CHAPTER 4: ANALYSIS OF NUCLEIC ACID BINDING ACTIVITY OF AHSV-6 VP6

4.1 INTRODUCTION

An analysis of the AHSV VP6 sequence data indicated the presence of a helicase motif. This suggests that the protein may have helicase activity similar to BTV VP6. Mechanisms for helicase action propose an ATP-driven conformational rearrangement that generates energy. At least two nucleic acid binding sites are required in this model (Gibson and Thompson, 1994; Kadaré and Haenni, 1997). The two sites would facilitate binding to two single-stranded nucleic acid regions or one single-stranded and one duplex region at an unwinding junction. Helicases may obtain multiple nucleic acid binding sites by oligomerization (Lohman and Bjornson, 1996). In the case of dsRNA viruses, such as AHSV, it may be predicted that the putative helicase would have both dsRNA and ssRNA binding activity. The first question to be addressed was whether AHSV VP6 binds nucleic acids.

It has been reported that BTV VP6 binds viral dsRNA, ssRNA and dsDNA (Roy *et al*., 1990). These authors also localized VP6 in infected cells to the matrix of the virus inclusion bodies (VIBs), virus like particles in the VIBs as well as in the cytoplasm. In 1994, Hayama and Li demonstrated that two domains of BTV VP6, which include three of six identified antigenic epitopes, were required by BTV VP6 to bind dsRNA and dsDNA by electrophoretic mobility shift assays. In 1997, BTV VP6 was found to exhibit an RNA-dependent ATPase activity proposed to be coupled to a dsRNA helicase activity (Stäuber *et al*., 1997).

Numerous assays have been employed for the investigation of nucleic acid binding activity. The nucleic acid binding activity of BTV VP6 was investigated by means of nucleic acid overlay protein blot (north western) assays. Further questions were answered using electrophoretic mobility shift assays (EMSA). Two expression systems were utilized by the groups working on BTV VP6, namely a baculovirus system (Roy *et al*., 1990, Kar and Roy, 2003) and a bacterial system (Hayama and Li, 1994).

Results described in the previous chapter indicated that baculovirus expressed AHSV VP6 is N-glycosylated. The addition of a carbohydrate group to a protein could affect the conformation of the protein and as such affect characteristics of the protein such as the stability, solubility, protein transport, membrane interaction and the biological recognition of the protein. This raised the question of whether N-glycosylation influences the function of the protein with respect to immunological recognition and nucleic acid binding activity of AHSV VP6.

Nucleic acid binding may arise as a result of charge, conformation dependent domains or linear epitopes involved in binding. The mechanism of nucleic acid binding for AHSV

VP6 has as yet not been demonstrated. Hayama and Li (1994), used truncated deletion mutants to map and characterize antigenic epitopes and nucleic acid binding domains of BTV VP6. In order to investigate possible regions of importance for nucleic acid binding in AHSV VP6, deletion mutation analysis was performed. The conservation between two of the regions identified by Hayama and Li (1994) on BTV VP6 (70 and 71) and corresponding regions on AHSV VP6 is low. The epitope designated 75 is well conserved. Common conserved motifs found in other nucleic acid binding proteins were not identified in AHSV VP6. As a result, a series of deletion mutants were generated using restriction endonuclease sites located in the genome segment encoding VP6. The epitopes identified by Hayama and Li (1994) in BTV VP6 were accommodated within this strategy. These truncated proteins were also used to determine immunologically important regions on the AHSV VP6 protein.

The aim of the work described in this chapter was to investigate the binding activity of AHSV VP6. The effect of conditions such as salt concentration, pH and N-glycosylation on binding activity were also investigated. In an attempt to identify regions of importance for binding, a series of truncated peptides were produced and tested for binding activity.

4.2 MATERIALS AND METHODS

4.2.1 NUCLEIC ACID OVERLAY PROTEIN BLOT ASSAYS

Nucleic acid overlay protein blot assays (otherwise known as north/south western binding assays) were performed according to a combination of the methods described by Roy *et al*. (1990) and Mears and Rice (1996). Initially, PAGE gel purified proteins were used for binding assays, later partially purified proteins (by sucrose gradient fractionation) were separated by 15% PAGE. Samples containing an estimated 1μg of the desired protein and an equivalent amount of wildtype proteins were used in each assay. For each assay, samples were electrophoresed in repeats of five. The proteins were transferred to a nitrocellulose membrane using an EC140 mini submerged blotter as described previously (3.2.12). The membrane was cut into 5 identical strips containing a marker, a control and a VP6 sample. Each membrane was placed in a bag and transferred proteins were allowed to renature at 4°C overnight in standard binding buffer (SBB) (Mears and Rice, 1996). Basic standard binding buffer [50mM NaCl; 1mM EDTA; 10mM Tris-HCl, pH 7; 0.02% Ficoll (Sigma); 0.02% polyvinylpyrollidone (Sigma) and 0.02% BSA (Merck)] was prepared using DEPC UHQ and varied in a) NaCl concentration between 50 and 150mM and b) pH. Membranes were allowed to adjust to RT for 30min in fresh SBB. Radioactively labelled probes were applied to membranes and incubated with shaking for 1hr at RT. The SBB containing the probe was subsequently discarded and the membrane was washed three times for 10min each at RT with shaking. The membrane was air-dried and autoradiographed.

4.2.2 COMPETITION ASSAYS

Competition assays using unlabelled nucleic acids as competition agents were performed as follows. Initially, probe saturation points were determined by producing 10 identical membranes as described above (4.2.1). Several ssRNA and dsRNA probes were produced and pooled. The

specific activities of the pooled probes were determined and the probe concentration calculated. Each repeat was incubated with an increasing increment of either labelled ssRNA or dsRNA from 0.05ng to 0.5ng. Following autoradiography, the binding was quantified as described below and the saturation point for ssRNA and dsRNA was determined.

For the first competition assays, 4 x 5 identical repeat membranes were produced. For each set of 5, one membrane was probed with 0.25ng of ssRNA (control). The other four membranes were first incubated with 0.25ng, 0.5ng, 0.75ng or 1μg of either unlabelled ssRNA, dsRNA, ssDNA or dsDNA with shaking for 1hr at RT prior to the addition of the probe. Thereafter, 0.25ng of labelled ssRNA probe was added to each membrane and incubated for a further 1hr at RT with shaking. Membranes were washed with 50mM SBB followed by autoradiography and quantification. The percentage reduction in binding was calculated as a percentage of the control.

Binding was quantified in a manner similar to the method described by Lemay and Danis (1994) and Wang *et al*. (1996b) using a GS300 transmittance / reflectance scanning densitometer (Hoefer Scientific Instruments). Peaks were generated using Gelcompar software (Applied Maths, Kortrijk Belgium). Peaks were weighed and expressed as a percentage of the total window. Three repeats of each assay were quantified and for each repeat the peaks were weighed three times and averaged.

4.2.3 PREPARATION OF SINGLE AND DOUBLE-STRANDED NUCLEIC ACID PROBES

AHSV and BTV dsRNA (large segments) were kindly supplied by Dr M. van Niekerk and Prof H. Huismans respectively. Approximately 1μg of dsRNA was end labelled using T4 polynucleotide kinase (PNK) (Amersham) with 100μCi γ ^{32}P ATP (ICN). The final reaction volume of 50μl contained 500mM Tris-HCl, pH 7.6; 100mM $MgCl_2$; 100mM 2-mercaptoethanol and 6U of T4 PNK. The reaction was incubated at 37°C for 30min and terminated by G-75 Sephadex column chromatography.

ssRNA templates were produced by *in vitro* transcription of the genome segment encoding VP6 of AHSV-3 in pBS. Transcription occurred from the T3 promoter in the presence of 25μCi α^{32} CTP (Amersham) as the only source of CTP, as described in section 3.2.1.

DNA probes were generated by nick translating 1μg of AHSV VP6 gene DNA in the presence of 20μCi of α^{32}dCTP (Amersham). All the probes were purified from unincorporated nucleotides by G-75 Sephadex chromatography (Fernández and Garcia, 1996). The columns were washed with 1mM Tris pH 8.0 and the probes were washed through with 10mM Tris-HCl, 0.1% SDS. Twelve fractions of 100μl were collected and counted in the Beckman LS3801 scintillation counter. Peak fractions were pooled. Single-stranded DNA probes were produced by heating the dsDNA probes to 95°C for 5min followed by rapid cooling on ice immediately prior to use.

4.2.4 SPECIFIC ACTIVITY CALCULATIONS

The amount of label incorporated into each probe was determined by selective precipitation (Doyle, 1996). 1μl of each DNA and dsRNA labelling reaction was diluted 1:100 in 200mM EDTA, pH 8.0 and 3μl were spotted onto a 0.5cm^2 nitrocellulose membrane. SsRNA probes were diluted 1:10. 1μl was spotted onto a membrane. Each filter was air-dried and counted in the presence of 2.5ml scintillation cocktail. This determined the total cpm in the sample. 3μl of the same dilution was selectively precipitated with 0.5 volumes of 7.5M ammonium acetate

(RNA) or 1 volume of 4M ammonium acetate pH 4.5 (DNA) and 2 volumes of 96% ethanol.

Following a 30min incubation at -20°C and centrifugation for 5min at 15 000rpm, precipitated RNA was resuspended in 1 X TE and spotted onto a new membrane. The membrane was air-dried and counted with scintillation fluid in a Beckman LS3801 scintillation counter. This value represented the cpm incorporated into the sample.

The cpm incorporated value for DNA probes was obtained after incubating the precipitation mix on ice for 15min. This was followed by 2min at 37°C to redissolve the free dNTPs which precipitated in the previous step. The precipitated probe was collected by centrifugation for 15min at 15 000rpm. The pellet was washed in 0.5ml 0.67M ammonium acetate, pH 4.5 and 67% ethanol at RT with gentle shaking. The pellet was washed a second time in 90% ethanol, and resuspended in 1 X TE. The sample was spotted onto a new membrane, air-dried and counted in 2.5ml scintillation fluid.

The following formulas were used to calculate the percentage incorporation and the specific radioactivity of each product using the values obtained above.

$$\frac{\text{cpm incorporated}}{\text{total cpm}} \times 100 = \%\ \text{incorporation} \qquad (1)$$

$$\frac{\text{cpm incorporated} \times 33.3 \times \text{total volume of reaction}}{\text{mg input DNA}} = \text{specific activity (cpm/}\mu\text{g)} \qquad (2)$$

$$\%\ \frac{\text{incorporation} \times \text{total cpm added to reaction}}{\text{mg of dsRNA substrate in reaction}} = \text{specific activity (cpm/}\mu\text{g)} \qquad (3)$$

$$\%\ \text{incorporation} \times 99\text{ng} = \text{total ng ssRNA made} \qquad (4)$$

Equation (1) was used for the calculation of the percentage cpm incorporated for DNA (nick translation), dsRNA (5' end labelling) and ssRNA (*in vitro* transcription) probes. The specific activity of the dsDNA probes was calculated using equation (2) where 33.3 is derived from the use of 3µl of a 1:100 dilution. The specific activity of the dsRNA probes was calculated using equation (3). The specific activity of ssRNA probes was calculated using equation (4). The 99ng is derived from the following: if 1µl of a 1:10 dilution was precipitated, 10 x the value represents the cpm/µl. In a total reaction volume of 20µl, the total cpm would be 20 x this value. If 30µCi of labelled CTP at 400µCi/nmole were used, then $^{30}/_{400}$ = 0.075 nmoles of CTP were added to the reaction. If 100% incorporation occurred and CTP represents 25% of the nucleotides in the probe, then 4 x 0.075 = 0.3nmoles of nucleotides were incorporated and 0.3nmoles x 330ng/nmole (where 330ng/nmole = average molecular weight of a nucleotide) = 99ng of RNA synthesized (Titus, 1991).

4.2.5 N-GLYCOSIDASE F DEGLYCOSYLATION

A deglycosylation assay using N-Glycosidase F (Roche) enzyme as described by Hedges *et al*. (1999) was performed. Briefly, sucrose gradient purified baculovirus expressed VP6 samples were made up to a final volume of 100µl with endoglycosidase buffer (50mM sodium phosphate, pH 6.8; 20mM EDTA; 1% NP-40; 0.15% SDS, 1% β-mercaptoethanol and a protease inhibitor

cocktail of aprotinin; leupeptin and pepstatin A at 1μg/ml and phenylmethane sulfonyl fluoride (PMSF) at 5μg/ml). 200mU of N-Glycosidase F (Roche) were added to the mixture and incubated at 30°C for 16hrs in a shaking incubator. The results were analyzed by PAGE followed by PAS staining.

