Characterisation of the genome of

Nudaurelia Omega Virus

by

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ABSTRACT

Nudaurelia ω virus (NωV) is a small RNA virus belonging to the Family Tetraviridae. NωV was successfully isolated from field collected larvae of the pine emperor moth, *Nudaurelia cytherea capensis*. By polyacrylamide gel electrophoresis it was possible to determine the size of the capsid proteins. Anti-NωV antiserum was raised by inoculating a rabbit with purified virus.

RNA was extracted from the purified virus using a phenol\chloroform extraction procedure. It was possible to separate the viral RNA into its constituent species using sucrose density gradient centrifugation. The sizes of both species of RNA was accurately determined by agarose gel electrophoresis. These sizes corresponded to the replicative form of the RNA which was extracted from infected host tissue. The absence of a poly(A) tract on the RNA was shown through poly(U) sepharose chromatography.

Cell-free translation of the viral RNA elucidated the sizes of proteins encoded *in vitro* in a rabbit reticulocyte lysate system. Optimal conditions for *in vitro* translation of NωV were determined for a range of conditions. Immunoprecipitaion of viral encoded proteins with anti-NωV antiserum suggested that the putative coat protein of the virus was encoded by RNA 2, as a precursor polypeptide which underwent post-translational cleavage.

Reverse transcription - polymerase chain reaction (RT-PCR) was used to successfully produce a radiolabelled probe which could detect dot-blotted viral RNA. The efficacy of this probe in detecting the presence of NωV RNA in infected tissue was also tested.

ACKNOWLEDGEMENTS

ABBREVIATIONS

A	amperes
amp	ampicillin
bp	base pairs
°C	degrees Celsius
Ci	curies
cpm	counts per minute
Da	daltons
DNA	deoxyribonucleic acid
ds	double stranded
E. coli	*Eschericia coli*
EDTA	ethylenediaminetetraacetic acid
Fig.	Figure
g	grams
h	hours
kb	kilobase pairs
kDa	kilodaltons
LB	Luria broth
l	litre
M	molar
MDa	megadaltons
mg	milligrams
min.	minutes
ml	millilitres
mM	millimolar
µCi	microcuries
µg	micrograms
µl	microlitres

μM	micromolar
nm	nanometres
ng	nanograms
NβV	*Nudaurelia* beta virus
NωV	*Nudaurelia* omega virus
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcription
SDS	sodium dodecyl sulphate
ss	single stranded
TMV	tobacco mosaic virus
U	units
UV	ultraviolet
V	volts
X-gal	5-bromo-4-chloro-3-indoyl-b-galactosidase

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PROJECT MOTIVATION

The larvae of the Saturniid, *Nudaurelia cytherea capensis* (pine emperor moth), have important economic implications in the Southern and Western Cape of South Africa where they are responsible for defoliation in pine plantations, particularly of the *Pinus radiata* species. These larvae periodically succumb to a disease caused by the virus, NωV. This makes the virus very attractive in terms of its potential use as a biological control agent of the larvae. Very little though is known about the virus as it is not possible to culture it in any known insect line, and thus much research is required into the biology of this virus.

The first phase of this project was therefore aimed at investigating the virus more closely at the molecular level by studying both the single stranded and replicative forms of the viral genome. To gain a greater understanding of the proteins encoded for by the viral genome, cell-free-translation and immunoprecipitation experiments were carried out.

The second phase of the project was intended to produce a cDNA probe, of sufficient sensitivity to detect the presence of viral RNA in infected insect tissue. This probe was produced through reverse transcription of the viral genome. Such a probe would prove useful in detecting the level of virus in a particular population of larvae from year to year. Such knowledge would be useful in determining the necessity of spraying the pine plantations with insecticide. This second phase of the project was carried out prior to the publication of the sequence of the RNA2 by Agrawal and Johnson (1992). Use of this sequence could therefore not be made.

This thesis illustrates the progress made in attempting the above mentioned goals.

CHAPTER 1 : Review of Literature

1.1 RNA Viruses of insects

The viruses of insects are at present attracting considerable interest as a result of their potential use as field control agents of pest species. It is however unlikely that these viruses may be used for biological control until exhaustive studies have been carried out so as to ascertain potential adverse factors. These include host specificity and their potential pathogenicity to other organisms especially mammals and in particular humans. Understanding of the possible dangers of the use of such organisms in biological control may prevent a potential ecological catastrophe arising from their misuse. Harrap and Payne (1979) divided insect viruses into five distinct groups:

(a) Baculoviruses

(b) Cytoplasmic Polyhedrosis Viruses

(c) Entomopoxviruses

(d) Iridoviruses

(e) Small Isometric Viruses

Of these viruses only the small isometric viruses contain a single stranded RNA genome. Viruses containing an RNA genome are amongst the most ubiquitous of all viruses. These viruses are found in vertebrates, invertebrates, plants, fungi and several prokaryote species (Matthews, 1982).

1.1.1 Small Isometric Viruses

The small isometric viruses may be recognised under the electron microscope both when negatively stained and in section. Electron microscopy further allows an estimation of the diameter of the virus, the arrangement of the capsomeres and the identification of any structural feature which may be present (Harrap and Payne, 1979). While the small RNA viruses of insects appear to have a wide diversity of morphology and genome size (Moore *et al.*, 1985), only three groups of these viruses have been studied in detail. These are the insect picornaviruses, the Nodaviridae and the Tetraviradae, the latter which until recently was referred to as the *Nudaurelia* β virus group (Moore, 1991 a).

a. Picornaviruses:

In excess of 30 small RNA viruses found to infect insects have been termed insect picornaviruses or insect enteroviruses as a result of their analogy with mammalian viruses of the same name (Longworth, 1978). Picornaviruses all have one molecule of single-stranded, positive sense RNA which is polyadenylated an the 3' end and has a protein attached to the 5' end (Moore, 1991 b). Three of the best studied members of this group are Cricket paralysis virus (CrPV), *Drosophila* C virus (DCV) and *Gonometa* virus (Matthews, 1982). Relatively little is known of the pathology of these viruses or how they are transmitted in field conditions. *Gonometa* virus was shown to be an effective field control agent of *Gonometa podocarpi* in the forests of Uganda, where the larvae were the causative agent of extensive defoliation of *Pinus patula* (Harrap *et al.*, 1966).

b. Nodaviridae:

The type member of this group is Nodamura virus first isolated from the *Culex*
tritaeniorhynchus mosquito in Japan (Scherer and Hurlbut, 1967). This virus has
been shown to replicate in both animal and insect cells. Newman and Brown
(1973), demonstrated that this virus contained two species of RNA both of which
were contained in the same particle, were not polyadenylated and had molecular
weights of $1x10^6$ and $0.5x10^6$ (Newman and Brown, 1975; 1978). The members
of this group, of which there are six in all, are all unenveloped, icosahedral viruses of
approximately 30 nm in diameter, with a bipartite genome (Hendry, 1991). The
Nodaviridae are all strictly insect pathogens with the exception of Nodamura virus
which is also pathogenic for mammals (Scherer and Hurlbut, 1967). The capsid
proteins of the Black Beetle Virus (BBV), another member of the group, have been
shown to be arranged according to a $T=3$ icosahedral symmetry (Hendry, 1991).

c. Tetraviridae:

This group of viruses was originally termed the *Nudaurelia* β virus group after the
type member *Nudaurelia* beta virus (NβV), first purified by Hendry *et al* (1968).
The name was later changed to Tetraviridae (Brown, 1989), on the basis of its
icosahedral symmetry as the capsid proteins of the viruses are arranged in a unique
T=4 quasi symmetry (Finch *et al.*, 1974; Olson *et al.*, *1990*).

Sixteen viruses are classified, or tentatively classified, as members of this group as a
result of their capsids and protein subunits being of a similar size (Hendry *et al.*,
1985). NβV was isolated from the larvae of the Pine Emperor Moth, *Nudaurelia*

cytherea capensis (Hendry *et al.*, 1968). Several other Tetraviridae, designated α, δ, ϵ ,γ and most recently ω, have since been isolated from this larvae. All of the viruses with the exception of NωV have a single molecule of ssRNA with a molecular weight of approximately 1.8×10^6 (Matthews, 1982). NωV was demonstrated as having a bipartite genome (Hendry *et al.*, 1985). The viruses are thought to contain neither lipid nor carbohydrate. The host range appears to be limited to Lepidoptera, principally Saturniid, Limacodid and Noctuiid moths where the viruses are found to replicate primarily in the gut cells of the larvae (Matthews, 1982). The lack of a cell culture system which will support the replication of these viruses has meant that little is known of their replicative strategy.

