CHAPTER 5:CONCLUDING REMARKS

The broad aim of this study was to characterize the structure and nucleic acid binding function of AHSV VP6. A number of short term aims were directed at addressing important questions such as: whether there is evidence suggesting that VP6 is the AHSV helicase; whether there are any specific motifs in the amino acid sequence which would suggest its function within the virus. What properties does the protein exhibit i.e. is it soluble, is there evidence of post- or co-translational modification. Whether there is any evidence that VP6 binds nucleic acids and if so, by what mechanism. Is there sequence specificity or is binding only as a result of charge? Whether there is a difference in the binding of ssRNA, dsRNA and DNA.

Analysis of the primary structure showed the glycine rich nature of AHSV VP6. This is in agreement with other *Orbivirus* VP6 sequences. Variation between serotypes is low and between serogroups is high.

Double-stranded RNA helicases should theoretically demonstrate some homology. The homology of VP6 in terms of available *Orbivirus* sequences was investigated. Exhaustive maximum parsimony methods applied to the nucleotide data yielded a single tree in which BTV and AHSV VP6 genes grouped together with a boodstrap value of 100. The results of functional constraint analysis were inconclusive as different results were obtained depending on the treatment of the gaps. In many protein alignments, gaps would not be as relevant, however, in the VP6 alignment gaps play a prominent role. As a result of the difference in length of the various *Orbivirus* VP6 genes and/or proteins, their value in phylogenetic analyses is questionable.

Analysis of the primary structure identified motifs common to glycine rich proteins. As mentioned above, in biting midge borne *Orbivirus* VP6 proteins investigated to date, the most prevalent amino acid in terms of amino acid content is glycine. Various glycine motifs common to glycine rich proteins have also been identified.

The positions of three domains identified in BTV VP6 thought to be important for binding (Hayama and Li, 1994) were located on AHSV VP6. These authors proposed that BTV VP6 bound nucleic acids in a conformational manner as a result of the juxtapositioning of these three epitopes. Only one of these epitopes showed conservation between AHSV and BTV VP6 proteins. These regions are, however, characterized by between 55 to 60% basic amino acid residues.

Regions associated with helicase activity such as a conserved region found in the Rep helicase of *E. coli*, the “A” and “B” sites of the Walker box involved in ATP binding and hydrolysis as well as two motifs common to SF2 helicases were identified. From the primary structure analysis it may be predicted that VP6 of AHSV is the most likely candidate for helicase activity. Several post translational modification signals were identified, including a N-glycosylation signal. Hydrophillicity plots predicted a soluble

protein. Specific secondary structures characteristic of nucleic acid binding proteins were not identified in AHSV VP6.

In order to function as a helicase, AHSV VP6 must bind and hydrolyse ATP for energy. In order to unwind double-stranded nucleic acid, it must bind nucleic acids. Numerous amino acid sequences or motifs have been associated with nucleic acid binding. In BTV VP6, Kar and Roy (2003), have shown the importance of an arginine residue in a highly positively charged domain that is similar to the conserved helicase motif VI. This domain is not conserved in AHSV VP6, Chuzan virus VP6 or St Croix River virus VP6. No clear indication of nucleic acid binding motifs with the exception of generally distributed glycine rich motifs were identified in AHSV VP6.

Evidence of nucleic acid binding by AHSV VP6 was sought using baculovirus and bacterially expressed VP6. Partially purified proteins were successfully applied in a RNA overlay protein blot binding assay for the demonstration of dsRNA binding of baculovirus expressed VP6. Bacterially expressed VP6 did not bind the dsRNA probe in this assay. There are two major differences between baculovirus and bacterially expressed VP6 protein. The first of those is that glycosylation of baculovirus expressed VP6 is not found in bacterially expressed VP6. The other difference is an increase in the sedimentation value of bacterially expressed VP6 as compared to baculovirus expressed VP6. This suggests a difference in either folding or oligomerization of the bacterially expressed protein.

In order to characterize the nucleic acid binding activity of AHSV VP6, a number of questions were addressed. Based on the findings of Roy *et al*. (1990), the first question was whether VP6 has an affinity for different types of nucleic acids. It was established by north western assay that AHSV VP6 binds both single and double-stranded RNA and DNA of AHSV origin as well as dsRNA of BTV. In the electrophoretic mobility shift assays (EMSA) performed, bacterially expressed VP6 bound both viral specific and non-specific DNA as well as dsRNA.