4.2.6 TUNICAMYCIN DEGLYCOSYLATION

Sf9 cells seeded at 1.8 x 10^6 in a six well tissue culture plate were infected with VP6 gene recombinant baculovirus stocks at 10 pfu/cell and treated with tunicamycin similar to the methods of Grubman and Lewis (1992) with modifications described by Theron *et al*. (1994). Briefly, 1h.p.i. the infections were treated with increasing concentrations of tunicamycin from 1μg; 2.5μg; 5μg; 7.5μg to 10μg/ml. Cells were harvested as usual and proteins were separated by PAGE followed by PAS staining. The optimal concentration of tunicamycin was determined and expression was scaled up to 75cm^2 flask infections.

4.2.7 DELETION MUTATION ANALYSIS

Using 5' modified VP6 gene of AHSV-6 cloned in the SP6 orientation as source plasmid, truncated gene fragments were generated using restriction endonuclease sites within the genome segment encoding VP6 (refer to figure 2.6). Restriction endonuclease digestions were performed according to manufacturers protocols (Roche).

Following ligation of the prepared fragments to the prepared vectors, ligation mixes were transformed into XL1blue cells and plated onto ampicillin and tetracycline containing plates. Recombinant pFASTBAC Ht molecules were selected following restriction endonuclease digestion and used for transposition in DH10BAC cells (refer to 3.2.4 – 3.2.7).

4.2.8 SCREENING OF COMPOSITE BACMID DNA BY PCR

Composite bacmid DNA was screened for inserts of the correct size by a standard PCR reaction. Primers annealing to the composite bacmid at the polyhedrin promoter [5' TTC CGG ATT ATT CAT ACC 3'] (polyhedrin primer) and the standard M13 reverse primer which anneals to the opposite side of the mini-*att*Tn7 allow amplification of a wildtype region of approximately 700bp which incorporates part of the *Lac*Z gene and MCS. If an insert were present the amplification product would be the size of the insert plus the wildtype band of 700bp.

The composite bacmids were tested in a Perkin Elmer 9600 system using the following programme: 3min at 93°C to denature the template followed by 30 cycles of 94°C for 45sec; 63°C for 45sec and 72°C for 5min. Amplification products were resolved on a 1% 1 x TAE gel and compared to a wildtype control.

The truncated and full-length control proteins were expressed in Sf9 cells as previously described (3.2.9 – 3.2.11). Sf9 cell lysates were separated by PAGE and transferred to a nitrocellulose membrane for immunological screening by means of a western blot as previously described (3.2.12).

4.2.9 ELECTROPHORETIC MOBILITY SHIFT ASSAYS (EMSA)

EMSA were performed as described by Hayama and Li (1994). Briefly, partially purified bacterially expressed full-length AHSV-6 VP6 protein samples containing increasing amounts of protein were prepared as follows: 1μg; 2μg; and 4μg. Two negative controls namely a) of the corresponding wildtype fraction containing bacterial proteins (1μl and 20μl) and b) 1μg of BSA (Roche). Each sample was incubated with 125ng of AHSV-6 dsRNA segments 1 – 3 (pool 1 purified on two sucrose gradients) in 100mM Tris-HCl; 50mM NaCl and 2.5mM $CaCl_2$, pH 7.0 at

30°C for 30min. The mobility shift of the dsRNA after incubation with increasing amounts of protein was assayed by 0.8% 1 x TAE agarose gel electrophoresis, stained with Ethidium bromide and visualized on a UV transilluminator (312nm tube, Vilber Lourmat).

4.3 RESULTS

In order to function as a helicase, AHSV VP6 would need to have nucleic acid binding activity. Such activity has been demonstrated for BTV VP6 (Roy *et al*., 1990, Hayama and Li, 1994).

In the previous chapter, the expression of AHSV VP6 in a baculovirus and a bacterial system was described. The first objective was to test for nucleic acid binding activity using AHSV VP6 expressed in both systems.

4.3.1 NUCLEIC ACID OVERLAY PROTEIN BLOT ASSAYS

Numerous groups have successfully employed nucleic acid overlay protein blot assays (north / south western blots) for the investigation of nucleic acid binding (Roy *et al*., 1990; Brantley and Hunt, 1993; Labbé *et al*., 1994; Lemay and Danis, 1994; Fernández *et al*., 1995; Bleykasten *et al*., 1996; Mears and Rice, 1996; Wang *et al*., 1996b) to name a few.

Roy *et al*. (1990), reported the use of BTV VP6 that was partially purified by sucrose gradient fractionation for RNA overlay protein blot (north western) assays. Proteins were accordingly partially purified by sucrose gradient purification, separated by PAGE and blotted to nitrocellulose membranes. Probes were generated by radioactively labelling single-stranded and double-stranded RNA and DNA.

AHSV-6 VP6 was expressed in baculovirus and bacterial systems. A preliminary binding assay with γ^{32}-P labelled AHSV dsRNA (end labelled by the addition of a γ^{32}-P ATP molecule) was performed to determine whether AHSV VP6 was able to bind to the dsRNA (figure 4.1). The results show that the baculovirus expressed VP6 was able to bind to the dsRNA with no background binding in the negative control. However, the bacterially expressed VP6 did not bind to the dsRNA in the same assay. All possible controls were in place to ensure the integrity of the bacterially expressed VP6 protein. The same 5' modified VP6 gene was used as source DNA to clone into both baculovirus and bacterial expression vectors. Restriction enzyme analysis was followed by sequencing of the 5' terminal to verify the reading frame. Unique proteins of the same size were obtained. A Western blot on bacterially expressed VP6 could not be performed successfully (refer to section 3.3.4, figure 3.5). Both Western blotting and nucleic acid overlay protein blot assays rely on transfer of proteins separated by SDS PAGE to a membrane. This could be the common underlying factor. Alternatively, there could be something different about the proteins expressed in the two different systems, for example, post- or co-translational modification. Both possibilities were investigated and will be described later. The effect of glycosylation on AHSV VP6 nucleic acid binding activity was investigated (refer to section 4.3.5) and electrophoretic mobility shift

assays (EMSA) were used to determine whether bacterially expressed proteins retained nucleic acid binding activity (refer to section 4.3.12).

The first question addressed was, however, the affinity of the baculovirus expressed VP6 for different nucleic acids.

4.3.2 AFFINITY OF AHSV VP6 FOR DIFFERENT NUCLEIC ACIDS

Roy *et al*. (1990), demonstrated that sucrose gradient purified BTV VP6 bound single-stranded and dsRNA and dsDNA. The objective was to investigate the affinity of AHSV VP6 for AHSV single and double-stranded RNA and DNA as well as a non-specific nucleic acid such as BTV dsRNA. Binding assays were performed as described in section 4.2.1 in SBB (standard binding buffer) with 50mM NaCl, pH 7.

DsRNA probes were prepared by end labelling with γ^{32}P ATP using T4 polynucleotide kinase, ssRNA probes were prepared by *in vitro* transcription of the genome segment encoding VP6 of AHSV-3 in the presence of α^{32}P CTP and DNA probes were prepared by nick translation of the genome segment encoding VP6 of AHSV in the presence of α^{32}P dCTP. SsDNA probes were prepared by denaturing nick translated dsDNA at 95°C for 5 minutes prior to application. All probes were purified from unincorporated free nucleotides by G75 Sephadex chromatography. Specific activities were determined for each type of probe and found to be in the order of dsRNA: $\pm$ 3 x 10^{10} cpm/μg; ssRNA: $\pm$ 1 x 10^{6} cpm/μg; dsDNA and ssDNA: $\pm$ 3 x 10^{6} cpm/μg.

In order to test how the different nucleic acids reacted with AHSV VP6, a nucleic acid overlay protein blot assay was performed. Five repeats of sucrose gradient purified protein were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The five repeats were separated and each membrane was equilibrated in SBB. Each membrane was probed with a different labelled nucleic acid probe. AHSV VP6 was found to bind single-stranded and dsRNA and DNA as well as BTV dsRNA in (figure 4.2). This is not a quantitative experiment and strict standardization of the amount of labelled probe and uniform specific activity of each different probe added to each membrane was not applied. Due to differences in labelling method, isotope and specific activities of the probes, the five lanes (A-E) are not comparable. What this result shows is that AHSV VP6 binds different types of nucleic acids well.

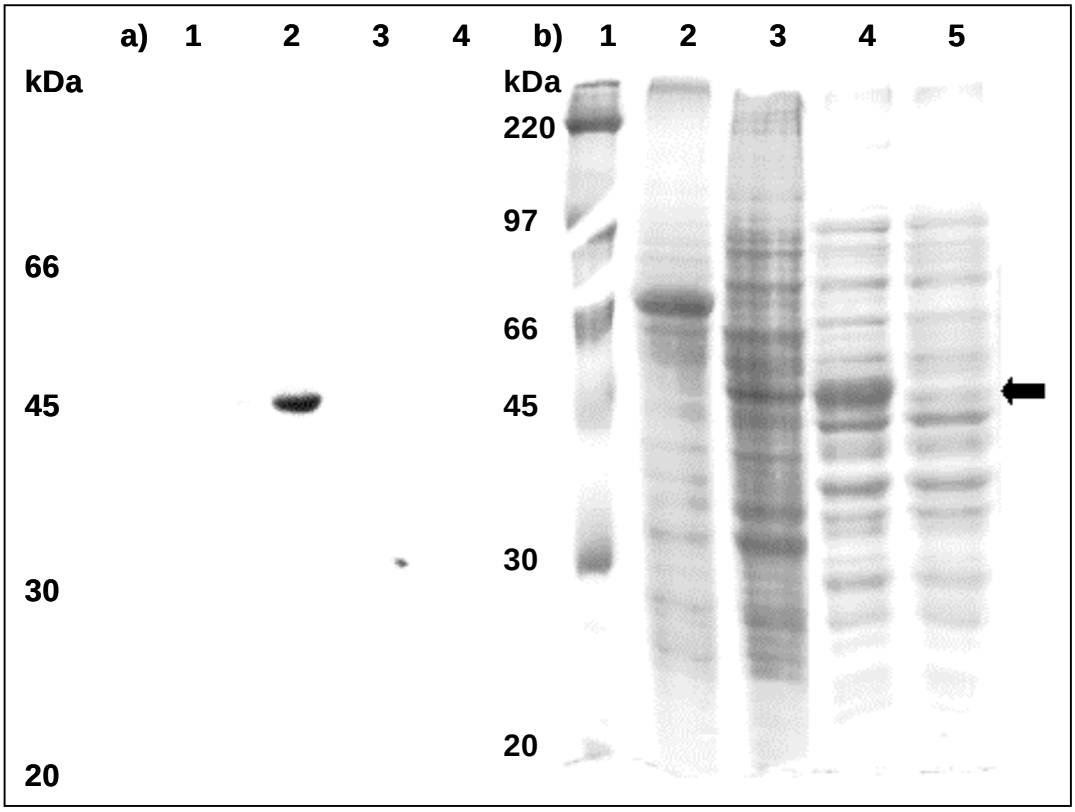

Figure 4.1 Comparison of nucleic acid binding activity of baculovirus and bacterially expressed AHSV VP6.

(a) Nucleic acid overlay protein blot assay of baculovirus and bacterially expressed VP6 probed with a dsRNA probe and (b) Coomassie brilliant blue stained SDS-PAGE control gel.

In a) Lane 1: wildtype infected Sf9 cells;

lane 2: Sf9 cells infected with a baculovirus recombinant expressing VP6;

lane 3: *E. coli* cells expressing VP6 and

lane 4: control *E.coli* cells.

In b) Lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lane 3: Sf9 cells infected with a baculovirus recombinant expressing VP6;

lane 4: *E. coli* cells expressing VP6 and

lane 5: control *E.coli* cells.

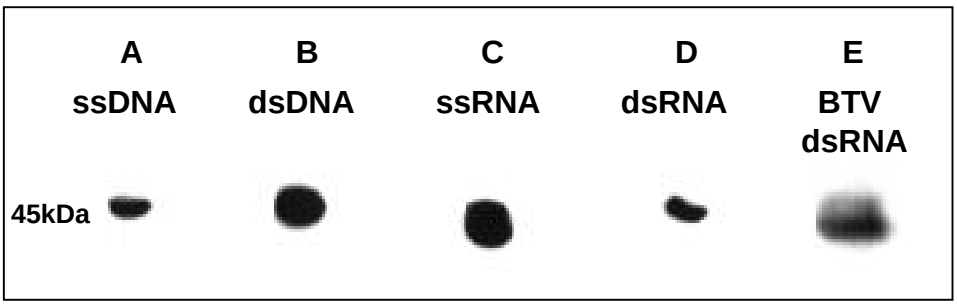

Figure 4.2 Autoradiogram of an assay to determine the affinity of AHSV-6 VP6 to various nucleic acid probes.

Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted onto individual membranes. Nucleic acid overlay protein blots membranes were incubated in SBB with 50mM NaCl, pH 7.