1.2 Characteristics of the Tetraviridae

1.2.1 Isolation

Tooke and Hubbard (1941) reported that large numbers of the larvae of the Pine Emperor Moth periodically succumbed to a disease which they referred to as polyhedral wilt. This was seen to manifest itself by flaccidity and internal liquification of the larvae. Hendry *et al.* (1968), successfully isolated an non-inclusion virus from the diseased larvae which was shown to be a polyhedral virus with a diameter of approximately 35 nm in the electron microscope. This virus termed was *Nudaurelia* beta Virus (NβV). Morris *et al.*(1979), in the isolation of *Trichoplusia ni* virus (*Tni*V) from *Trichoplusia ni* larvae developed a three fold protocol which has since been largely adopted for the isolation of Tetraviridae from infected larvae. This protocol involves the homogenisation of the larvae,

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precipitation with polyethylene glycol and purification of the virus on a sucrose density gradient. Hendry *et al.*(1985) used this method for the purification of the NωV with an additional CsCl isopycnic centrifugation step. Tripconey (1970) succeeded in the isolation of NβV from both the adult and the pupal forms, and showed that infected larvae which pupated, often died before assuming the adult form, whilst infected pupae which emerged as adults generally exhibited retarded development such as malformed wings.

1.2.2 Members of the Tetraviridae

Since the isolation of NβV from the larvae of the Pine Emperor Moth (Hendry *et al.*, 1968), a further four viruses were isolated, namely α, δ, γ, and ε (Juckes, 1970). Of these only NβV has been extensively characterised. In a comparison of the biophysical properties of NβV and NεV, Juckes (1979) found that although no morphological characteristics were evident in the electron microscope, NεV was slightly larger and less dense. The viruses were further found to be serologically distinct (Juckes, 1979).

Grace and Mercer (1965) were successful in the isolation of a new virus from the Saturniid, *Antheraea eucalypti*, Scott.(Gum Emperor Moth) which was later shown by Juckes *et al.*(1973) to be serologically indistinguishable from NβV. Reinganum *et al.*(1978) isolated three viruses belonging to the Tetraviridae. Two of the viruses were isolated from Limacodid moths, *Darna trima*, a pest of several crops in South East Asia, and *Thosea asigna*, a pest of oil palm in Malaysia. The third virus was isolated in England from a hybrid laboratory culture of the Saturniid, *Philosamia*

cynthia x *ricini*. All of these viruses are serologically related but distinct from NβV (Reinganum *et al.*, 1978).

Morris *et al.*(1979) isolated an RNA virus, *Tni*V, from *Trichoplusia ni* larvae infected with a baculovirus, *Autographa californica*. The virus was found to have a bouyant density in CsCl of 1.3 g / ml and a sedimentation coefficient of 200 s. The ssRNA genome has a molecular weight of 1.9×10^6 Da and the coat protein sub-unit a molecular weight of 67 000 Da (Morrris *et al.* , 1979). The virus *Dasychira pudibunda* virus (*Dp*V) isolated by Greenwood and Moore (1981), from a single moth of *Dasychira pudibunda* was found to be serologically related to *Tni*V.

Chao *et al.* (1983) isolated an icosahedral RNA virus later ascribed to the group from the Soybean Looper, *Pseudoplusia includens*. This was found to contain a single polypeptide of molecular weight 55 000 Da, to be 40nm in diameter and to be serologically unrelated to other Tetraviruses including NβV , *Tni*V and *A. eucalypti* RNA virus.

The most recent member of the group to be isolated was NωV from larvae of *Nudaurelia cytherea capensis* (Hendry *et al.*, 1985). The virus is serologically unrelated to NβV . NωV has a buoyant density of 1.285 g / ml in CsCl and contains a single, major polypeptide of 62 000 Da (Hendry *et al.*, 1985; Agrawal and Johnson, 1992). The virus is morphologically distinct from NβV and tryptic peptide analyses have shown it to differ from both NβV and NεV in respect of its polypeptide (Hendry *et al.*, 1985).

1.2.3 Structure

Polson *et al.* (1970) employed the particle counting method using haemocyanin as a reference substance and estimated the molecular weight of NβV at approximately 16.3×10^6 Da. Tripconey (1970) found NβV had a particle density of 1.28 g / ml and a sedimentation coefficient of 210s. Struthers and Hendry (1974) found NβV to be an extremely stable virus *in vitro* and demonstrated its ability to resist degradation by treatment with mild alkali, warm and cold salt and 69% acetic acid.

Investigations by Finch *et al.* (1974) of the capsid structure of NβV yielded the first example of icosahedral surface symmetry of $T=4$. This model was based on the quasi-equivalence theory proposed by Casper and Klug (1963) as a basis for the classification of protein shells in terms of their Triangulation number (T). In this theory, T may have values of 1, 3, 4, 7, etc.. The $T=4$ lattice proposed by Finch *et al.* (1974) was based on three dimensional image reconstructions from electron micrographs of negatively stained NβV . This group postulated the capsid to be built of 240 structure units clustered in Y-shaped trimers.

More recent work by Olson *et al.*(1990) using frozen hydrated virus particles is consistent with the findings of Finch *et al.*(1974). This group found the NβV capsid to consist of 240 copies of a 61 kDa protein. The construction of models has suggested the existence of a large and a small domain of 40 kDa and 21 kDa respectively within each subunit. The large domain which has a cylindrical shape is thought to associate with the large domains of two neighbouring subunits to

produce a Y-shaped trimeric aggregate in the outer capsid surface. Each of the 20 planar faces is then made up of four of these trimers. The small domains associate at a lower radii to form a contiguous non-spherical shell. The ssRNA genome is thought to be loosely packed within the capsid (Olson *et al.*, 1990).

Cavarelli *et al.* (1991) found the biophysical similarities between NβV and NωV make it almost certain that NωV is also $T=4$. This however has not yet been confirmed by electron microscopy.

Recently, Agrawal and Johnson (1992) confirmed the presence of a second protein of approximately 8 kDa in NωV, in addition to the single major polypeptide described by Hendry *et al.* (1985). It would appear that these two proteins are produced as a result of post-translational cleavage of a 70 kDa precursor.

1.2.4 Genome

The genome of the Tetraviridae is a single stranded, positive sense RNA. Struthers and Hendry (1974) confirmed the NβV contained an RNA genome through the orcinol and diphenylamine reaction which gave positive and negative reactions respectively. The molecular weight of the genome was estimated at 1.8×10^6 with a total RNA content of 11 %. The phosphorus content of the virus was found to be 1.03% and the base ratios shown to consist of 27.8 % guanylic acid, 22.6 % uridylic acid, 25.5 % cytidilic acid and 24.1 % adenylic acid (Struthers and Hendry, 1974).

King and Moore (1985) attempted to compare the degree of relationship of NεV

and *Tni*V at the genomic level using cDNA in liquid hybridisation studies. There was virtually no hybridisation between *Tni*V cDNA and NεV RNA, and no detectable hybridisation at all between NεV cDNA and *Tni*V RNA. The absence of a poly (A) tract in both NεV or *Tni*V RNA was demonstrated through chromatography on an oligo (dT) cellulose column (King and Moore, 1985).

Morris *et al.*(1979) found *Tni*V to contain approximately 10-15 % RNA from its OD_{260nm}/OD_{280nm} ratio. This is approximately the same as that found for NβV . Similar results have been found for other Tetraviridae with OD_{260nm}/OD_{280nm} ratios of between 1.32 (*Thosea asigna* V.) and 1.45 (NβV) (Olson *et al.*, 1990).

Du Plessis *et al.*(1991) isolated dsRNA from larval tissue infected with NβV in an attempt to detect the presence of double-stranded replicative forms (RF) of potential sub-genomic messenger RNA's. The method used was that developed by Morris and Dodds (1979). It was hoped that if any sub-genomic messenger RNA's were present, they would be more readily observed in the double-stranded form which is more resistant to degradation. A single dsRNA molecule was isolated with a molecular weight of 3.6×10^6 Da. Such a molecule would be expected, representing the replicative form of the NβV genome which has a molecular weight of 1.8×10^6 Da.

Hendry *et al.*(1985) used electrophoresis in a composite agarose-acrylamide gel to characterise the genome of the most recently isolated Tetravirus, NωV. They

suggested the virus had a bipartite genome approximately 60% of the size of NβV with molecular weights of approximately 0.9 x 10^6 and 1.8 x 10^6 (Hendry *et al.*, 1985).

Agrawal and Johnson (1992) were able to determine the sequence of the RNA 2 and showed it to consist of 2 448 nucleotides consisting of a single open reading frame which had the capacity to encode a putative 644 amino acid residue protein with a molecular weight of 70 000 Da.