The basic amino acid content of AHSV VP6 suggested a possible role for charge in the nucleic acid binding mechanism of the protein. Accordingly, the effect of salt concentration on the affinity of AHSV VP6 for nucleic acids was determined. VP6 was assayed for binding activity with all four different AHSV specific nucleic acid species at increasing salt concentrations. As salt concentration increased, the affinity of VP6 for each nucleic acid decreased. Due to the effect of salt in the electrostatic screening of nucleic acids and proteins, this result supports a role for charge in the nucleic acid binding mechanism of VP6.

Most models proposed for helicase activity assume that the helicase should be capable of binding to both single and double-stranded nucleic acids. The hypothesis was, therefore, proposed that AHSV VP6 as the putative helicase of a dsRNA virus, should preferentially bind RNA as opposed to DNA. This was demonstrated by competition assays with a radioactively labelled ssRNA probe. An attempt to establish a preference

between single and double-stranded RNA was made using a radioactively labelled dsRNA probe. The results suggested that VP6 may exhibit a slight preference for binding dsRNA over ssRNA.

Although the glycosylation of baculovirus expressed VP6 may be an artefact of the expression system, it could have affected the dsRNA binding studies. Therefore, the role of N-glycosylation in immunological recognition and binding ability of AHSV VP6 was investigated. Following tunicamycin treatment to inhibit glycosylation of VP6 expressed in insect cells, a western immunoblot revealed that immunological recognition is not determined by the presence of a N-linked glycan. A dsRNA overlay protein blot assay demonstrated that baculovirus expressed VP6 treated with tunicamycin retained its affinity for dsRNA. N-glycosylation may play a role in the regulation of VP6 protein function as N-glycosylation lowers the isoelectric point (pI) of a protein (Fivaz *et al*., 2000).

To investigate regions that may be important in nucleic acid binding activity, deletion mutation analyses were performed. Following the analysis of AHSV VP6 aligned to BTV VP6, a series of truncated proteins were generated which accommodated regions of AHSV VP6 which correspond to the three epitopes identified by Hayama and Li (1994). The results suggested that a specific sequence of amino acids was not the determining factor for nucleic acid binding as truncated proteins with the same overlap did not necessarily all bind. One major difference that could explain the differences in binding is the isoelectric points of the respective truncated proteins.

The hypothesis was, therefore, proposed that binding of denatured AHSV VP6 in a RNA overlay protein blot binding assay is as a result of charge. This hypothesis was tested by repeating the assays at pH 10 where the proteins should have a net negative charge, and pH 6 where the proteins should have a net positive charge. It may be proposed that the nucleic acid binding activity of AHSV VP6 is determined by a series of positively charged amino acids (localized between residues 190 and 289) that constitute a domain that determines the nucleic acid binding characteristics of the peptide. The results suggest a role for electrostatic binding in non-conformational nucleic acid binding activity of AHSV VP6. These results were supported by the effect of increasing concentrations of NaCl on the nucleic acid binding affinity of VP6.

Amino acid regions predicted to bind did not necessarily bind. A stretch of amino acids may be predicted to bind based on amino acid composition. However, if it is attached to a region rich in acidic (negatively charged) amino acids it no longer binds nucleic acids. Binding can be reestablished by manipulating the pH of the binding conditions. Peptides which do not contain the binding domain, show no binding activity at all regardless of the binding assay conditions. An argument could be made for the use of smaller peptides. However, even if smaller peptides are used, the basic amino acid content and resulting pI of the peptide would still be relevant to its capacity for binding nucleic acids. It is as a result of the use of overlapping truncated proteins that the charge effect was identified. Theoretically, if a region rich in basic amino acids was removed from a

protein, the rest of the protein may not bind nucleic acids as the amino acid content of the peptide has changed. On the other hand, a strongly acidic residue containing peptide added to a peptide shown to bind nucleic acids may cause the resulting peptide to no longer bind. Truncated protein VP6mt116-289 used in this study (172 amino acids) has a theoretical pI of 8.29 and has been demonstrated to bind nucleic acids at pH 7. Truncated protein VP6mt116-369 has an additional peptide of 80 amino acids added to truncated protein VP6mt116-289. The additional 80 peptide region has a predicted pI of 5.04. The predicted pI for truncated protein VP6mt116-369 (total of 254 amino acids) is 6.27 which means that it has an overall negative charge at pH 7. Truncated protein VP6mt116-369 does not bind nucleic acids at pH 7. The addition of the acidic residues in the 80 amino acid peptide has changed the character of the peptide by changing the charged amino acid content resulting in a loss of binding activity.