Membrane A was probed with α^{32}P labelled ssDNA;

membrane B was probed with α^{32}P labelled dsDNA;

membrane C was probed with α^{32}P labelled ssRNA,

membrane D was probed with γ^{32}P labelled AHSV dsRNA and

membrane E with γ^{32}P labelled BTV dsRNA.

4.3.3 EFFECT OF SALT CONCENTRATION ON BINDING ACTIVITY

Nucleic acid binding activity may be as a result of conformation dependent domains, linear domains or may be related to charge. Macromolecules such as nucleic acids and proteins may carry a substantial net charge depending on the pH of the solution. These molecules attract oppositely charged small ions resulting in the neutralization of net charge on the molecule (Mathews and van Holde, 1991). High salt concentrations will, therefore, affect charge and play an important role in the efficiency of charge related binding.

Our objective was to determine the effect of NaCl concentration on the nucleic acid binding activity of AHSV VP6. The binding to single-stranded and double-stranded RNA and DNA at a series of NaCl concentrations was assayed (figure 4.3a). For each type of nucleic acid, five repeats of Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted to generate five identical membranes. Each membrane was incubated in standard binding buffer with one of five NaCl concentrations ranging from 50mM to 150mM. Each series of membranes was probed with equal amounts of labelled probe. Following washing, drying and autoradiography, binding was quantified using a GS300 Transmittance / Reflectance Scanning densitometer (Hoefer Scientific Instruments) and Gelcompar software (Applied Maths, Kortrijk, Belgium). In figure 4.3,

a) is a sample assay and b) is the average of the quantification of three repeats of the same assay.

Maximum binding for all four nucleic acid species occurred at a concentration of 50mM NaCl (figure 4.3a and b). Therefore, decrease in binding activity was measured as a percentage of binding at 50mM. Binding decreased continuously with increased NaCl concentration. At 75mM, AHSV VP6 bound about 80% of the dsRNA, ssRNA and ssDNA that was bound at 50mM salt concentration. At 75mM, 69% of the dsDNA was bound by VP6. At 125mM, VP6 no longer bound ssDNA while dsRNA, dsDNA and ssRNA bound at ± 39%, 27% and 20% respectively of that bound at 50mM. At 150mM, dsRNA and dsDNA were bound at ±19% and 17% respectively while ±6% ssRNA was bound (figure 4.3b).

Salt concentration has a marked effect on the binding activity of AHSV VP6. Most nucleic acid overlay protein blot assays are performed at 50mM salt concentration. These results have shown that when the salt concentration in this binding assay is increased, the nucleic acid binding is strongly affected.

Having established that AHSV VP6 binds various nucleic acids and this binding is negatively influenced by increased salt concentrations, the next step was to investigate whether a preference for one type of nucleic acid over another could be established.

4.3.4 INVESTIGATION OF NUCLEIC ACID PREFERENCE

If VP6 functions as the helicase of AHSV, a preference for binding RNA over DNA may be expected. The objective was to determine whether a nucleic acid preference could be determined for AHSV VP6.

Saturation points were estimated for ssRNA and dsRNA. For both probes, ten repeats of Sf9 cells infected with a baculovirus recombinant expressing VP6 purified by sucrose gradient fractionation were separated by SDS-PAGE and blotted to produce ten identical membranes. Each membrane was incubated in standard binding buffer with 50mM NaCl, pH 7. Each membrane was probed with an increasing amount of labelled probe. Following washing, drying and autoradiography, binding was quantified. Binding saturation points for 1μg of VP6 were estimated at 0.2ng for dsRNA and 0.25ng for ssRNA.

Competition assays were performed in SBB with 50mM NaCl, pH 7 with either a ssRNA probe or a dsRNA probe. Initially, a labelled ssRNA probe was used in the presence of increasing amounts of unlabelled ssRNA, dsRNA, ssDNA and dsDNA. Unlabelled nucleic acid was added before the probe was applied to the membrane according to the saturation point established for the two probes as follows: equal amounts of unlabelled nucleic acid (1 x); twice as much unlabelled nucleic acid (2x); three times (3x) and four times (4x) as much unlabelled nucleic acid. The purpose was to investigate whether a

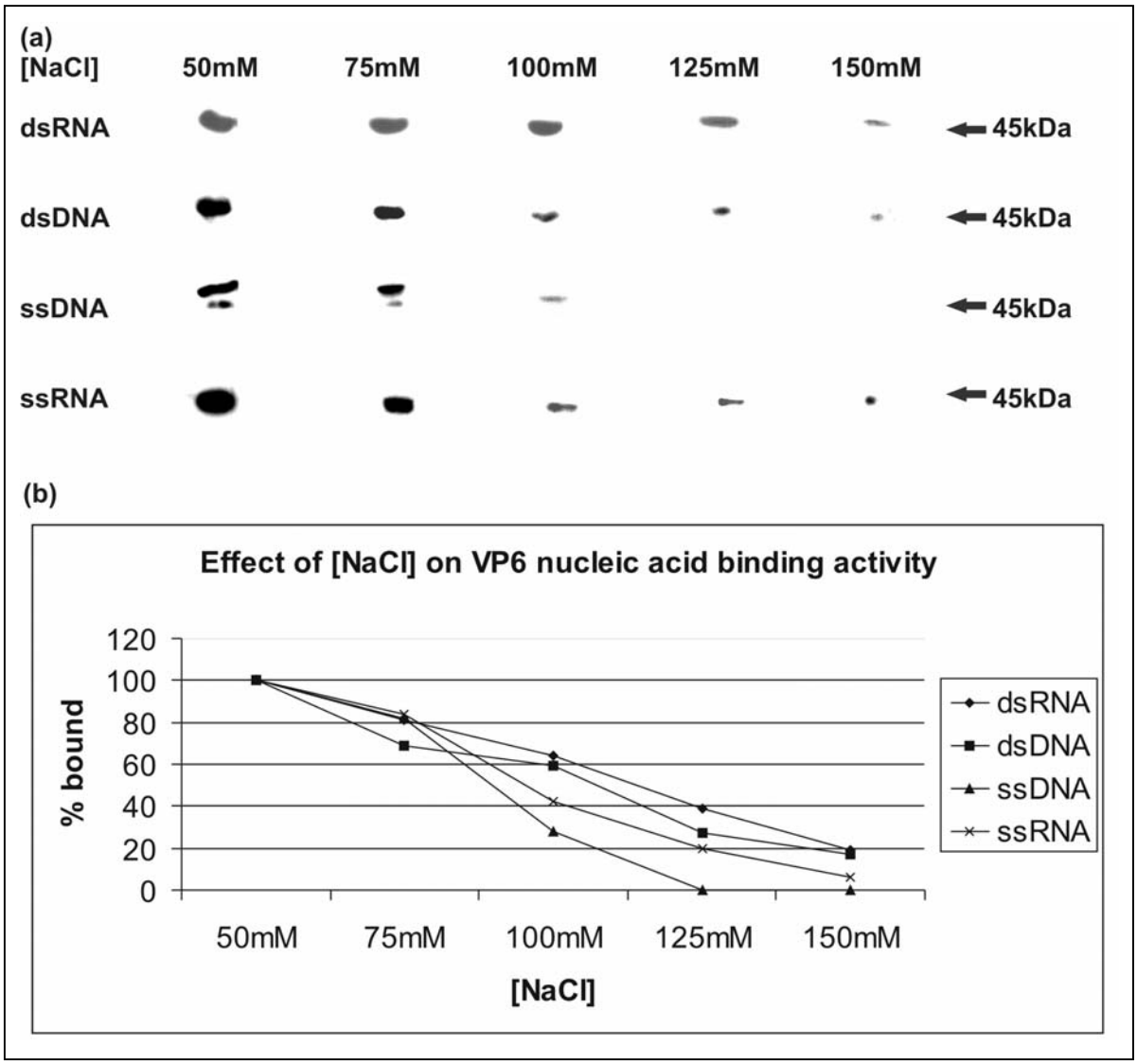

Figure 4.3 Investigation of the effect of salt concentration on VP6 nucleic acid binding activities.

(a) Autoradiograms of nucleic acid overlay protein blots using dsRNA, dsDNA, ssDNA and ssRNA probes at 5 NaCl concentrations ranging from 50mM to 150mM. Sf9 cells infected with a recombinant baculovirus expressing AHSV-6 VP6 were loaded in 5 repeats alongside markers and separated by SDS-PAGE. Proteins were blotted onto membranes and 5 strips of rainbow marker and VP6 were cut off. For each nucleic acid, an equal amount of probe was added to each membrane at a different salt concentration. Horizontal strips represent the area on the autoradiograph corresponding to the 45kDa ovalbumin of Rainbow marker with the 5 membranes aligned according to the marker. (b) Graph representing the effect of NaCl concentration on AHSV-6 VP6 nucleic acid binding quantified using a densitometer and expressed as a percentage of the binding at 50mM NaCl (average of three repeats).

preference for RNA over DNA could be established. In the second competition assay a dsRNA probe was applied in the presence of unlabelled ssRNA and unlabelled dsRNA to determine whether a preference for ssRNA or dsRNA could be established. Each assay was repeated three times. In both competition assays, reduction of binding was measured as a percentage of the control which in each assay consisted of the probe without the competition agent. Binding was quantified using a GS300 Transmittance / Reflectance Scanning densitometer (Hoefer Scientific Instruments) and Gelcompar software (Applied Maths, Kortrijk, Belgium).

A ssRNA probe was applied and unlabelled ssRNA, dsRNA, ssDNA and dsDNA were used as competition agents (figure 4.4). Both single-stranded and double-stranded DNA did not result in a large reduction in the binding of the ssRNA probe (figure 4.4). After the addition of 4x dsDNA, binding was reduced by less than 30%. The addition of 3x unlabelled DNA resulted in a reduction of less than 20% in the binding. The addition of increasing amounts of unlabelled ssRNA caused a continuous decrease in the binding of labelled ssRNA as expected. DsRNA caused an initially larger decrease in labelled ssRNA binding (1x dsRNA reduced binding by 33% as opposed to a 22% reduction caused by the addition of the same amount of unlabelled ssRNA). This decrease evened out with a 62% (dsRNA) and 63% (ssRNA) reduction in binding of labelled ssRNA after the addition of 4x competition agent. From these results it appears that VP6 has a preference for binding RNA over DNA. This is in line with what would be expected of a putative RNA helicase.

With the objective of comparing AHSV VP6 affinity for ssRNA and dsRNA, competition assays were performed with a labelled dsRNA probe and unlabelled ssRNA and dsRNA (figure 4.5). The reduction of binding was consistent for both ssRNA and dsRNA with the addition of unlabelled nucleic acid. There was once again initially a greater decrease of binding following the 1x and 2x unlabelled dsRNA than found using unlabelled ssRNA. When 4x dsRNA was added, binding was reduced by 41%, the equivalent addition of ssRNA as competition agent resulted in a 35% decrease in binding. These results suggest the possibility of a slight preference for binding dsRNA by AHSV VP6.

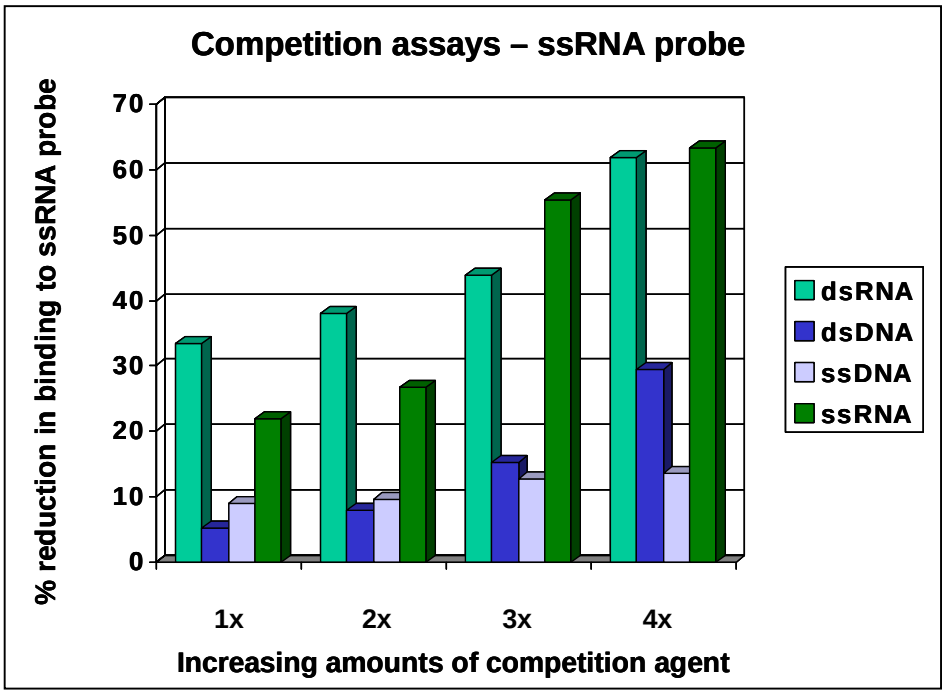

Figure 4.4 Competition assays to investigate a nucleic acid binding preference for AHSV VP6 using a ssRNA probe.