1.2.5 *In vitro* translation products

Due to the lack of a cell line capable of supporting viral replication, it is necessary to rely on *in vitro* translation experiments to investigate viral encoded proteins. Several cell-free protein synthesising systems are available for the translation of messenger RNA's. Of these systems the rabbit reticulocyte lysate and wheat germ extract are the most readily used as they are easy to prepare and available commercially (Clemens, 1984). Other lysate systems have been used in the translation of insect virus RNA's. These include a *Drosophila melanogaster* cell free lysate developed from cultured cells and used by Guarino *et al.*(1981) for the translation of Black Beetle Virus (BBV) RNA. As it is possible to culture BBV in a *Drosophila* cell line, such a lysate system might have advantages in the translation of the BBV RNA. The Tetraviruses which lack an insect cell line which will support their replication, are however adequately translated in a rabbit reticulocyte lysate system (King *et al.*, 1984; Reavy and Moore, 1984; Du Plessis *et al.*, 1991).

The *in vitro* translation products of *Tni*V and NεV have been well studied (King *et al.*, 1984; Reavy and Moore, 1984). Using rabbit reticulocyte lysate systems it was found that RNA from both these viruses would stimulate protein synthesis with maximal synthesis being obtained following 60 min. incubation (King *et al.*, 1984). The levels of protein synthesis however were much lower than those obtained from other insect viruses *in vitro*, such as Cricket Paralysis Virus (Reavy and Moore, 1984) which gave approximately a 5-10 fold greater protein synthesis than the endogenous RNA in the lysate (King *et al.*, 1984).

When the radiolabelled, viral encoded proteins were resolved on polyacrylamide gels and autoradiographs produced, a number of proteins were detected. Both *Tni*V and NεV RNA have molecular weights of approximately 2.0×10^6 Da and therefore have the capacity to encode protein totaling approximately 200.0 kDa. The largest protein encoded by both viruses was 140 kDa (Reavy and Moore, 1984; King *et al.*, 1984).

When immunoprecipitation studies were carried out on *Tni*V proteins synthesised *in vitro*, no protein corresponding to the capsid protein was found; however a protein of molecular weight 70 kDa was immunoprecipitated. This protein may represent a precursor from which the coat protein is produced via a post-translational event (King *et al.*, 1984).

In vitro translation studies of NβV RNA in a rabbit reticulocyte lysate system showed that NβV gave 30 times greater protein synthesis then the endogenous

messenger. A single major translation product of 71 kDa was produced which could not be immunoprecipitated. This would suggest that this protein was not the capsid protein precursor (Du Plessis *et al.*, 1991) unlike the 70 kDa protein immunoprecipitated by King *et al.*(1984) from lysates containing *Tni*V RNA. Du Plessis *et al.*(1991) stated however that this 71 kDa protein could have arisen as a result of a spurious initiation site being activated due to fragmentation of the RNA. Unlike *Tni*V and NεV (Reavy and Moore, 1984; King *et al.*, 1984), no large protein of 140 kDa was observed for NβV; however Du Plessis *et al.* (1991), found that in some overexposed autoradiographs, proteins exceeding 71 kDa were present.

1.3 cDNA Synthesis and Cloning

1.3.1 cDNA Synthesis

The cloning of cDNA is a useful tool for the analysis of genomes of organisms. Rougeon and Mach (1976) demonstrated the biosynthesis of globin genes *in vitro* from globin mRNA. The technique has since been refined with Okayama and Berg (1982), eliminating the S1 nuclease mediated cleavage step of the hairpin loop thus reducing the loss of clones encountered previously. Gubler and Hoffman (1983) were able to improve upon the Okayama-Berg method by linking the oligo (dT) primed first strand synthesis with RNase H DNA polymerase mediated second strand synthesis. Using this method it was possible to generate up to 10^6 clones per μg mRNA.

In addition to the synthesis of cDNA from mRNA it is also possible to generate

cDNA from dsRNA. This was shown by Cashdollar *et al.*(1982) who used Reovirus dsRNA as a template for the synthesis of cDNA.

Agrawal and Johnson (1992) demonstrated that cDNA to RNA 2 could be synthesised through reverse transcription using degenerate primers based on the capsid protein amino acid sequence.

1.3.2 Polymerase Chain Reaction

The amplification of DNA through the polymerase chain reaction (PCR), has greatly contributed to recent advances in recombinant DNA technology. PCR is a method by which the double stranded target DNA is heat denatured in the presence of an excess of two primers which flank the sequence to be amplified and are complementary to the opposite stands of the target. The primers are allowed to hybridise to the target DNA at reduced temperatures and then are extended towards one another by a thermostable DNA polymerase. These extension products are then dissociated from the template by heating. Each extension product may serve as a template and therefore after n cycles of denaturation, annealing and extension, the target sequence may be amplified 2^n times (Birkenmeyer and Mushahwar, 1991). Saiki *et al.*(1985) were amongst the first to demonstrate the usefulness of this technique in their amplification of the β-globin genomic sequences. This technique has since been used for a wide variety of applications and requires only minute amounts of DNA as demonstrated by Puchta and Sanger (1989) with DNA reverse transcribed from viroid RNA.

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The scope of PCR for the amplification of DNA was further enhanced when the use of arbitrary primers was demonstrated by Welsh and McClelland (1990) and Williams *et al.*(1990). These two groups used conditions of very low stringency with annealing temperatures lower than 40°C and were able to amplify genomic DNA using non-specific primers. They showed however, that temperatures in excess of 40°C in the thermal cycling profile prevented amplification of some of the oligonucleotides tested.

1.3.3 Cloning Vectors

Over the last few years a number of expression vectors have been developed for the cloning of both prokaryotic genomic and eukaryotic cDNA. One of the most commonly used type of vectors are plasmids. Several types of plasmids are available including the pBR322 plasmid which is a 4362 base pair, double stranded DNA plasmid designed for the simple and rapid cloning of cDNA. The plasmid contains tetracycline and ampicillin resistance genes and several restriction sites which allow the cloning and sub-cloning of restriction fragments. When *E.coli* is successfully transformed with the plasmid, antibiotic resistance will be conferred upon the bacterium (Davis *et al.*, 1986).

In addition to the plasmids, bacteriophages may also be used as vehicles for cloning. The M13 mp series are derivatives of the M13 *E.coli* bacteriophage. These vectors contain sequence of restriction sites which have been inserted into the amino terminus of the *lacZ* gene which encodes the enzyme β-galactosidase which metabolises galactose sugars (Davis *et al.*, 1986).

A series of plasmids which contain features of both M13 and the pBR plasmids are the pUC plasmids. These plasmids, which are approximately 2.7 kb in size, contain the ampicillin resistance gene and the origin of replication of pBR322 and a portion of the *lacZ* gene of E.coli. Like M13, the *lacZ* gene contains a polylinker sequence of unique restriction sites. These plasmids grow in high copy number and when DNA fragments are inserted into their cloning sites the *lacZ* gene is inactivated. This feature may be used for the selection of transformed cells as, when appropriate *E.coli* such as JM103 or JM109 which are *lac*⁻ are transformed with plasmids containing an insert and the cells grown on media containing X-gal and IPTG, the transformed cells will give rise to white colonies, while those cells which are not transformed will appear blue (Davis *et al.*, 1986). The pUC 18 and pUC 19 plasmids are identical except that they contain polycloning sites in opposite orientations. These plasmids lack the *rop* gene which is involved in the control of copy number and is usually located near the origin of replication. As a result these plasmids replicate at a higher copy number than most other plasmids (Sambrook *et al.*, 1989).

1.3.4 Transformation of *E.coli* cells

The basis of cloning rests upon the ability to transfer the vector plasmid into the host cell. The most commonly used host is *E.coli* and the process is known as transformation. The ability to transform *E.coli* with a plasmid was first demonstrated by Cohen *et al.* (1972) based on the observation of Mandel and Higa (1970), who showed that *E.coli* cells could be transfected with bacteriophage n the

presence of $CaCl_2$ at 0^oC. It has since been demonstrated that in the presence of divalent cations at low temperatures *E.coli* and DNA will productively interact. A number of factors have been seen to improve the frequency of transformation and these include heat shock, the addition of a monovalent ion to the buffer, the addition of hexamine cobalt (III) chloride, treatment of the cells with solvents and sulphydryl reagents and growth in media containing 10-20 mM magnesium ions (Hanahan, 1986).

1.4 Hybridisation Studies

The technique of nucleic acid hybridisation has evolved from a method for determining the copy number of genes to one of the most important techniques in the isolation and manipulation of genes. Hybridisation techniques have been modified from the initial methods of liquid hybridisation to procedures involving DNA and RNA immobilisation including Southern Blots, Northern Blots and Dot Blots (Berent *et al.*, 1985). The development of non-radioactive probes has done much to increase the range of uses for hybridisation. Non-radioactive probes such as those labelled with Photobiotin, an analogue of biotin, have been shown to be equally sensitive to radiolabelled probes (Habili *et al.*, 1987). These probes have the added advantages in that they are not limited by a short half-life and stringent safety precautions do not have to be taken.

