et al., 1993). We have found that deletion of the nine-amino-acid motif did not alter the cytoplasmic compartmentalization of the protein although the deletion did seem to affect VIB formation. This was evidenced by the lack of structural integrity of the bodies observed in mutant baculovirus-infected cells, compared with those in cells infected with the wild-type NS2 baculovirus recombinant. These results differ from those reported for mutant BTV NS2 proteins lacking N-terminal amino acids up to 92 residues in length (Zhao et al., 1994).

In further investigations of the amino acid 75 to 83 domain, site-specific mutants were expressed as soluble fusion proteins in E. coli using pGEX-1 expression vectors and characterized for ssRNA-binding at different salt concentrations. It was found that a single substitution exhibited on average 10 to 15 % less affinity for the nucleic acid substrate than shown by the wild-type NS2, while the other mutant proteins had drastically reduced affinities over the salt concentrations investigated, providing further evidence for the participation of the substituted residues in a functionally important structure. When three or more amino acids were substituted, RNA-binding was almost completely abolished, similar to what has been displayed for the NS2 deletion mutant. The impaired RNA-binding ability of the substitution mutant GST--NS2 proteins could indicate that amino acids from this region form part of an RNA-binding domain or alternatively that these amino acids are important for the formation of the secondary and tertiary structure necessary for ssRNA-binding. We favour the latter explanation, since this sequence does not conform to the generalized RNA recognition motif which has been identified in several RNA-binding proteins (Dreyfuss et al., 1988) and the NS2 protein does not contain any other conserved motifs associated with RNA-binding proteins (Burd & Dreyfuss, 1994). Furthermore, although the short stretch of amino acids contains acidic (D) and hydroxylated (S) amino acids which could potentially form hydrogen bonds with nucleic acid bases (Kenan et al., 1991), this domain is predominantly hydrophobic with low surface probability and the residues would therefore not be available to form interactions with the nucleic acid bases. Northwestern blotting assays are often used for the characterization of RNA-binding proteins (Boyle & Holmes, 1986; McCormack et al., 1992; Ribas et al., 1994; Labbé et al., 1994). Using such a blotting technique, Dennis (1991) has shown that the RNA-binding of baculovirus-expressed BTV NS2 is dependent on protein conformation, as is the nucleic acid-binding ability of BTV VP6 (Hayama & Li, 1994). We therefore conclude that mutations within the conserved motif (amino acids 75 to 83) can affect the structural integrity of NS2 to such an extent that its ability to bind ssRNA may be totally impaired.

Studies regarding the RNA-binding activity of NS2 reported only non-specific ssRNA-binding activity and it is still not known whether specific interaction between NS2 and viral mRNA occurs. Whether different domains are responsible for specific or non-specific binding is also not known at present. If the actual role of NS2 is to select the 10 ssRNA segments for replication, specificity in NS2--mRNA interaction should be demonstrable.

We acknowledge the technical assistance of Julian Jaftha and we thank Alan Hall for providing assistance in the electron microscopic analysis. This work was funded with a grant from the Foundation for Research Development.

References


(Received 6 July 1995; Accepted 22 September 1995)