Graph indicating the percentage reduction in binding of labelled ssRNA by VP6 in the presence of increasing amounts of competition agent compared to the binding of the control (average of three repeats). Binding was assayed by nucleic acid overlay protein blots. The control membrane was probed with 0.25ng of ssRNA probe. 1x, 2x, 3x and 4x indicate the percentage reduction in binding of the probe to VP6 with prior incubation with 0.25ng, 0.5ng, 0.75ng and 1ng of unlabelled nucleic acid respectively.

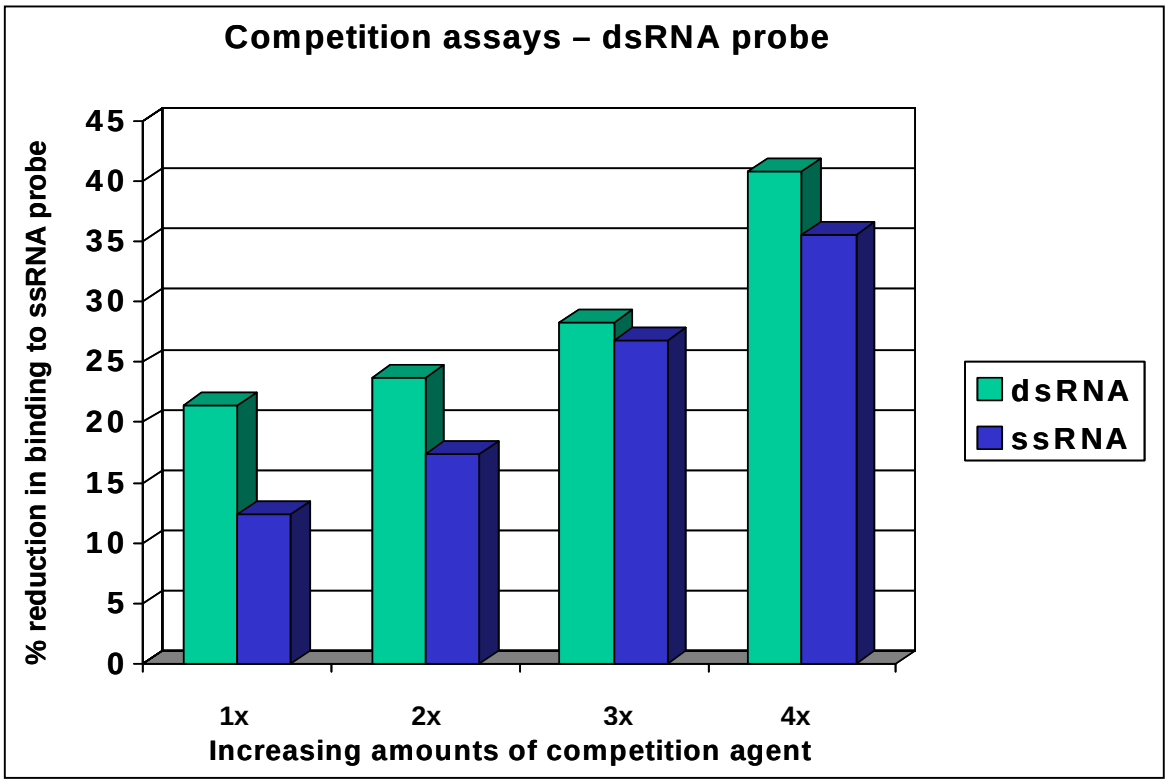

Figure 4.5 Competition studies for binding preference between ssRNA and dsRNA by AHSV VP6.

Graph indicating the percentage reduction in binding of labelled dsRNA by VP6 in the presence of increasing amounts of competition agent compared to the binding of the control (average of three repeats). Membranes were probed with 0.2ng of labelled dsRNA with increasing amounts of unlabelled ssRNA and dsRNA as competition agents {i.e. 0.2ng (1x); 0.4ng (2x); 0.6ng (3x) and 0.8ng (4x)}.

4.3.5 DEGLYCOSYLATION OF BACULOVIRUS EXPRESSED VP6

Soulard *et al*. (1993), described a glycosylated RNA binding protein P43 (hnRNP G). They suggested that the glycosylation may be important in the regulation of binding activity. AHSV VP6 expressed in a baculovirus system is glycosylated. Bacterially expressed AHSV VP6 is not glycosylated as glycosylation does not occur in prokaryotes. It was found that bacterially expressed VP6 did not bind a dsRNA probe in a nucleic acid overlay protein blot assay (refer to section 4.3.1). In view of the work done on P43 and the problems experienced with nucleic acid binding assays using bacterially expressed VP6, the question arose of whether glycosylation of AHSV VP6 plays a role in the binding activity of the protein.

In order to investigate the role of glycosylation in AHSV VP6 binding activity, the baculovirus expressed VP6 was deglycosylated and its binding activity was compared to that of the glycosylated VP6. The first step was to successfully deglycosylate baculovirus VP6. Two strategies for deglycosylating baculovirus expressed VP6 were pursued. Initially, a deglycosylation assay using N-Glycosidase F (Roche) enzyme as

described by Hedges *et al*. (1999) was performed. N-Glycosidase F cleaves asparagine bound N-glycans under certain conditions. It is, however, difficult to determine whether a protein has been entirely deglycosylated or whether it has only been deglycosylated sufficiently to avoid detection by PAS staining. Another difficulty arose as a result of the 16hr incubation at 30° C, which in spite of the protease inhibitor cocktail present, resulted in significant protein degradation.

In the light of these obstacles, tunicamycin was used to block the addition of all N-linked side chains to usually glycosylated cellular proteins (Gallagher *et al*., 1992). Tunicamycin may inhibit protein synthesis, however, all cell systems are not tunicamycin sensitive (Elbein, 1987).

To establish optimal concentrations of tunicamycin required for inhibition of the N-glycosylation pathway and to establish the effect of tunicamycin (if any) on VP6 protein synthesis in Sf9 cells, cells were inoculated with VP6 recombinant bacmid in the presence of increasing amounts of tunicamycin (1μg; 2.5μg; 5μg; 7.5μg and 10μg/ml). Coomassie brilliant blue staining revealed that there was no inhibition of protein synthesis by the addition of tunicamycin. Moreover, 2.5μg/ml was sufficient for inhibition of glycosylation of VP6 expressed in Sf9 cells as evidenced by PAS staining (results not shown).

4.3.6 EFFECT OF DEGLYCOSYLATION ON VP6 SOLUBILITY

According to Elbein (1987), N-glycosylation may increase the solubility of a protein and as such the removal of the glycosyl group may decrease the solubility. Baculovirus expressed VP6 in its glycosylated form was found to be soluble and was recovered from four fractions (11 – 14) at the top of the sucrose gradient (refer to section 3.3.5, figure 3.6a). The solubility of tunicamycin treated AHSV VP6 was also investigated by sucrose gradient fractionation (figure 4.6). The deglycosylated VP6 was also found at the top of the gradient (fractions 8 – 10, lanes 11 – 13 in figure 4.6) indicating that deglycosylation has no effect on the solubility of the protein.

4.3.7 INVESTIGATION OF THE ROLE OF N-LINKED GLYCOSYLATION OF VP6

Levels of protein synthesis may be negatively affected by Tunicamycin treatment (Elbein, 1987). Figure 4.7a shows expression of baculovirus VP6 untreated and treated with tunicamycin. It is clearly evident that the expression levels are not affected. In order to investigate a possible role for glycosylation in the function of AHSV VP6, immunological recognition and nucleic acid binding of deglycosylated baculovirus expressed VP6 were assessed. A Western immunoblot was performed to establish whether glycosylation contributed to recognition of VP6 by guinea pig anti-AHSV-3 antiserum (figure 4.7b). In the western immunoblot, tunicamycin treated VP6 reacted positively indicating that antibody recognition on a western blot is not influenced by the addition of carbohydrate to the protein. In order to assess the role of glycosylation in the nucleic acid binding activity of VP6, a nucleic acid overlay protein blot binding assay

with dsRNA as a probe was performed. Both glycosylated and unglycosylated VP6 bound the dsRNA probe with equivalent efficiency (figure 4.7c). This result indicates that glycosylation has no direct role in the binding activity of baculovirus expressed AHSV VP6. Post- or co-translational modification is, therefore, not the explanation for the lack of binding activity of bacterially expressed VP6 in a nucleic acid overlay protein blot binding assay.

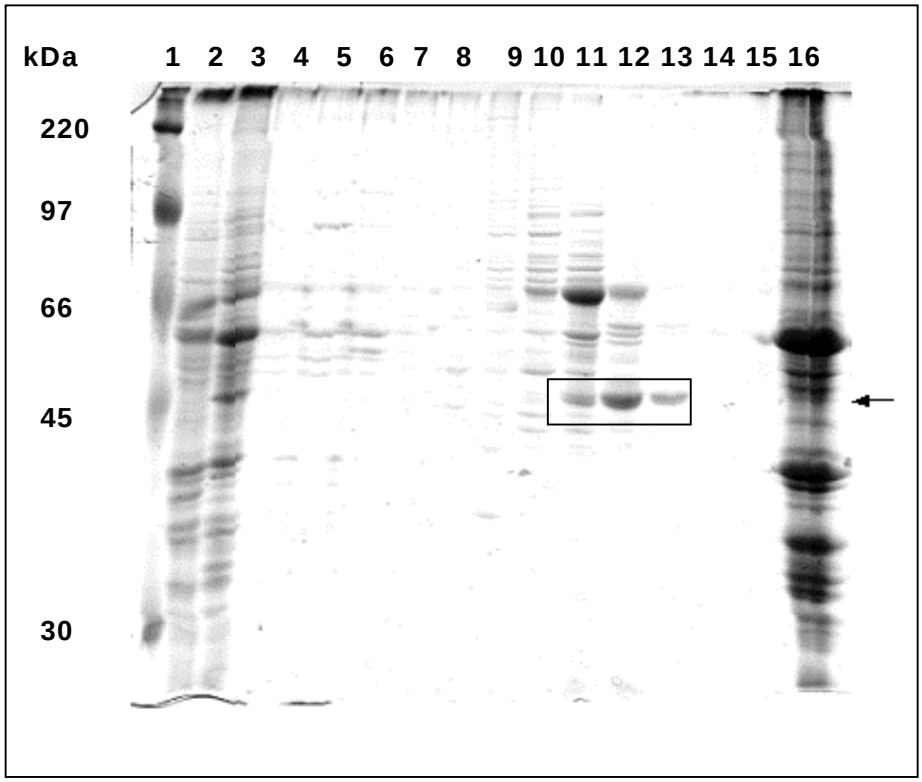

Figure 4.6 Sucrose gradient fractionation of baculovirus expressed VP6 treated with tunicamycin.

A Coomassie brilliant blue stained SDS-PAGE gel of fractions collected from a sucrose gradient where samples were loaded onto a linear 10 – 40% sucrose gradient and centrifuged for 16hrs at 40 000rpm in a SW50.1 rotor in a Beckman L-70 ultracentrifuge.

Lane 1:	Rainbow marker;
lane 2 and 3:	wildtype vector infected cell lysate and tunicamycin treated recombinant baculovirus expressing VP6 infected cell lysate respectively;
lanes 4 – 15:	sucrose fractions 1- 12 (where fraction 1 was collected from the bottom of the gradient and fraction 12 from the top);
Lane 16:	is a sample from the pellet.