DNA hybridisation studies have frequently been used for the detection of virus within insects, whether it be an insect pathogen itself or a plant pathogen within an insect vector. A DNA hybridisation assay for the detection of Gypsy Moth Nuclear

Polyhedrosis Virus (NPV) in infected gypsy moth larvae was developed by Keating *et al.*(1989). These larvae, which cause defoliation of forests occur in periodic outbreaks often followed by epizootics of the virus. Keating *et al.*(1989) detected the viral DNA in larvae using a Dot-Blot Hybridisation procedure based on the method developed by Kaftos *et al.*(1979). DNA was extracted from the larvae and vacuum blotted onto nitrocellulose. Viral DNA was labelled with [^{32}P]dCTP and used to probe the Nitrocellulose filters blotted with DNA extracts under stringent conditions. The presence of the bound probe was detected through autoradiography (Keating *et al.*, 1989).

Similarly Boulton and Markham (1985) developed the squash blot technique, a modification of the dot-blot procedure which allowed the rapid and sensitive detection of plant pathogens in their insect vectors. The insects were squashed onto nitrocellulose filters and hybridised with [^{32}P]- labelled cDNA. They were able to demonstrate using Maize Streak Virus (MSV) that the degree of nucleic acid hybridisation was linear with respect to DNA concentration within the range of 20pg to 10 ng.

The Dot-Blot Hybridisation technique is less time consuming than either Northern or Southern blot analyses and can be used for partially degraded RNA samples with semi-quantitative results (Davis *et al.*, 1986).

CHAPTER 2: METHODS AND MATERIALS

2.1 Extraction and purification of NωV

2.1.1 Virus purification

The *Nudaurelia* ω virus was extracted from frozen, naturally infected pine emperor moth larvae collected from pine (*Pinus radiata*) plantations near George, in the South Western Cape, South Africa. The method used was a variation of that devised by Morris *et al.*(1979) for the extraction of *Trichoplusia ni* virus from infected larvae. This purification method is a four fold procedure involving:

(i) Homogenisation of the infected larvae.

(ii) Precipitation of the virus from the homogenate using a PEG/NaCl precipitation method.

(iii) Purification by centrifugation on a sucrose density gradient

(iv) Further purification by isopycnic centrifugation on a caesium chloride gradient (Hendry *et al.*,1985).

The purity of the preparation was assayed using ultra-violet (UV) spectrophotometry which allowed the calculation of the concentration of virus in the sample in addition to the RNA content. SDS-PAGE made estimation of the molecular weight of the virus capsid proteins possible .

(i) Homogenisation of the larvae:

A total of 25 g infected larvae was thawed and then homogenised using a Du Pont Sorvall Omnimixer at speed setting 6 for 1 minute in 90 ml 0.5 M Tris / 0.01 M ethylenediaminetetraacetic acid (EDTA) buffer at pH 7.5 with 0.2% 2-mercaptoethanol and 25 ml carbon tetrachloride (CCl_4). The homogenisation was carried out on ice and the homogenate clarified by centrifugation in the Sorvall JA14 rotor at 8 000 rpm for 15 minutes at $4^{o}C$.

(ii) Virus precipitation:

The virus was precipitated overnight from the clarified supernatant in the presence of 8 % polyethylene glycol (PEG) and 0.1 M NaCl. The precipitated virus was pelleted by centrifugation in the Beckman JA14 rotor at 8000 rpm for 10 minutes at $4^{o}C$ and resuspended in 20 ml 0.1 M Tris / 0.01 M EDTA. A further clarification step was carried out by centrifugation at 8 000 rpm for 10 minutes at $4^{o}C$. The supernatant was retained, and the virus pelleted through 1 ml 30% sucrose at 25 000 rpm $10^{o}C$ for 3 hours at $10^{o}C$ in a Beckman Type 30 rotor.

(iii) Sucrose Density Gradient Centrifugation:

The virus pellet was resuspended in 2 ml 0.1 M Tris / 0.01 M EDTA buffer and 1ml samples layered onto a 15 - 45 % linear sucrose gradient made up in 0.1 M Tris / 0.01 M EDTA. The gradients were centrifuged in a Beckman type SW28 rotor at 28 000 rpm for 110 minutes at $10^{o}C$. The gradients were fractionated using an Isco Density Gradient Fractionator, and the pooled fractions diluted 1:3 in 0.1 M Tris /

0.01 M EDTA buffer. These fractions were centrifuged in a Beckman Type 30 rotor at 25 000 rpm for 3 hours at 10^oC, and the pellets resuspended in 1 ml of the same buffer. The concentration of the virus was determined spectrophotometrically using the extinction coefficient for NωV. A sample of this virus preparation was electrophoresed on SDS-PAGE as outlined below.

(iv) Purification on a CsCl Gradient:

The method used follows that of Hendry *et al.* (1985), in which 1.0 mg virus in 0.1 M Tris / 0.01M EDTA buffer was adjusted to 39% (wt/vol.) with respect to CsCl. The samples were centrifuged in a Beckman SW50 rotor at 30 000 rpm for 16 hours at 10^oC. The gradients were fractionated as for the sucrose gradient. Pooled virus-containing fractions were pooled and dialysed against 0.1 M Tris / 0.01 M EDTA overnight. The concentration of the virus was calculated by UV spectrophotometery.

2.1.2 PAGE

Approximately 10 μg virus was loaded per lane, on a 4 % : 10 % polyacrylamide vertical slab gel (see Appendix 1A). Each sample was mixed with dissociation buffer containing 0.2 % bromophenol blue and heated to 80^oC in a water bath for 3 minutes prior to loading. The samples were electrophoresed together with samples of NβV and molecular weight marker proteins (see Appendix 1B) at 40V for 16 hours. Following electrophoresis the gel was placed in a staining solution containing 0.2% coomassie blue (see Appendix 1A) for 45 minutes and destained in several changes of destaining solution (see Appendix 1A). The gels were dried

under vacuum using a heated, slab gel drying apparatus.

Attempts were also made to fix the proteins so as to prevent loss of resolution due to diffusion, by soaking the gels in glacial acetic acid : methanol : water (10 : 20 : 70) prior to staining, as described by Sambrook *et al.* (1989).

2.1.3 Ouchterlony test

Antibodies were raised against NωV following two intra-muscular injections of a rabbit with Freunds adjuvant. A two fold dilution series of the antiserum was made in phosphate buffered saline up to 1/512. Ouchterlony tests were carried out to determine the titre of the antiserum raised using virus at a concentration of 2mg / ml. The plates were stored overnight and precipitin bands observed.

2.2 Extraction and electrophoresis of the NωV genome

Having obtained pure virus by sucrose density gradient and caesium chloride centrifugation, intact viral nucleic acid was extracted using a basic phenol / chloroform extraction procedure, following treatment with Proteinase K (Du Plessis *et al.*, 1991). The success of the extraction was determined using UV spectrophotometry and agarose gel electrophoresis. The dsRNA isolated was electrophoresed on a non-denaturing 1% agarose gel which allowed an estimation of the molecular weights of these intermediate forms relative to molecular weight markers.

The presence of a poly (A) tract was investigated following the procedure outlined

by Clemens (1984).

In the following experiments, gloves were worn at all times and all equipment and solutions were autoclaved at 15 psi for 20 min. to avoid degradation of the viral genome by ribonucleases (RNase), particularly those produced by the skin.

2.2.1 Phenol / Chloroform Extraction

The virus was diluted to a concentration of approximately 1 mg/ ml in 0.1 M Tris buffer (pH 7.5). The solution was made up to a final concentration of 0.25 % sodium dodecyl sulphate (SDS), 0.1 M NaCl and 1 mM dithiothreitol (DTT). Proteinase K was added at a concentration of 50 μg / mg virus and the resulting solution was incubated at 37°C overnight. The concentration of SDS was increased to 1% and the solution incubated at 37°C for 1 hour. If opalescence remained, the solution was heated to 80°C for 5 minutes.

An equal volume of buffer-saturated phenol (see Appendix 2 A) was added and the solution vortexed for 1 minute. An equal volume of chloroform : isoamylalcohol (24 : 1) was then added and the solution was again vortexed. The solution was microfuged at 10 000 rpm for 5 minutes. The lower-protein containing phenol phase was removed and the upper aqueous phase retreated as before. The aqueous phase was extracted and treated with three washes of equal volumes chloroform : isoamylalcohol (24 : 1). To the final aqueous phase, 2.5 volumes 95% ethanol was added and the solution was made 0.3 M with respect to sodium acetate and stored at -20°C overnight to precipitate the RNA.

The solution was spun at 10 000 rpm for 15 minutes. The RNA pellet was dried under vacuum and resuspended in 400 µl sterile, distilled water. The concentration of the RNA was determined from its absorbance at 260 nm. The RNA was reprecipitated, pelleted, dried as before and then resuspended in 50 µl sterile, distilled water.

2.2.2 Agarose Gel Electrophoresis

The extracted RNA was electrophoresed on a agarose-formaldehyde gel system as described in Sambrook *et al.*(1989).