In order for a helicase to get near to the nucleic acid to be unwound, it must be basic as like charges will repel each other. The evidence presented in this study suggests that the manner in which AHSV VP6 binds nucleic acids necessary for its function as a helicase, is related to charge. Traditionally, it has been proposed that a specific “motif” or sequence of amino acids confers binding activity to the protein. If changes or mutations are introduced into that motif, binding activity is abolished. It may in fact be that the mutations are changing the character of the entire peptide and as a result of these changes, the isoelectric point changes. The overall charge of the protein at that pH changes, and the protein no longer binds nucleic acids. Therefore, total amino acid composition (and resulting pI) of the binding domains may be of more importance than the actual sequence of amino acids.

Most authors report binding studies performed at 50mM salt concentration and a neutral pH (pH 7.0 – 7.5). These results have shown that if either of these conditions are changed, the results are very different.

Bacterially expressed VP6 was demonstrated to bind dsRNA in an electrophoretic mobility shift assay (EMSA) as described by Hayama and Li (1994). These authors reported that nucleic acid binding was concentration dependent. Partial purification raised a potential problem with EMSA as concentration can only be estimated.

For further investigations of the functions of AHSV VP6 one could include an investigation into the role of the two regions rich in basic residues found in the region of amino acid residues from 190 – 289 which appears to be important in nucleic acid binding; a mechanism for specific nucleic acid binding; for ATP binding and hydrolysis and helicase activity, highly purified protein is necessary. A system such as the baculovirus system which results in the expression of native proteins with post-translational modifications, correct folding and conformation is required. However, another vector system supplying an alternative method of purification is desirable.

CHAPTER 6:RESEARCH OUTPUT

Refereed Journals:	**Turnbull, P.J.**, Cormack, S.B. and Huismans, H. 1996. Characterization of the gene encoding core protein VP6 of two African Horsesickness virus serotypes. J. Gen. Virol. 77, 1421 – 1423. **De Waal P.J.** and Huismans, H. Characterization of the nucleic acid binding activity of inner core protein VP6 of African horsesickness virus. Accepted on 4 April 2005 for publication in Archives of Virology.
International Congress Participation:	Cormack, S.B., **Turnbull, P.J.** and Huismans, H. (1995) Characterization of the Inner Core Protein VP6 of two Serotypes of African Horsesickness Virus. Fifth international symposium on double-stranded RNA viruses, March 1995, Jerba, Tunisia. Huismans, H. and **de Waal, P.J.** (1997) The Inner core protein VP6 of African Horsesickness Virus binds Nucleic Acids with a preference for dsRNA. International symposium on double-stranded RNA viruses, November 1997, Mexico. Huismans, H. and **de Waal, P.J.** (2003) Characterization of the nucleic acid binding activity of core protein VP6 of African horsesickness virus. 8th International Symposium on Double-Stranded RNA viruses. September 2003. Il Ciocco, Castelvecchio Pascoli, Italy.
Local Congress Participation:	Cormack, S.B., **Turnbull, P.J.** and Huismans H. (1994) Cloning and in vitro expression of two minor core proteins of African Horsesickness Virus type 3. Fourteenth Congress of the South African Genetics Society: GENETICS FOR AFRICA, June 1994, Pretoria. **Turnbull, P.J.**, Cormack, S.B. and Huismans, H. (1996) The cloning characterization and expression of a gene that encodes a possible African Horsesickness Virus helicase. Ninth Biennial Congress of the South African Society for Microbiology, July 1996, Pretoria. **De Waal, P.J.** and Huismans, H. (2000). The role of post translational modification in the nucleic acid binding property of the inner core protein VP6 of African Horsesickness virus. Seventeenth Congress of the South African Genetics Society. June 2000, Pretoria. **De Waal P.J.** and Huismans, H. (2005) Characterization of the nucleic acid binding activity of inner core protein VP6 of African horsesickness virus. XIXth South African Society of Biochemistry and Molecular Biology (SASBMB) Conference. January 2005, Stellenbosch.