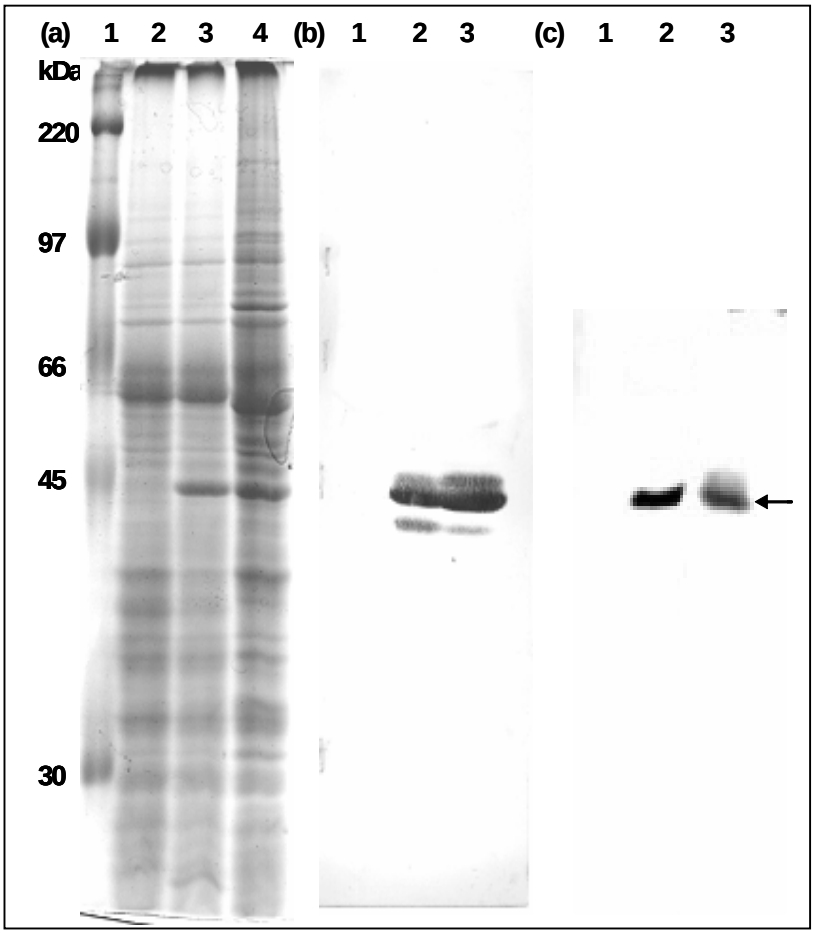

Figure 4.7 Analysis of the role of N-glycosylation in AHSV VP6 nucleic acid binding activity.

In (a), SDS-PAGE control gel stained with Coomassie brilliant blue showing protein expression.

Lane 1: Rainbow marker;

lane 2: wildtype baculovirus infected Sf9 cells;

lane 3: Sf9 cells infected with a recombinant baculovirus expressing VP6

lane 4: Sf9 cells infected with a recombinant baculovirus expressing VP6 treated with 2.5μg/ml tunicamycin.

In (b) and (c) where (b) Western immunoblot to assess immunological recognition and (c) Nucleic acid overlay protein blot assay using a dsRNA probe and proteins partially purified by sucrose gradient fractionation.

Lane 1: wildtype baculovirus infected Sf9 cells;

lane 2: Sf9 cells infected with a recombinant baculovirus expressing VP6

lane 3: Sf9 cells infected with a recombinant baculovirus expressing VP6 treated with 2.5μg/ml tunicamycin.

4.3.8 PREPARATION OF BACULOVIRUS RECOMBINANTS THAT EXPRESS DIFFERENT TRUNCATED VP6 PEPTIDES.

Truncated proteins are useful in the identification of regions involved in nucleic acid binding. In order to investigate which regions are important for nucleic acid binding, a series of VP6 gene deletions were cloned and the truncated proteins expressed in Sf9 cells.

Analysis of the primary structure of AHSV VP6 did not reveal common motifs found in other nucleic acid binding proteins. Two of the domains involved in binding identified by Hayama and Li (1994) in BTV VP6, namely 70 and 71, were not well conserved in AHSV VP6. The third domain designated 75, is well conserved in AHSV, BTV, Chuzan and St Croix River virus VP6. A series of deletion mutants were generated using restriction enzyme sites in the gene (refer to figure 2.6). Deletion mutants were constructed that lack the amino terminal, the carboxyl terminal or both. Constructs VP6mt1-189 and VP6mt190-369 represent the amino and carboxyl halves of the full-length protein respectively. Truncated proteins VP6mt1-289 and VP6mt116-289 incorporate regions equivalent to domains 70 and 71 identified by Hayama and Li (1994). Truncated proteins VP6mt116-369 and VP6mt190-369 incorporate regions corresponding to all three domains (namely 70, 71 and 75).

Figure 4.8 summarizes the cloning strategy. Six truncated proteins were generated utilizing restriction endonuclease sites within the genome segment encoding VP6. The 5' modified VP6 gene of AHSV-6 cloned in the SP6 orientation of pGEM, was used as source plasmid for the generation of the truncated proteins. The full-length NS2 gene (segment 8, negative control) and the full-length VP6 genes (positive control) were digested out of their source plasmids using *Bam*HI and cloned into suitably prepared pFASTBAC Hta and Htb vectors respectively. AHSV NS2, which binds single-stranded RNA (Huismans *et al*., 1987a) but which does not bind dsRNA (Theron and Nel, 1997), was cloned and expressed for use as a control. Putative pFASTBAC Ht recombinants were screened using restriction endonucleases for insert and orientation. Recombinants were used for the generation of recombinant bacmids by transposition in DH10BAC cells. The equivalent positions of the regions identified as being important in dsRNA binding of BTV VP6 (regions 70,71 and 75) (Hayama and Li, 1994) are also indicated in figure 4.8. Of these, only region 75 is well conserved in different orbiviruses.

Composite bacmid DNA was screened for inserts by means of a standard PCR reaction using the polyhedrin promoter and the M13 reverse primers. A negative control containing no template DNA and a positive control using wildtype bacmid DNA as template were performed. Agarose gel electrophoresis showed the expected wildtype band in the order of 700bp (figure 4.9). The full-length NS2 and VP6 gene clones yielded the expected 1866 and 1852bp amplification products respectively. Clones for the expression of truncated proteins VP6mt1-115, VP6mt1-289, VP6mt1-189, VP6mt116-289, VP6mt116-369, and VP6mt190-369 yielded amplification products corresponding to the segment cloned plus the wildtype 700bp band namely: 1046bp;

1565bp; 1267bp, 1219bp; 1462bp and 1243bp respectively. Bacmids confirmed as positive recombinants using PCR were used for the transfection of Sf9 cells.

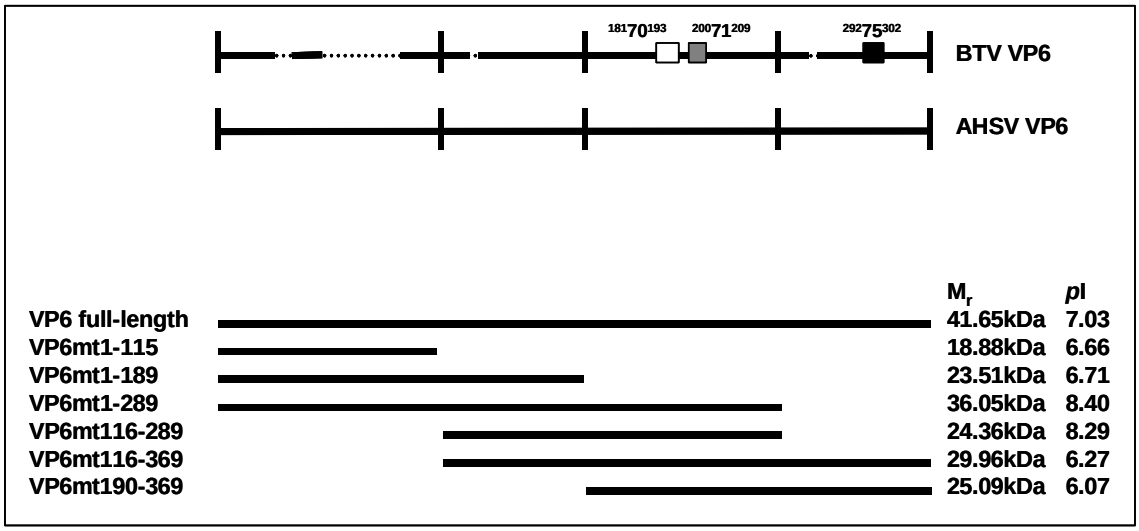

Figure 4.8 Schematic diagram illustrating the cloning strategy for the production of truncated VP6 peptides.

The positions of the binding domains identified on BTV VP6 (Hayama and Li, 1994) are indicated relative to AHSV VP6. Molecular weights and pI values were estimated for the expressed peptides including non-viral generated extensions using Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998) available through EXPASY (expert protein analysis system) hosted by the Swiss Institute of Bioinformatics.

4.3.9 EXPRESSION OF TRUNCATED PROTEINS IN A BACULOVIRUS SYSTEM AND IMMUNOLOGICAL SCREENING BY WESTERN BLOT

A series of truncated proteins with non-viral generated N-terminal (His-tag domain) and in some cases C-terminal extensions (fragments without the VP6 C-terminal) were expressed in *Spodoptera frugiperda* cells. Full-length VP6 and NS2 were used as positive and negative controls respectively. Figure 4.10a and b show the SDS-PAGE profile of the six truncated VP6 constructs prior to sucrose gradient purification. The truncated VP6 proteins expressed at comparatively similar levels. The proteins all exhibited slower electrophoretic mobility than predicted for the size of the genome segment encoding VP6 fragments and non-viral vector generated extensions. Both the full-length proteins of AHSV and BTV (Roy *et al.*, 1990) have been found to show slower electrophoretic mobility than predicted during SDS-PAGE. All the truncated VP6 proteins were found to be soluble and accordingly were partially purified by sucrose gradient fractionation. The truncated proteins were recovered from different fractions in the sucrose gradients in correspondence to the size of each peptide. There are, therefore, differences in the non-VP6 background proteins of the different VP6 peptides used in the binding assays. In a western immunoblot analysis, truncated proteins

VP6mt1-289, VP6mt116-289, VP6mt116-369 and VP6mt190-369 reacted positively to the anti-AHSV-3 antibodies (figure 4.10c). These truncated proteins have a region of amino acids from 191 – 289 in common. This result suggests a linear epitope within that region or a region exposed on the surface of the protein in its native conformation that is involved in immunological recognition.

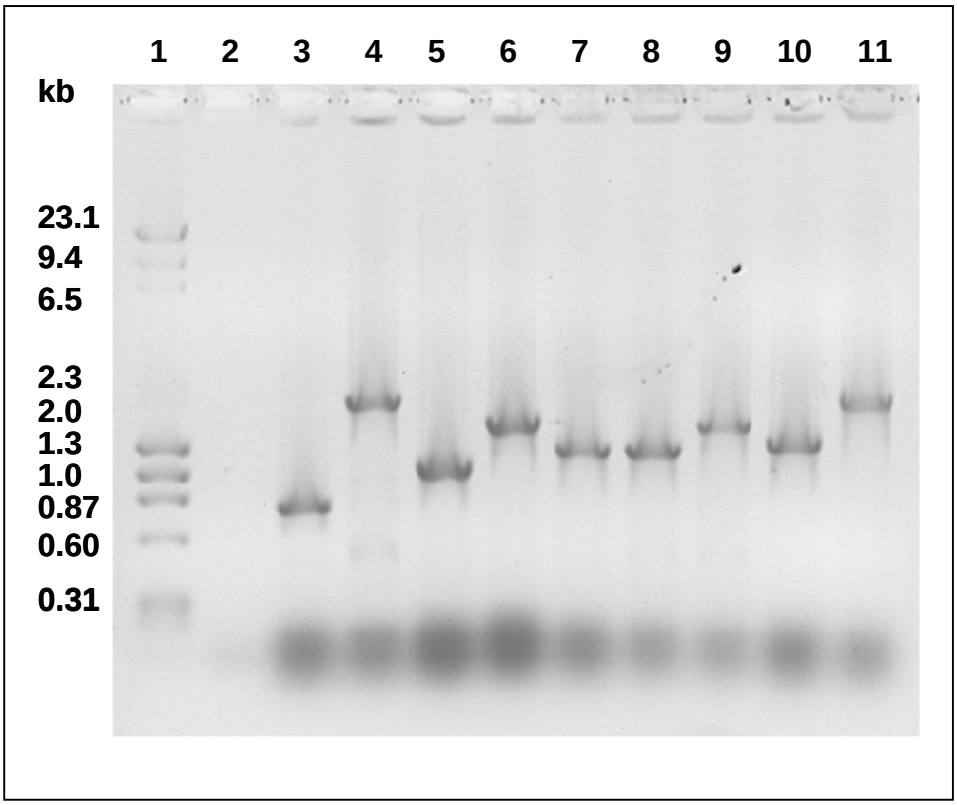

Figure 4.9 PCR screening of recombinant bacmid DNA used for transfection.

PCR products were separated by 1% 1 x TAE agarose gel electrophoresis.

Lane 1: DNA molecular weight marker II (Roche) and ϕX (Promega).

Lane 2: negative control (no template);

lane 3: wildtype bacmid DNA;

lane 4: VP6 recombinant;

lane 5: truncated VP6mt1-115;

lane 6: truncated VP6mt1-289;

lane 7: truncated VP6mt1-189;

lane 8: truncated VP6mt116-289;

lane 9: truncated VP6mt116-369;

lane 10: truncated VP6mt190-369 and

lane 11: NS2 recombinant bacmid.