The gel was prepared (see Appendix 1C) and samples of approximately 10 µg RNA were dissolved in sterile, distilled water and made up to 20 µl with sample solution to which 5 µl loading buffer was added (see Appendix 1C). The solution was loaded on the gel and electrophoresed for 3 hours at 40V together with RNA markers. The gels were washed with several changes of water to remove formaldehyde, prior to staining in a solution containing 0.5 µg / ml ethidium bromide for 20 minutes and observed under UV light using a transilluminator.

2.2.3 Sucrose Density Gradient Centrifugation

Approximately 100 µg RNA was layered on top of a 5 to 25% sucrose (RNase-free) gradient prepared in a 0.1 M Tris - acetate (pH 7.5), 0.002 M EDTA, 0.1 % SDS buffer. The gradients were centrifuged in a SW41 rotor at 40 000 rpm for 5 hours 45 minutes at $10^{\circ}C$, and fractionated using the Isco Density Gradient

Fractionator. The fractions containing each of the separated RNA species were pooled. The RNA was precipitated as before and the pellet resuspended in 20 µl sterile distilled water. The concentration of each of the species was determined spectrophotometrically. The separated RNA was analysed on formaldehyde agarose gels.

2.2.4 Extraction of ds RNA

Approximately 10 g infected larval tissue was frozen with liquid nitrogen and ground to a fine powder with a mortar and pestle. The powder was allowed to thaw and ground further with 10 ml extraction buffer (see Appendix 2B). The solution was poured into a centrifuge tube and equal volumes of phenol and of chloroform / isoamylalcohol (24 : 1) were added. After vortexing for 30 seconds and incubating on ice for 15 minutes with occasional shaking, the solution was centrifuged at 8 000 rpm for 20 minutes at 4°C in a JA20 rotor and the supernatant fluid carefully removed. If the supernatant volume was too low, the pellet was re-extracted as before. Absolute ethanol was slowly added to the supernatant with stirring to give a final concentration of 16%. This was achieved by adding 1ml 95% ethanol per 5 ml supernatant fluid.

A total of 1 g Whatman's CF11 cellulose powder was added to the supernatant fluid and vortexed. The solution was added to a small glass column and allowed to settle and dry. The column was washed with 100 ml washing buffer (Appendix 2B). An 8 ml sample of ethanol-free elution buffer was run through the column so as to elute the dsRNA and samples were collected directly into a centrifuge tube. The RNA

was precipitated as for the single-stranded RNA by the addition of 2.5 volumes 95% ethanol and 3 M sodium acetate to a final concentration of 0.3 M, and stored at -20°C overnight. The RNA was pelleted by centrifugation at 10 000 rpm for 15 minutes at 4°C, and the pellet dried under vacuum. The pellet was resuspended in 400 μl 10 x STE Buffer (Appendix 2B) and a further precipitation step carried out. The RNA pellet obtained from this step was resuspended in 40 μl sterile, distilled water.

2.2.5 Non-Denaturing Agarose Gel Electrophoresis

A 1 % agarose gel was prepared (Appendix 1E), and samples containing approximately 10 μg dsRNA were mixed with loading buffer to give a final volume of 20 μl and loaded on the gel together with DNA markers (Appendix 1F). The samples were electrophoresed at 50V for 2 hours and the gel stained with ethidium bromide as for the single stranded RNA gels. These were then viewed using the transilluminator and the molecular weights of the dsRNA calculated relative to the DNA molecular weight markers (Appendix 1F).

2.2.6 Affinity chromatography on poly(U) sepharose

A total of 0.5 g poly(U) sepharose-4B(Pharmacia) was suspended in 2.5 ml elution buffer (Appendix 2C). The suspension was poured into a sterile, silanized pasteur pipette containing a glass wool plug. The column was washed with 3 ml elution buffer followed by 3 ml loading buffer (Appendix 2C). 60 μg mixed NωV RNA was dissolved in 60 μl loading buffer and added to the top of the column. The column was washed three times with 1 ml loading buffer, and six times with 0.25 ml

elution buffer. 400 μl aliquots were collected subsequent to the addition of the RNA. The RNA containing fractions were determined spectrophotometrically from their absorbance at 260 nm and pooled. The RNA was precipitated as before.

2.3 Cell-free translations and immunoprecipitations

In these experiments single stranded (ss) NωV RNA extracted previously, was translated in both its mixed and separated forms, in a rabbit reticulocyte lysate system (Amersham). [^{35}S]-methionine (Amersham) incorporated into the translated proteins was used to monitor the course of the viral protein synthesis.

This rabbit reticulocyte lysate is optimised for the tobacco mosaic virus (TMV) mRNA and thus optimal conditions for NωV RNA translation were investigated taking the following three factors into account:

(i) RNA concentration

(ii) magnesium and potassium concentrations

(iii) Labelled amino acid concentration

During the optimisation experiments, incorporation of the label into the translated proteins was determined using a filter disc method whereby aliquots of the translation mix were spotted onto pretreated paper filters (Appendix 3) which were subsequently counted in a scintillation counter. Synthesised proteins contained in the translation mix were resolved by SDS-PAGE and autoradiography.

Immunoprecipitation was attempted in order to identify the radiolabelled coat protein of NωV, synthesised during the cell free translation of the NωV RNA.

The Amersham Rabbit Reticulocyte Lysate (nuclease treated, message dependent) code N.90 was used in these experiments and was stored at -70°C in small aliquots which were thawed when needed. The NωV RNA used was that extracted previously and as before precautions were taken to avoid its degradation.

2.3.1 General Translation Procedure

A preliminary translation experiment was carried out to compare the translation efficiency of NωV RNA (mixed, large and small species) with that of tobacco mosaic virus (TMV) RNA, for which the system is optimised. Each 30 μl reaction mixture consisted of a minimum of 21 μl lysate and contained per μl lysate: 0.05 μg RNA; 1 μCi [^{35}S]-methionine; 33ng t-RNA. All additions were carried out on ice. The translation mixture was vortexed and microfuged briefly and 2 μl samples were spotted onto pretreated Whatman 2.5cm filter paper discs (Appendix 3A) at time zero. The lysate mixture was incubated at 30°C and further 2 μl samples taken at 5 , 10 , 15, 30 and 60min..

The paper discs were dried using an infra-red (IR) lamp and then treated further (Appendix 3A) so as to remove any unincorporated radiolabel. The discs were again dried using the IR lamp, transferred to a pre-counted scintillation vial containing 5 ml non-aqueous scintillation cocktail (see Appendix 3B), and the incorporation of [^{35}S]-methionine into translated proteins was measured in a liquid scintillation counter.

2.3.2 Preparation of the translation mixture for PAGE

After the 1 hour incubation period, the translation reaction was terminated with the addition of an equal volume of 2% SDS, 1% 2-mercaptoethanol, and stored at 4°C. A volume of each lysate mixture containing approximately 50 x 10^3 cpm was made up to 30 µl with dissociation buffer (Appendix 1), heated to 80°C for 4 minutes and layered onto a 4 % : 10 % polyacrylamide slab gel (Appendix 1A). The gel was electrophoresed at 40 V for 16 hours and placed in staining solution (Appendix 1A) for 2 hours, followed by destaining. The gel was dried, overlaid with an x-ray film and exposed for 72 hours to produce an autoradiograph.

2.3.3 Optimisation of the lysate

(i) RNA concentration:

The optimal concentration of RNA was determined over the range 0.05 to 0.25 µg RNA / µl lysate. The remainder of the lysate conditions were maintained as for the preliminary translation procedure and the incorporation of [^{35}S]-methionine determined as before.

(ii) Potassium and Magnesium concentrations:

The endogenous concentration of potassium (K$^+$) and magnesium (Mg^{2+}) in the undiluted lysate, as stated by the manufacturer, was 142.2 mM and 2.95 mM respectively. To determine the optimal K$^+$ concentration for the translation of the NωV RNA, the Mg^{2+} concentration was held constant at 2.4 mM and the concentration of the K$^+$ varied between 100 mM and 230 mM by either dilution of

the lysate or addition of 800 mM potassium acetate. Similarly, the optimum Mg^{2+} concentration was determined by maintaining the K^+ concentration at 130 mM and varying the Mg^{2+} concentration from 1.5 mM to 2.5 mM by either dilution of the lysate or addition of 25 mM magnesium acetate. In all tests, the RNA concentration was maintained at 0.2 µg RNA / µl lysate (ie: the optimum determined previously). All other lysate conditions were as for the general translation procedure. The amount of [^{35}S]-methionine incorporated into NωV encoded proteins was determined as before.

(iii) Radiolabel concentration:

In this experiment between 3.75 µCi and 30 µCi [^{35}S]-methionine was added to 30 µl of the optimised lysate and the amount of incorporated label determined as previously.

2.3.4 Immunoprecipitation

Cell free translations were carried out as before except that no 2% SDS, 1% 2-mercaptoethanol was added following incubation. To each 30 µl lysate mixture, 30 µl antiserum (raised against NωV in the rabbit) was added either undiluted or at appropriate dilutions of RIPA buffer (Appendix 3C).