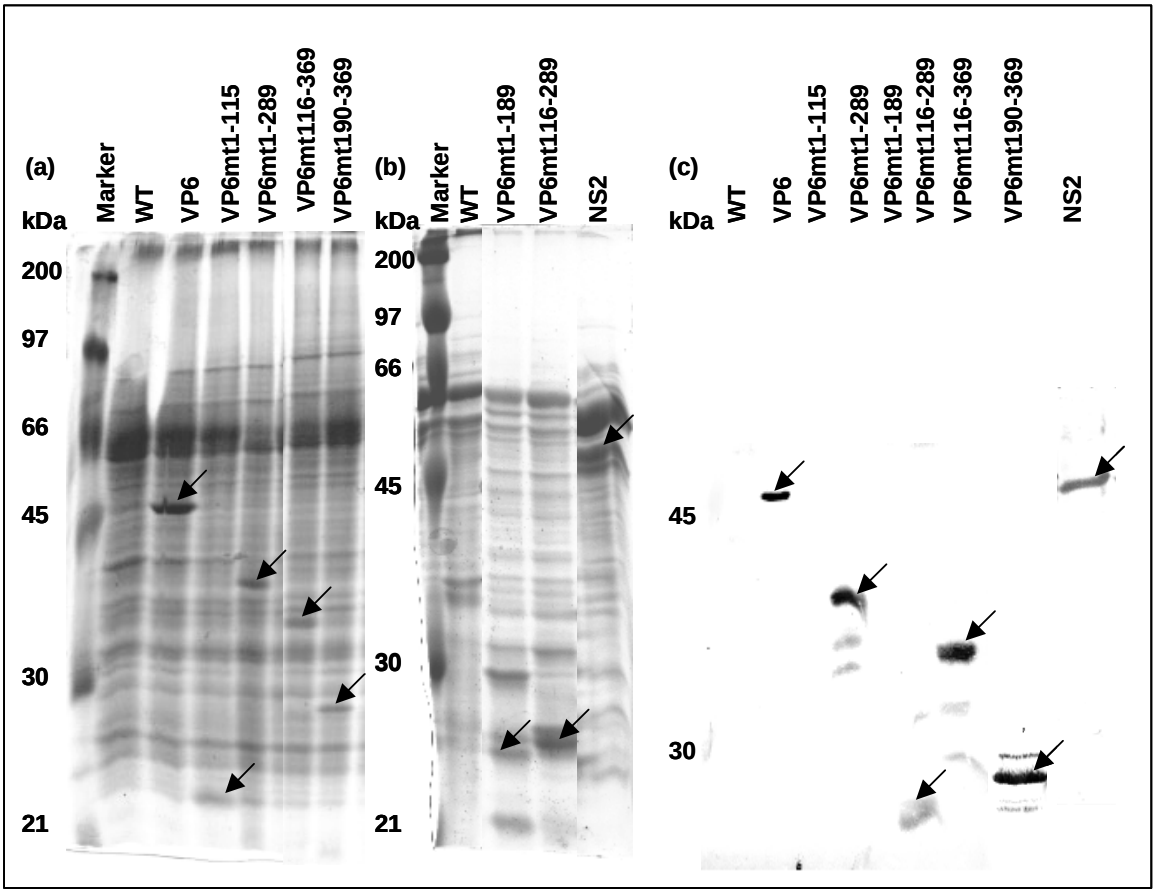

Figure 4.10 Expression and immunological screening of truncated proteins expressed in Sf9 cells.

(a and b) Coomassie stained SDS-PAGE analysis of the expression of truncated proteins in Sf9 cells by means of baculovirus recombinants prior to sucrose gradient purification. (c) Western immunoblot of truncated VP6 proteins using α-AHSV-3 guinea pig antiserum.

Fig 4.10(a):

lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lane 3: full-length VP6;

lanes 4 - 7: truncated VP6 proteins

Fig 4.10(b):

lane 1: Rainbow marker;

lane 2: wildtype infected Sf9 cells;

lanes 3 - 4: truncated VP6 proteins

lane 5: NS2 protein control

Fig 4.10(c):

lane 1: wildtype infected Sf9 cells;

lane 2: full-length VP6;

lanes 3 - 8: truncated VP6 proteins

lane 9: NS2 protein control

4.3.10 BINDING OF DOUBLE-STRANDED RNA BY TRUNCATED VP6 PROTEINS

Samples of sucrose gradient purified truncated proteins, were separated by SDS-PAGE, blotted onto a membrane and incubated in a 50mM NaCl standard binding buffer pH 7.0 in the presence of 1µg of labelled dsRNA (specific activity of 3 x 10^{10} cpm/µg). The membrane was washed as described in materials and methods (refer to section 4.2.1). Full length AHSV VP6 was used as a positive control and nonstructural protein NS2 of AHSV, which binds ssRNA, but not in a blot assay and does not bind dsRNA, was used as a negative control. Following autoradiography, it was evident that truncated proteins VP6mt1-289 and VP6mt116-289 bound the dsRNA (figure 4.11). The positive VP6 full-length control bound well and no binding was observed in the wildtype baculovirus infected Sf cells control or to AHSV NS2. The two VP6 mutants that bound the dsRNA probe (VP6mt1-289 and VP6mt116-289) have the region between amino acids 116 and 289 in common. However, this region is also present in VP6mt116-369 which does not bind dsRNA (figure 4.11b). There is also a partial overlap with VP6mt190-369 which also does not bind dsRNA. This result suggested that the addition of the C-terminal flanking region to truncated protein VP6mt116-289 eliminated binding of the protein. The amino acids in the C-terminal flanking region may have influenced the overall charge of the peptide.There may be specific domains involved in binding. These domains may have a highly positive charge. The VP6 binding domain could however, also be affected by the overall charge of the peptide, masking the effect of the smaller binding domain. The charge of the peptide is reflected by the isoelectric point. Isoelectric point (pI) estimations revealed that truncated proteins VP6mt1-289 and VP6mt116-289 have basic pI values of 8.4 and 8.29 respectively while truncated proteins VP6mt116-369 and VP6mt190-369 have acidic pI values of 6.27 and 6.07 respectively (figure 4.8). This raised the possibility that nucleic acid binding activity of VP6 assayed by nucleic acid overlay protein blot is strongly affected by differences in the net charge of the protein.

4.3.11 EFFECT OF pH ON DOUBLE-STRANDED RNA BINDING OF TRUNCATED VP6 PROTEINS

In order to investigate this further, the truncated protein binding assay was repeated with the truncated proteins above in 50mM NaCl SBB at a pH of 10.0 (figure 4.12a) and pH 6.0 (figure 4.12b). As expected, when the pH was raised above the estimated pI values of the proteins so that the proteins had a net negative charge, no binding was observed with the exception of some background in the wildtype control (figure 4.12a). When the pH was lowered to 6.0, the truncated proteins theoretically had a net positive charge and were induced to bind dsRNA. An increase in background was also observed at a lowered pH, therefore, the positions of the truncated proteins were verified using log graphs. The results appear to suggest a region on VP6 which contains domains that are important for dsRNA binding. This binding is abolished if the binding domain is linked to an amino acid sequence that lowers the pI. Binding to such a peptide can be

restored if the pH during the binding assay is lowered to give the peptide a net positive charge and this suggests that some level of binding specificity is maintained. From the results, it would appear that nucleic acid binding of AHSV VP6 can be manipulated by changing the binding assay conditions. This would suggest an important role for charge in the nucleic acid binding activity of the denatured VP6 protein of AHSV.

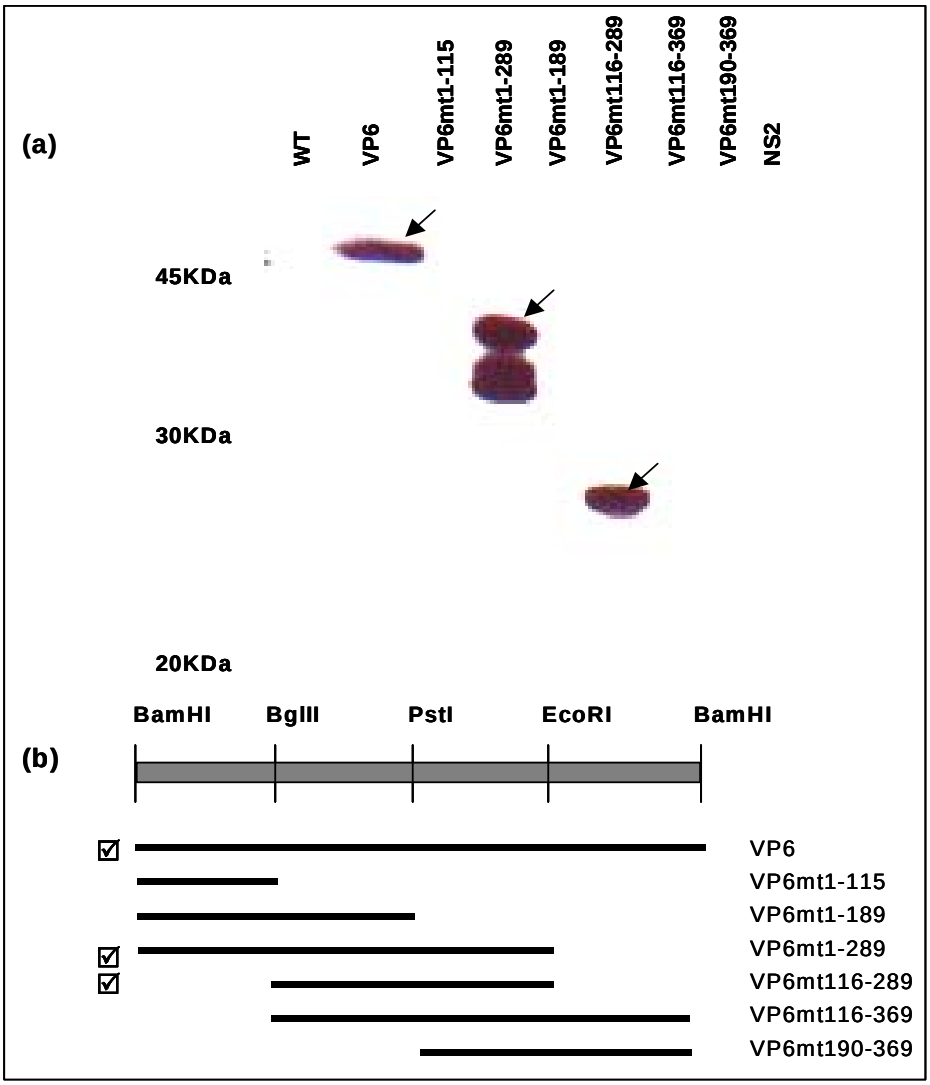

Figure 4.11 DsRNA binding by truncated VP6 proteins.

Autoradiogram of dsRNA binding activity of sucrose purified truncated AHSV-6 VP6 proteins assayed by nucleic acid overlay protein blots at pH 7.

Lane 1: wildtype baculovirus infected Sf cell proteins;

lane 2: full-length VP6 protein;

lane 3 - 8: truncated VP6 proteins;

lane 9: NS2 protein control.

Schematic diagram illustrating which truncated proteins bound dsRNA.

☑ indicates binding

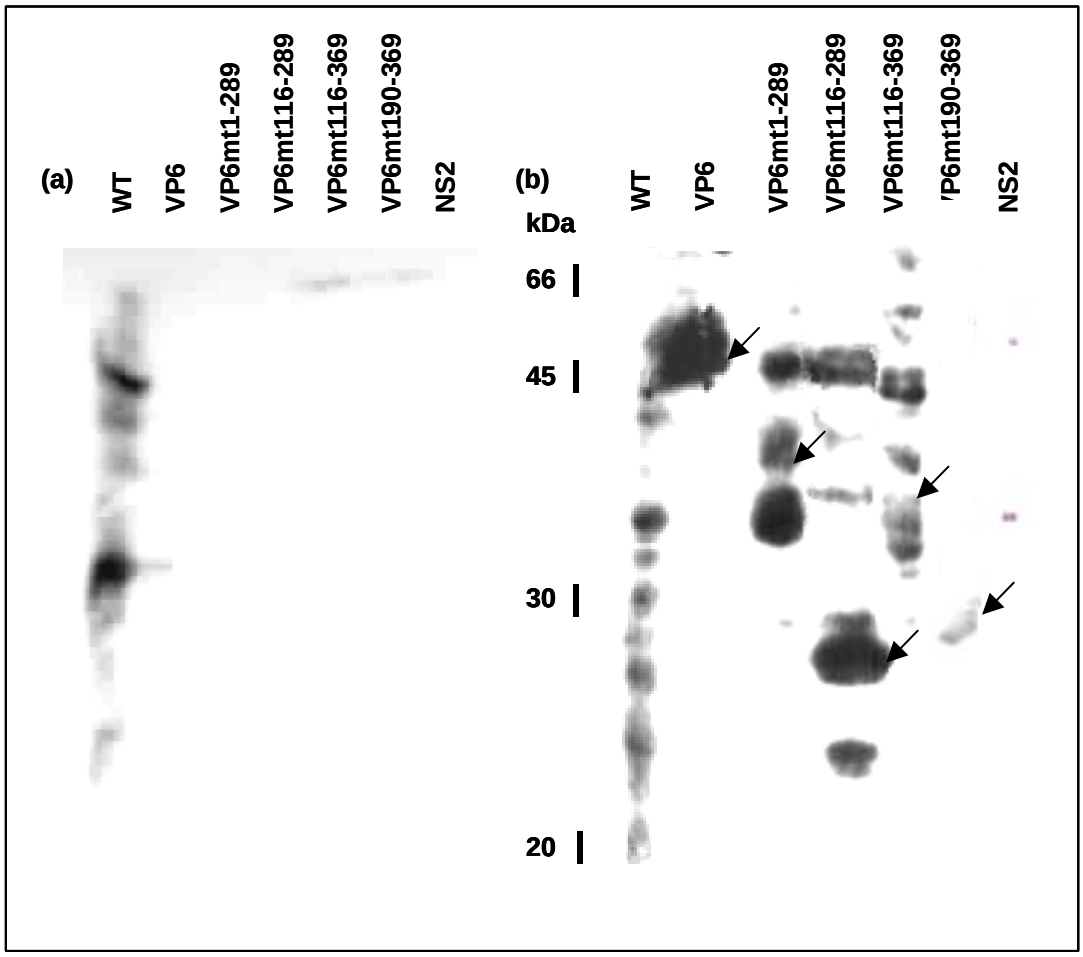

Figure 4.12 The effect of pH on dsRNA binding activity of the truncated AHSV-6 VP6 peptides.

Binding of sucrose purified truncated peptides was assayed by nucleic acid overlay protein blots at (a) pH 10.0 and at (b) pH 6.0. Positions of truncated proteins were verified using log graphs.

In both a) and b),

lane 1: wildtype baculovirus infected Sf cell proteins;

lane 2: full-length VP6;

lanes 3 – 6 : truncated VP6 proteins

lane 7: NS2 protein control.

4.3.12 DEMONSTRATION OF BINDING ACTIVITY OF BACTERIALLY EXPRESSED VP6 BY EMSA

Hayama and Li (1994), demonstrated the presence of domains required for the conformational binding of nucleic acids by BTV VP6 expressed in bacterial cells. A question remained as to whether the lack of binding of nucleic acids by bacterially expressed AHSV VP6 was due to a technical artefact or some aspect of the protein itself. In order to clarify this, partially purified bacterially expressed VP6 was used in an electrophoretic mobility shift assay (EMSA) using AHSV-6 dsRNA according to the method described by Hayama and Li (1994). Increasing amounts of VP6 protein from ±1μl to 4μl each of bacterial cell lysate expressing VP6 (as in figure 4.13a lane 3) were incubated with 125ng of AHSV dsRNA (segments 1 – 3 pool 1) followed by 0.8% agarose gel electrophoresis. Bacterially expressed VP6 was found to bind dsRNA as evidenced by an increased mobility shift with the addition of increasing amounts of protein (figure 4.13b). The controls of 1μl (lane 1) of bacterial cells (as in figure 4.13a lane 2); 20μl of bacterial cells (lane 5) and 1μg of BSA (lane 7) did not retard the mobility of the dsRNA (figure 4.13b). The problems experienced with attempts to demonstrate nucleic acid binding with bacterially expressed VP6 using a nucleic acid overlay protein blot can most probably be attributed to some aspect of the assay, most probably the blotting of the proteins to the membrane which may be influenced by the blotting conditions.

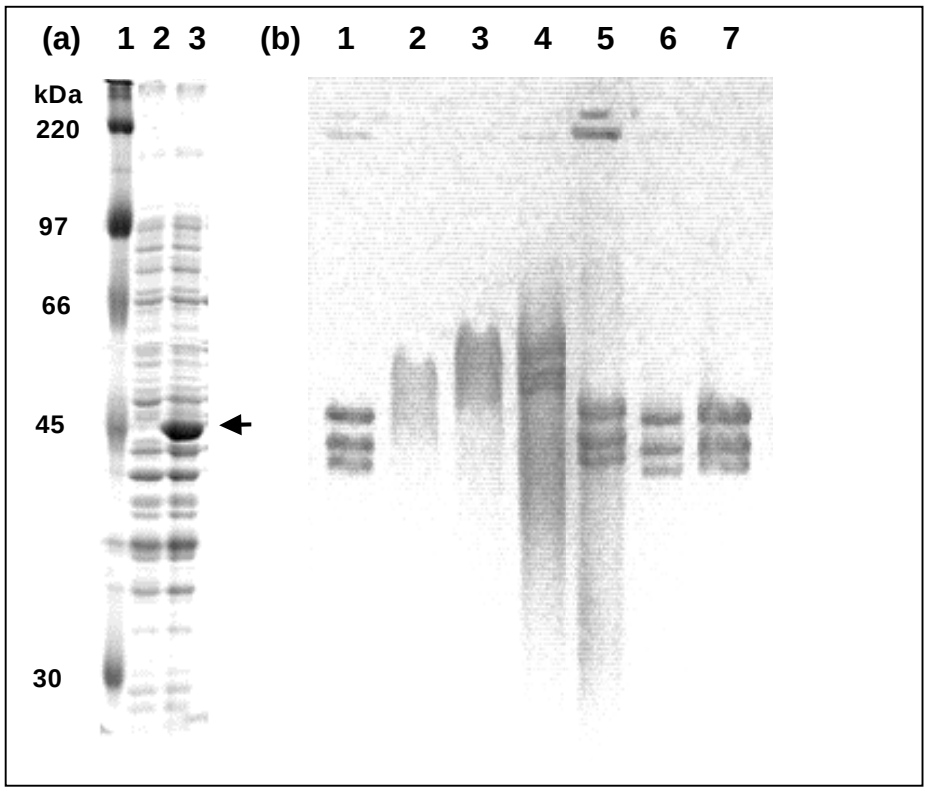

Figure 4.13 Nucleic acid binding activity of bacterially expressed AHSV-6 VP6.

(a) SDS-PAGE separation of bacterially expressed proteins. (b) Nucleic acid binding activity of bacterially expressed VP6 determined by electrophoretic mobility shift assay (EMSA).

(a) Coomassie blue stained SDS-PAGE gel of bacterially expressed proteins.

Lane 1: Rainbow marker;

lane 2: bacterial cells and

lane 3: VP6 expressed in bacterial cells.

The arrow indicates the VP6 protein.

(b) An electrophoretic mobility shift assay (EMSA) of 125ng of AHSV-6 dsRNA segments 1 – 3 (pool 1) incubated with increasing amounts of bacterially expressed VP6 protein and analyzed on a 0.8% TAE agarose gel.

Lane 1: dsRNA incubated with1μl of bacterial cell lysate;

lane 2 - 4: dsRNA incubated with 1μl, 2μl and 4μl of bacterial cell lysate expressing VP6 (±1 μg/μl) respectively.

lane 5: dsRNA incubated with an excess of 20μl of bacterial cell lysate without VP6;

lane 6: AHSV-6 dsRNA control and

lane 7: dsRNA incubated with 1μg of BSA.

4.4 DISCUSSION

The function of BTV VP6 has been investigated by various research groups. Aspects of nucleic acid binding activity have been elucidated and it has been demonstrated that BTV VP6 has ATPase linked helicase activity (Roy *et al.*, 1990; Hayama and Li, 1994; Stäuber *et al*., 1997). Based on sequence similarity, the equivalent protein in AHSV is considered to be the putative helicase for the virus. As a dsRNA helicase, VP6 could be expected to bind both single-stranded and double-stranded RNA (Lohman and Bjornson, 1996). As yet, no functional analyses have been performed on the AHSV VP6 protein. The objective of the work described in this chapter was the demonstration and characterization of nucleic acid binding activity of VP6 with the aim of proposing a mechanism responsible for this activity. In order to address this aim, the initial step was to demonstrate nucleic acid binding activity.

Nucleic acid binding was demonstrated by means of nucleic acid overlay protein blots or north western assays as described by Roy *et al*. (1990) and Mears and Rice (1996). Roy *et al.* (1990) successfully used BTV VP6 that had been partially purified by sucrose gradient fractionation for nucleic acid overlay protein blot assays. Accordingly, all further binding studies were performed using partially purified proteins which were prepared by sucrose gradient fractionation.

Baculovirus expressed AHSV VP6 was demonstrated to bind dsRNA by nucleic acid overlay protein blot assays. Bacterially expressed VP6 did not bind dsRNA in the same assay. Some difficulty was previously identified during western blotting of bacterially expressed VP6 which also relies on transfer of proteins to a nitrocellulose membrane. No valid reason could be identified to offer an explanation for the lack of blotting of the bacterially expressed VP6 protein to the membrane. Differences in two characteristics of AHSV VP6 expressed in a baculovirus and a bacterial system that may influence binding activity have been identified. Firstly, bacterially expressed VP6 appears to have a higher sedimentation value than baculovirus expressed VP6 (refer to section 3.3.5). This may indicate different oligomeric structures or different protein folding for VP6 expressed in the two systems. These differences should, however, be eliminated during SDS-PAGE. Secondly, it has been demonstrated that the baculovirus expressed VP6 is glycosylated (refer to section 3.3.7). Both possibilities were evaluated but characterization of AHSV VP6 nucleic acid binding was focussed on baculovirus expressed VP6.

Having demonstrated that AHSV VP6 binds dsRNA, the next objective was to evaluate whether baculovirus expressed AHSV VP6 could bind various types of nucleic acid by means of a north western binding assay. Each type of nucleic acid was labelled in a different manner. The specific activities were calculated to be in the order of 10^6cpm/μg for ssRNA, and DNA probes. The dsRNA probe was calculated to have a specific activity of approximately 10^{10}cpm/μg. AHSV VP6 was found to bind AHSV and BTV

dsRNA as well as virus specific ssRNA generated by *in vitro* transcription. VP6 was also found to bind both single-stranded and dsDNA. It seems, therefore, that unlike NS2 of BTV (Huismans *et al.*, 1987a), EHDV (Theron *et al*., 1994) and AHSV (Uitenweerde *et al*., 1995) which only bind ssRNA, VP6 is capable of binding all four types of nucleic acid including dsRNA from BTV. This corresponds with evidence for nucleic acid binding activity of BTV VP6 presented by Roy *et al*. (1990) where it was demonstrated to bind single-stranded and double-stranded RNA and DNA.

In order to characterize nucleic acid binding activity, the effect of various salt concentrations was assayed. Salt concentrations of 50mM through to a higher concentration of 150mM were used for this assay. With all four types of nucleic acid probes, a continuous decrease in binding was observed as the salt concentration increased. SsDNA bound least efficiently with less nucleic acid bound at the onset (50mM NaCl) and no binding by 125mM NaCl. VP6 nucleic acid binding activity is strongly affected by an increase in salt concentration. This may be because the increase in ion concentration changes the net charge on the proteins and nucleic acids involved and directly influences the level of electrostatic binding.

As a putative dsRNA virus helicase, VP6 may bind single-stranded and dsRNA and could exhibit a preference for RNA over DNA. In order to investigate this hypothesis, competition assays were performed. Competition assays were used by Lemay and Danis (1994) to establish nucleic acid affinity for the reovirus λ1 protein. Finerty and Bass (1997), successfully applied competition assays to establish dsRNA binding by a zinc finger protein of *Xenopus*. Labelled ssRNA and dsRNA were prepared and the specific activities determined. A fixed amount of VP6 was then probed with increasing amounts of the labelled nucleic acid until a saturation point for the binding of 1μg of VP6 to ssRNA and dsRNA was established. In the first round of competition assays, labelled ssRNA was used as probe. DNA (both single and double-stranded) proved a poor competitor to ssRNA binding. Although the addition of dsRNA as competition agent caused an initially larger decrease in binding of the ssRNA probe, both single and double-stranded RNA resulted in a decrease in the order of 62 – 63% following the addition of 4x the unlabelled competition nucleic acid. These results suggest that VP6 has a greater affinity for RNA than DNA. In order to determine whether a preference for single or double-stranded RNA could be established, competition assays using a labelled dsRNA probe were performed. Initially, unlabelled dsRNA resulted in a greater decrease in binding of the dsRNA probe. After the addition of 4x the unlabelled competition nucleic acid, dsRNA decreased the binding by 6% more than ssRNA. This may indicate a slight preference for VP6 binding of dsRNA over ssRNA. A dsRNA helicase may bind both single-stranded and double-stranded regions at an unwinding junction depending on the mechanism of helicase action (refer to section 1.10.1). AHSV VP6 binds both ssRNA and dsRNA more efficiently than it binds DNA. This is in line with what would be expected of a RNA helicase.