A control was prepared containing no antiserum (i.e. containing 30 µl RIPA buffer). These mixtures were incubated at 4°C overnight for Ab-Ag complex formation. Following incubation, 40 µl protein A solution (Appendix 3D) was added to a final concentration of 3.5% and allowed to stand for 20 minutes with occasional

vortexing to permit binding of the antibody-antigen complex to the Protein A. The solution was microfuged for 30 seconds to pellet the Protein A complex, the supernatant discarded and the tubes drained. 500 µl RIPA buffer was added to each pellet, which was allowed to stand at room temperature for 15 minutes for softening of the Protein A. The solution was again vortexed and microfuged. A further 500 µl RIPA buffer added to the pellet. This washing step was repeated three times. To the final pellet, 50 µl 1% SDS, 1% 2-mercaptoethanol was added and the solution boiled to release the Protein A. The solution was again microfuged to pellet the Protein A and the supernatant retained.

2.3.5 Electrophoresis and Autoradiography

The supernatant was made up to 30 µl with dissociation buffer and boiled for 4 minutes. Electrophoresis on a 4 % :10 % polyacrylamide slab gel (Appendix 1A) followed by autoradiography was carried out as before.

2.4 DNA Synthesis, PCR Amplification and Cloning

An attempt was made to synthesise and clone cDNA from mixed viral RNA using both a cDNA synthesis kit and RT-PCR

2.4.1 cDNA Synthesis

A Boehringer-Mannheim cDNA Synthesis Kit was used adhering to the provided protocol.

The following were added to a sterile Eppendorf tube:

4 μl 5x buffer I (for first strand synthesis)

1 μl RNase inhibitor (25 units)

2 μl deoxynucleotide triphosphate mixture (10mM)

2 μl random hexanucleotide primer

2 μl [^{32}P] - dCTP (approximately 20 μCi)

7 μl mixed RNA (approximately 1 μg)

2 μl AMV reverse transcriptase (40 units).

Following each addition the solution was vortexed. The mixture was microfuged following the addition of the reverse transcriptase and incubated for 60 min. at 42°C. After incubation, the following additions were made to the solution on ice:

40 μl buffer II (for second strand synthesis)

1 μl RNase H (1 unit)

34 μl redistilled water

5 μl E.coli DNA polymerase. (25 units)†

The solution was vortexed after each addition and microfuged after the final addition. The mixture was incubated for 60 min. at 37°C.

The blunt ends were "polished" further by the addition of 6 μl deoxynucleotide triphosphate mixture (10 mM), 6 μl 0.15 M MgCl$_2$ and 3 μl T4 DNA polymerase and incubated for 15 min at 37°C. Following incubation, 8 μl 0.5M EDTA was added to terminate the reaction. The cDNA was extracted with phenol/chloroform

and precipitated twice with ethanol. The precipitate was washed with 70 % ethanol The cDNA was resuspended in 500 µl TE Buffer (Appendix 4).

A 5 µl cDNA solution was spotted onto each of two Whatman GF/C glass fibre discs pinned on a polystyrene board. Both discs were dried under an infra-red lamp. One disc was washed 3 times (2 min. per wash) in a fresh solution of 5% TCA, 20 mM sodium pyrrophosphate. The filter was transferred briefly to 70% ethanol and then redried. Both the treated and untreated discs were placed in 10 ml non-aqueous scintillation cocktail and counted in a liquid scintillation counter. The incorporation of label into newly synthesised cDNA was determined as follows:

$$\frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} \times 100 = \% \text{ incorporation.}$$

The remainder of the cDNA solution was passed through a minicolumn of Sephadex G75 prepared by mixing 3 g Sephadex G75 with 25 ml 1 x TE Buffer (pH 8.0). The slurry was mixed well and allowed to stand overnight. The supernatant was decanted and an equal volume of 1 x TE Buffer pH 8.0 added. The slurry was pipetted into a silanised pasteur pipette with a glass wool plug to produce the mini-column.

Aliquots of 50 µl were collected and those containing cDNA were determined through Cerenkov counting in a scintillation counter.

Fractions containing cDNA were pooled and precipitated as before. The precipitate was resuspended in 50 µl TE Buffer.

A volume of 10 µl cDNA was mixed with 10 µl alkaline loading buffer (Appendix 1G) and loaded on an alkaline-agarose gel (Appendix 1G). The gel was electrophoresed for 2.5 h. at 50 V and then placed in 7 % trichloroacetic acid (TCA) for 30 min. to fix the nucleic acids. By exposing X-ray film to the gel (sealed in a plastic bag) overnight, an autoradiograph was produced.

2.4.2 Polymerase Chain Reaction

It was initially attempted to amplify the amount of cDNA synthesised via reverse transcription using the Polymerase Chain Reaction, for use in cloning. In addition a Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) (Kawasaki, 1990) was carried out to produce amplified cDNA directly from the RNA.

As this PCR was carried out prior to the publication of the sequence of RNA 2 by Agrawal and Johnson (1992), no sequence of the genome was known and therefore arbitrary primers were used. Welsh and McClelland (1990), and Williams *et al.* (1990), demonstrated that through the use of low stringency PCR conditions, with annealing temperatures of less than 40°C, it was possible to amplify DNA using non-specific primers.

(i) PCR of cDNA

PCR was used to try and amplify the amount of cDNA produced using the cDNA synthesis kit. The following were added to a sterile Eppendorf Tube on ice:

1 µl bovine serum albumin (BSA)

5 µl 10 x PCR buffer (Promega)

0.5 µl Taq polymerase (2.5 units) (Promega)

3 µl 10 mM deoxynucleotide triphosphate mixture

0.1 µl 10mer primer (~ 3 mg / ml)

5.0 µl cDNA

The solution was made up to a final volume of 30 µl by the addition of sterile, distilled water. 500 µl liquid paraffin was added to the surface to prevent condensation on the sides of the tube during the thermal cycling.

A two stage reaction was followed with a first stage of 3 cycles and a second stage of 40 cycles. Initial denaturation was carried out at 94°C for 240 seconds. The remainder of the denaturation steps were carried out at 94°C for 30 seconds. Annealing was carried out for stage 1 at 40°C for 25 seconds and for stage 2 at 45°C for 30 seconds. Low annealing temperatures were used to ensure binding of the primers regardless of the Tm. The extension step was carried out at 72°C for 60 seconds with a final extension at 72°C for 300 seconds.

A total of 10 µl of the reaction mixture was electrophoresed on a 3% agarose gel

(in TAE Buffer) for 2.5 h. at 100V. Following electrophoresis the gel was stained with ethidium bromide.

(ii) RT-PCR

The protocol followed was that outlined by Kawasaki (1990).

Approximately 1 μg (0.1 μg / μl) RNA was heated to 90°C for 5 min and then chilled rapidly. The following was added to a sterile Eppendorf Tube on ice:

4   μl   10 x PCR buffer (Promega)

1   μl   RNase inhibitor (25 units)

4   μl   10 mM deoxynucleotide triphosphate mixture (Boehringer-Mannheim)

2   μl   [^{32}P] dCTP   (approximately 20 μCi) (Amersham)

5   μl   mixed RNA   (0.1 μg)

2   μl   random hexanucleotide primer

1 μl 10mer arbitary primer (~ 0.3 μg / μl)

2   μl   M-MuLV reverse transcriptase (20 units).

Following the addition of the reverse transcriptase, the solution was vortexed and microfuged. The mixture was incubated for 10 min. at 23°C and then 60 min. at 42°C. The reaction mixture was heated at 95°C for 5 minutes and quickly chilled to denature the RNA-cDNA hybrid and to inactivate the reverse transcriptase.

Samples of 1 μl of the PCR mix were spotted onto each of two Whatmann GF/C Glass Fibre discs. The incorporation of label into cDNA was determined as described previously. To the remainder of the solution 0.5 μl Taq polymerase was

added. The solution was made up to 30. μl with sterile distilled water. 500 μl liquid paraffin was added.

The PCR was carried out under the same thermal cycling conditions as described previously. 10 μl of the sample was electrophoresed on a 3% alkaline agarose gel as before.

The remainder of the PCR solution was eluted through a Sephadex G75 column and fractions containing cDNA pooled. This cDNA synthesised using RT-PCR was used as a probe for the detection of NωV RNA in subsequent hybridisation experiments.

2.4.3 Cloning

Cloning was attempted using a pUC cloning Kit (Boehringer-Mannheim).

(i) Preparation of Cleaved pUC Vector

The following were added to a sterile Eppendorf Tube on ice:

10 μl pUC DNA (approximately 1 μg)

2 μl 10 x restriction buffer A (Boehringer-Mannheim)

6 μl redistilled water

2 μl *Sma*I restriction endonuclease (4 units) (Boehringer-Mannheim).

Following the final addition, the solution was vortexed and microfuged. The mixture was incubated for 1h at 25°C. The plasmid was not dephosphorylated prior

to ligation. 205 µl TE Buffer was added and a phenol/chloroform extraction performed.

To the final aqueous phase, 25 µl sterile 4 M LiCl and 750 µl 95% ethanol were added. The solution was stored at -20°C overnight. The DNA was precipitated by centrifugation in a microfuge for 20 min. The supernatant was removed, and the precipitate washed with 1 ml 70% ethanol. The solution was recentrifuged for 10 min and the pellet dried under vacuum. The pellet was resuspended in 10 µl sterile, distilled water. 1 µl DNA was electrophoresed on an 0.8% agarose gel (TE) to ensure complete linearisation of the plasmid had occurred.

(ii) Ligation

A ligation reaction was attempted using both the cDNA generated via reverse transcription and that produced by RT-PCR. The following were added to a sterile Eppendorf tube on ice, vortexing after each addition:-

1 µl cleaved pUC DNA

9 µl cDNA

1 µl 10 x ligation buffer

1 µl T4 DNA ligase (1 unit).

The solution was microfuged after the final addition, and incubated at 22°C overnight. A control ligation reaction was carried out in which no cDNA was included to allow self-recircularisation of the cleaved pUC DNA. This was carried out to test the efficiency of the ligation reaction.

(iii) Preparation of Competent *E.coli* cells.

A single colony of *E.coli* JM 83 was picked from an L.B plate (Appendix 4) and inoculated into 5 ml L.B. liquid medium (Appendix 4). The culture was incubated overnight at 37°C with shaking. 40 ml L.B. liquid medium was inoculated with 0.4 ml overnight culture and incubated for approximatley 2.5h. at 37°C until an OD_{600} 0.2 was obtained. The cells were centrifuged in a pre-cooled JA 20 rotor at 4°C and 5000 rpm for 10 min. The pellet was resuspended in 4 ml ice cold 3M $CaCl_2$ and the cells stored on ice for 24h.

(iv) Transformation

The DNA, both the control plasmid and plasmid containing cDNA insert, was mixed with sterile 50 mM Tris-HCl (pH7.2) to give a final volume of 50 µl . The DNA was added to 300 µl competent *E.coli* cells and incubated on ice for 40 minutes. .The competency of the cells was determined by the addition of 5 ng uncleaved pUC DNA to 300 µl competent cells and counting the number of colonies grown following plating. The solutions were warmed to 42°C for 3 min and allowed to stand at room temperature for 10 minutes. A volume of 1 ml L.B. liquid medium was added to the cells which were incubated for 1h at 37°C with shaking.

Aliquots of 0.2 ml of transformed cells were spread on L.B. / Amp / x-gal / IPTG plates (Appendix 4) and incubated overnight at 37°C.

2.5 Hybridisation studies

2.5.1 Hybridisation to Viral RNA

The method used was an adaptation of that described by Church and Gilbert (1984).
Nitrocellulose membrane (Hoefer Scientific Instruments) was cut to fit a Dot-Blot
Manifold. The nitrocellulose was soaked briefly in water followed by washing for 1
h. at room temperature in 20 x SSC (Appendix 4A). The manifold was washed
with 0.1 N NaOH and rinsed with sterile distilled water. Two sheets of absorbent
paper wetted previously with 20 x SSC were placed on top of the vacuum unit.
The nitrocellulose membrane was layered on top of the paper and the manifold
clamped together.

Each of the slots of the manifold were filled with 20 x SSC and a vacuum applied
until all the fluid had passed through the membrane. The slots were filled with 10 x
SSC and the vacuum reapplied.

Mixed, viral RNA at a concentration of approximately 1 μg / μl was used together
with control RNA consisting of RNA molecular weight markers (Boehringer-
Mannheim). Four 10 μl dilutions of RNA (1/10, 1/5, undiluted and control) were
mixed with :

20 μl 100% formamide

7 μl 37% formaldehyde

2 μl 20 x SSC

The solution was incubated at 67°C for 15 min. and then cooled on ice. Two volumes of 20 x SSC were added to each of the samples which were pipetted into the manifold slots. A gentle suction was applied until all the fluid had passed through the membrane. The slots were rinsed twice with 1 ml 10 x SSC. Following the second rinse, the vacuum was applied for a further 5 min. to allow the membrane to dry partially and then air dried overnight. The RNA was fixed on the membrane by exposure to UV light for 5 min. prior to prehybridisation in Church Hybridisation Buffer (CHB) (Appendix 4A) for 5-10min at 65°C. The probe, synthesised using the RT-PCR technique, was heated to 95°C for 5 min. and then chilled rapidly on ice before being added to the CHB and allowed to hybridize overnight at 42°C. Following hybridisation the blot was washed twice in Wash Buffer A (Appendix 4A) (10 min. per wash) and four times in Wash Buffer B (10 min. per wash) (Appendix 4A). The blot was dipped in distilled water to remove SDS and then sealed in a plastic bag and subjected to autoradiography.

2.5.2 Squash Blot

A segment of gut tissue of both infected and healthy larvae which had been stored at -20°C was removed by dissection and a segment of this gut was used for the Squash-Blots. The gut tissue was not treated in any way prior to blotting. The blotting protocol followed was based on the procedure by Boulton and Markham (1985), with blotting conditions outlined by Brandsma and Miller (1980).

Approximately 1 mm^3 of gut tissue of both healthy and infected larvae was placed on a 1 cm^2 demarcated square on a nitrocellulose sheet previously soaked in 20 x

SSC. A drop of 0.5 M NaOH was added to the gut tissue which was then squashed onto the membrane with the round end of a sterile glass rod. The membrane was soaked in 0.5 M NaOH for 1 min., twice in 0.6 M NaCl / 1 M Tris-HCl (pH 6.8) for 1 min. and twice in 1.5 M NaCl / 0.5 M Tris-HCl (pH 7.4) for 1 min. The membrane was allowed to air dry for 20 min. The membrane was floated in 95% ethanol, air dried for 5 min., washed twice in $CHCl_3$ and allowed to dry for 15min. The membrane was rinsed in 0.3 M NaCl and allowed to air dry overnight. The blot was exposed to UV light for 5 min.

Hybridisation was carried out as described previously.

CHAPTER 3: Results

3.1 Extraction and Purification of Virus

3.1.1 Virus purification

Following homogenisation of the larvae and precipitation of the virus with PEG, it was possible to carry out density gradient centrifugation in order to further purify the virus. The basis of density gradient centrifugation is that all molecules of the same density will sediment at a specific point in the density gradient. Fractionation of the sucrose density gradients, produced a discrete, UV-absorbing, virus-containing zone (Fig.1.1). When the absorbance of the pooled fractions, read on a spectrophotometer, was plotted from 320 to 220 nm (Fig.1.2), a trace characteristic of uncontaminated nucleoprotein was produced. This could be ascertained due to the smooth curve lacking "shoulders" which are characteristic of contamination. Calculation of the virus concentration using Beer-Lambert's Law:

$$A = Ecl$$

where A = Absorbance at 260 nm

E = Extinction coefficient = 3.49

(at a concentration of 1 μg / μl)

c = concentration

l = light path = 1 cm

showed the solution to have a concentration of approximately 4.86 mg / ml. Further

purification of the virus on a CsCl gradient followed by fractionation again gave a discrete, virus containing band. The concentration of the virus was found to be approximately 0.85 mg / ml.

In order to estimate the percentage RNA content of the virus the E $_{280nm/260nm}$ ratio was calculated. This was found to be approximately 0.76 suggesting that the virus had an RNA content of about 8.0 % (Warburg and Christian, 1941).

3.1.2 PAGE

Electrophoresis of NωV and NβV on a polyacrylamide gel together with marker proteins of known molecular weight allowed an estimation of the molecular weights of the capsid proteins of these viruses to be made. The NωV capsid protein was found to have a molecular weight of approximately 65 kDa while that of NβV had a molecular weight of approximately 62 kDa (Fig.1.3). Gels which were fixed in acetic acid : methanol: water (10:20:70) prior to staining, so as to prevent diffusion of smaller proteins from the gel during the staining procedure. The ω virus was found to have a diffuse band of approximately 6 kDa, possibly representing a minor capsid protein. This faint protein band could be seen on the gel but was not evident in photographs.