It is thought that binding and dissociation of hnRNPs from RNAs and other proteins is regulated. It has been suggested that such interactions may be controlled by post-translational modification as most hnRNPs have been found to be post-translationally modified. These modifications include methylation, phosphorylation and glycosylation (Liu and Dreyfuss, 1995). N-linked glycoproteins are thought to play a role in among other things, recognition and regulation of protein receptors (Elbein, 1987). The first glycosylated RNA binding protein, P43 was described by Soulard *et al*. (1993). These authors speculated on a possible role for glycosylation in the function of this protein. As N-glycosylation is a feature of eukaryotes and eukaryotic virus proteins, it was deemed worthy of investigation. N-glycosylation would not be expected and was not found in bacterially expressed VP6. Studies using tunicamycin to evaluate the role of carbohydrate have revealed that the only unifying concept to explain all situations is that the addition of a carbohydrate affects the conformation of a protein. The oligosaccharide may affect the stability of the protein with regard to denaturation and proteolysis; may increase the solubility of the protein; cause the exposure of sites that involve transport, membrane interaction and / or function of the protein and may play a role in biological recognition on its own (Elbein, 1987). The glycosylation of VP6 may well be an artefact of the baculovirus expression system and be triggered by the known glycosylation motif that occurs at residues 48-51 on VP6. However, the glycosylation of the baculovirus expressed protein could have affected the dsRNA binding studies. For that reason, glycosylated and deglycosylated VP6 protein were compared with respect to dsRNA binding. N-Glycosidase F and tunicamycin treatment were used to obtain deglycosylated baculovirus expressed VP6 protein. Unsatisfactory results were obtained using N-Glycosidase F to deglycosylate VP6. In general, the baculovirus expressed protein was found to be fairly unstable and very susceptible to degradation following freeze-thawing. In comparison, the bacterially expressed VP6 and tunicamycin treated VP6 (to a lesser extent) were found to be more robust and resistant to the effects of freeze-thawing. This may be as a result of the N-glycosylation and its effect on the stability of the protein. Elbein (1987), suggested that tunicamycin may affect the synthesis and decrease the solubility of a protein. As a result, these two aspects were assessed. A relatively low concentration of tunicamycin was sufficient for inhibiting glycosylation of VP6 in Sf9 cells. Even as much as 10μg/ml had no negative effects on protein synthesis. No apparent change in solubility was observed in deglycosylated VP6 arising from tunicamycin treatment. A western blot revealed that immunological recognition of VP6 is not determined by the presence of a N-linked glycan. Tunicamycin deglycosylated VP6 retained its dsRNA binding activities. It would appear that glycosylation is not integral to the binding activity of AHSV VP6.

Hayama and Li (1994), used truncated deletion mutants to map and characterize antigenic epitopes and nucleic acid binding domains of BTV VP6. Truncated deletion mutants are frequently used for the purpose of identifying linear epitopes involved in nucleic acid binding (Brantley and Hunt, 1993; Labbé *et al*., 1994; Lemay and Danis, 1994; Fernández *et al*., 1995; Mears *et al*., 1995; Bleykasten *et al*., 1996; Fernández

and Garcia, 1996; Mears and Rice, 1996; Wang *et al*., 1996b). Numerous common motifs that are important for nucleic acid binding have been identified (Mattaj, 1993; Burd and Dreyfuss, 1994; Sachetto-Martins *et al*., 2000). Glycine rich nucleic acid binding proteins with specific consensus sequences have been identified. These sequences are usually found in conjunction with other binding motifs such as the dsRBDs or RNP sites (Sato, 1994; Zhang and Grosse, 1997; Sachetto-Martins *et al*., 2000). As is evident from chapter 2, there are few conserved sequences in VP6 that have been associated with nucleic acid binding. When AHSV; BTV, Chuzan and St Croix River virus VP6 proteins were compared (figure 2.11), with respect to regions important for binding identified by Hayama and Li (1994) for BTV VP6, the following was observed. Epitope/binding domain 70 is not conserved in AHSV; domain 71 shows some level of conservation and domain 75 is well conserved in the four *Orbivirus* VP6 proteins compared. Based on primary structure of the protein, there were no clear target areas for the development of truncated proteins. As a result a “shotgun” approach was followed whereby fragments representing sections of the gene were cloned and expressed. This incorporated the equivalent regions in AHSV VP6 that correspond with Hayama and Li’s domains (1994). The truncated proteins were used to investigate whether regions containing linear epitopes for immunological recognition and domains involved in nucleic acid binding activity could be identified. Western immunoblot analysis indicated a region of 98 amino acids between position 191 and 289 (from the first methionine) which may be important in immunological recognition. From the 6 overlapping truncated VP6 proteins (Fig 4.8), only two (VP6mt1-289 and VP6mt116-289) bound the dsRNA probe, whereas a third peptide (VP6mt116-369) which overlaps a shared region and a partially overlapping fourth peptide (VP6mt190-369), did not bind. The proteins which did not bind, effectively acted as negative controls for the involvement of the 6his tag in binding. However, in order to eliminate any possible involvement of the 6his tag, the binding experiment was also performed with the fragments for expression of truncated proteins VP6mt1-115; VP6mt1-289 and VP6mt1-189, which have the 5’ end intact and thus provide their own start codon, cloned into pFASTBAC (no 6his tag). The results were identical. The differences in binding of the truncated peptides could be explained by comparing the isoelectric points of the respective truncated proteins.

Molecules containing both acidic and basic groups are known as ampholytes. Proteins which are large molecules with many acidic and basic groups are called polyampholytes. At a particular pH the acidic and basic charges will cancel each other out and the net charge will be zero. This is the isoelectric point (pI) of the protein (Dunbar, 1987). If the majority of charged groups are acidic, the isoelectric point will be low (<7), while if basic groups predominate, the pI will be high (>7) (Mathews and van Holde, 1991). Predominantly acidic proteins have a negative charge at a neutral pH while predominantly basic proteins have a positive charge at a neutral pH (Campbell, 1995). Therefore, if a protein is in an environment with a pH higher than its pI, it will have a net negative charge while, at a pH lower than its pI, it will have a net positive

charge. The effects of pH are important in protein chemistry. It has been found that a small shift in pH may change the constellation of charges on a protein molecule to the extent that its behaviour may be significantly modified (Mathews and van Holde, 1991).

Peptides that did bind dsRNA had basic pIs of above 8 while VP6mt116-369 and VP6mt190-369 have predicted acidic pI values of just above 6. All four of the truncated proteins under consideration share an amino acid overlap from residue 190 to 289. The difference in pI values can be accounted for by the addition of the acidic C-terminal to the truncated proteins that bind dsRNA at pH 7. This region is rich in acidic residues and has a predicted pI of 5.04. The addition of this acidic 80 amino acid region to a basic or neutral peptide results in a change in the protein parameters of the truncated proteins and may affect binding activity because the total charge of the peptide has changed. This suggested that pH could play an important role in determining which peptides were likely to bind and which not. If a protein is in an environment with a pH higher than its pI, it will have a net negative charge while, at a pH lower than its pI, it will have a net positive charge. In order to investigate, the binding assay was carried out at a pH of 6.0 at which the peptides are assumed to have a largely positive charge and at pH 10.0 where they would have a net negative charge. When the pH was raised to 10, all binding activity was eliminated barring some background in the wildtype control. When the pH was lowered to 6, it would appear that binding could be induced by neutralizing the effect of the acidic region of amino acids joined to a basic or neutral peptide containing the AHSV VP6 binding domain.

Similar to BTV VP6, the VP6 protein of AHSV has numerous basic residues distributed throughout the protein. In the case of BTV VP6, it has the highest number of charged residues per unit length of 1000 amino acids (Fukusho *et al*., 1989). This number is comparable to what has been observed in AHSV VP6. It may be postulated that the dsRNA binding domain in AHSV VP6 is not a rigid consensus sequence of amino acid residues, but rather a sequence of positively charged amino acids that constitute a domain that determines the nucleic acid binding characteristics of the peptide. If a predominantly negatively charged peptide is added to this region, binding activity is abolished. Binding can be reestablished by manipulating the pH of the binding conditions. Peptides lacking the binding domain, show no binding activity at all regardless of the binding assay conditions. The regions on AHSV VP6 that corresponded to BTV VP6 binding domains 70 and 71 (Hayama and Li, 1994) were located between amino acid residues 190 to 289. The binding domains identified on BTV VP6 (Hayama and Li, 1994) are characterized by a high number of positively charged amino acid residues. When the overlapping region of the peptides which did bind was analyzed, two regions rich in basic residues were identified.

The effects of increasing salt concentration support the role of charge as the mechanism of binding. It has been demonstrated that salt concentration and pH have a marked effect on the nucleic acid binding activity of AHSV VP6. Similar results were obtained for the RNA binding domain of Southern cowpea mosaic virus (SCPMV) (Lee

and Hacker, 2001). In SCPMV, the binding domain has a number of basic residues, none of which are specifically required for RNA binding. Binding activity is determined by the overall charge of the domain. Electrostatic interactions are thought to be important for binding. Binding due to charge may erroneously be ascribed to an alternative domain specific mechanism, unless the pI of the peptide as well as the salt and pH effect on binding are taken into account.

Hayama and Li (1994), proposed that the nucleic acid binding domain in BTV may be single but conformational. They found that deletion of amino acid residues 292 – 302 (epitope 75) did not affect binding activity. This is the only epitope of the three that shows high levels of conservation in AHSV VP6. These authors found that deletion of the amino acid residues corresponding to domain 71 eliminated binding activity. This region is not well conserved in AHSV VP6. They further proposed that the three domains 70; 71 and 75, when juxtaposed, form a conformational domain that enables nucleic acid binding. These three domains are described as being rich in arginine and lysine which suggests a positive net charge. Six epitopes were identified by Hayama and Li (1994), generated as peptides and tested for binding activity. Of these, three bound nucleic acids and three did not. As a matter of interest, using the information supplied by Hayama and Li (1994), the isoelectric points of the six peptides representing six epitopes in BTV were estimated using Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998) available through EXPASY (expert protein analysis system) hosted by the Swiss Institute of Bioinformatics. The results are summarized in table 4.1.

Table 4.1 Estimated pI values of six epitopes identified in BTV VP6.

Epitope[a]	Bound nucleic acids[a]☑	Estimated pI[b]	Residue position in BTV VP6[a]	Peptide sequence[a]
72	☒	6.29	35 - 46	KENKTEPKEESK
74	☒	5.00	63 - 78	KEEGGKETKDADVDRR
70	☑	9.97	181 - 193	ERLRDLRRKEKSE
71	☑	10.9	200 - 209	ERGGRKQRKE
73	☒	4.37	216 - 227	REGVEEEKTSEE
75	☑	10.0	292 - 302	KEVAREASKKK

(a) Hayama and Li (1994); b) Compute pI/Mw (Bjellqvist *et al*., 1993; Bjellqvist *et al*., 1994; Wilkins *et al*., 1998).

BTV VP6 nucleic acid binding studies using EMSA were performed at pH 7.5 (Hayama and Li, 1994). Of the six domains identified in BTV VP6, three have basic isoelectric points and will have a net positive charge at pH 7.5. Peptides representing these same three domains i.e. 70; 71 and 75 bound nucleic acids. The remaining three have acidic

pIs resulting in a net negative charge at pH 7.5. Peptides representing these three epitopes i.e. 72; 74 and 73 did not bind nucleic acids. It is, therefore, possible that charge plays a role in nucleic acid binding in BTV VP6. Total amino acid composition (and resulting pI) of a binding domain, may in this instance be more important than its actual sequence.

N-glycosylation may play a role in the regulation of VP6 protein function by affecting the pI of the protein. Glycosyl phosphatidylinositol-anchored proteins have multiple isoforms with different isoelectric points which are consistent with extensive but differential N-glycosylation. GPI-anchored proteins with a lower M_r had a more basic pI. Therefore, the more heavily N-glycosylated a protein, the more acidic its pI (Fivaz *et al*., 2000).

The final question that was addressed was the failure of bacterially expressed VP6 to bind nucleic acids in a nucleic acid overlay protein blot assay. Having established that N-glycosylation played no direct role in the nucleic acid binding activity of VP6, the possibility remained that some artefact, possibly in the process of blotting, had resulted in the lack of binding. To avoid blotting, electrophoretic mobility shift assays were performed as described by Hayama and Li (1994). Using AHSV dsRNA segments 1 – 3 (pool 1), bacterially expressed VP6 bound the dsRNA as evidenced by the retardation of its movement through the agarose gel. Controls, of bacterial cell lysate which did not contain VP6 and BSA, did not bind the dsRNA. It appears that concentration plays a role as was described for BTV VP6 (Hayama and Li, 1994). It, therefore, seems fairly evident that the lack of binding of bacterially expressed VP6 in nucleic acid overlay protein blot assays was due to an artefact introduced during the blotting process and not a lack of binding activity *per se*.