3.1.3 Ouchterlony Test

The Ouchterlony test was used to determine the titre of the anti-NωV antiserum raised in the rabbit following intramuscular inoculation with purified NωV. A titre of 1/256 was obtained for the anti- NωV antiserum.
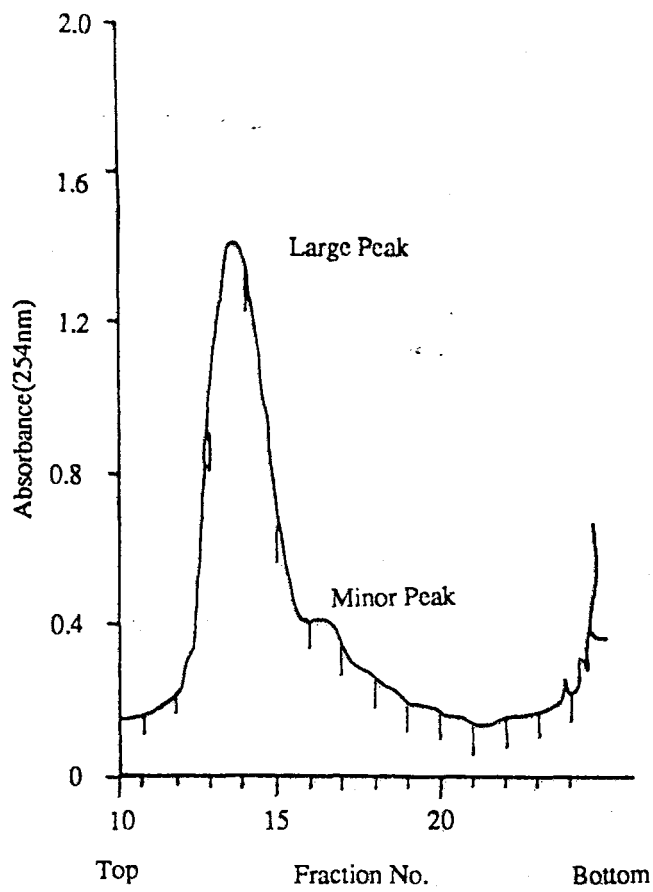
Fig. 1.1 Sedimentation Profile of NωV on a 15-45 % sucrose gradient.
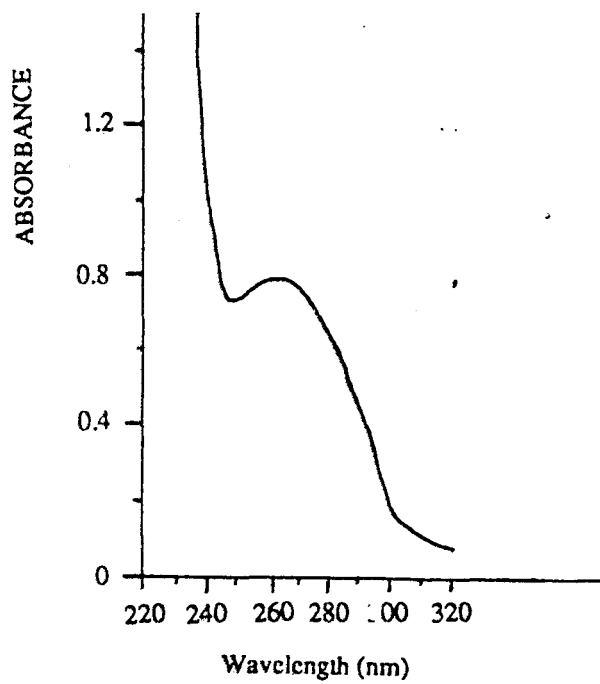
Fig. 1.2 Absorbance trace of NωV .
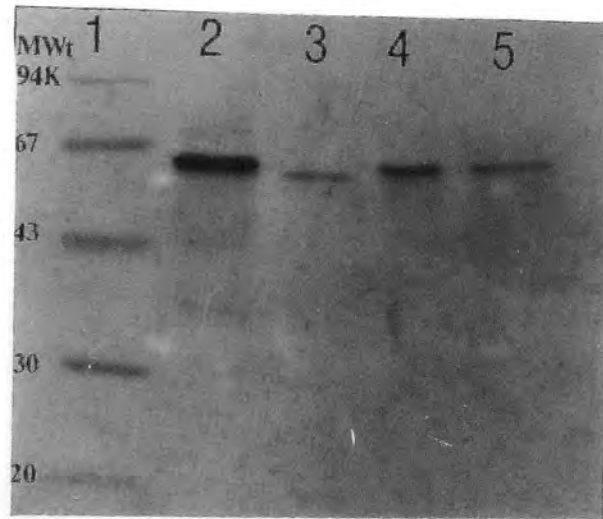
Fig. 1.3 SDS-Polyacrylamide Gel of NωV and NβV Capsid proteins (Lane 1 : Marker; Lane 2 ,4, 5 : NωV; Lane 3 : NβV).

3.2 Extraction and Electrophoresis of the NωV Genome

3.2.1 Extraction

Following proteinase K treatment of the virus and a phenol / chloroform extraction, genomic RNA was successfully obtained. When a scan of the absorbance of the extracted RNA from 320nm to 220nm was plotted, the trace produced was indicative of pure RNA free from protein contamination due to the absence of any "shoulders" on the trace (Fig. 2.1).
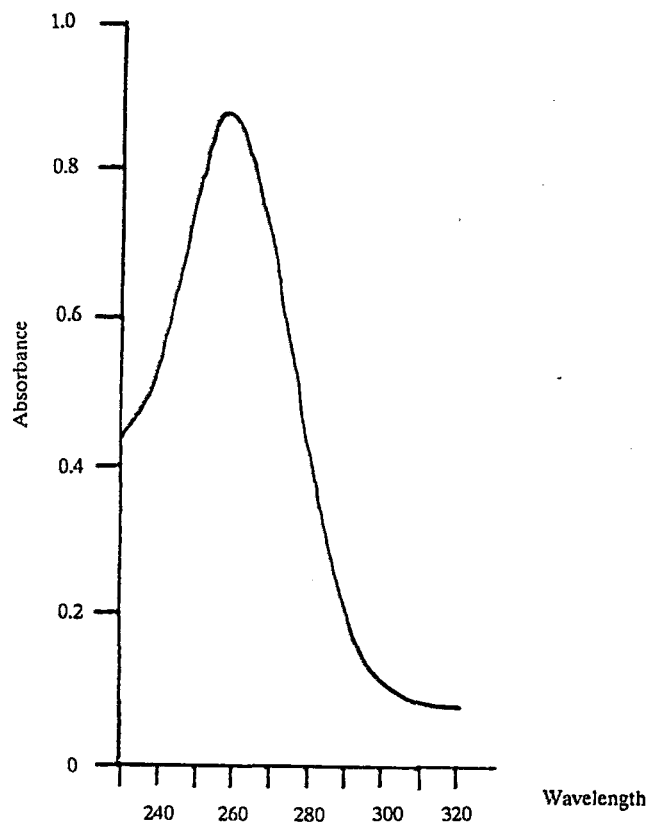
Fig. 2.1 Absorbance profile of NωV RNA
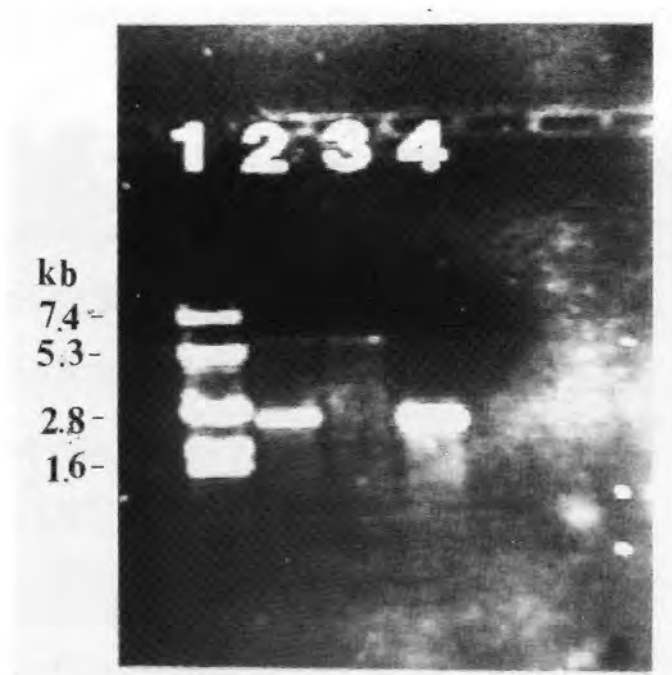
Fig 2.2 Electrophoresis of NωV RNA on an agarose-formaldehyde gel (Lane 1: RNA Markers (see Appendix 1D); Lane 2 : NωV mixed RNA; Lane 3 : NωV RNA 1; Lane 4 : NωV RNA 2).

3.2.2 Agarose Electrophoresis

Following electrophoresis of extracted ssRNA, together with RNA markers of known molecular weight the on an agarose-formaldehyde gel, two discrete bands were observed for the viral RNA representing the large (RNA 1) and small RNA (RNA 2) species. By comparison to the molecular weight markers, the sizes of these two bands could be estimated at approximately 5.5 kb and 2.4 kb (2.1 MDa and 1.8 MDa) for RNA 1 and RNA 2 respectively (Fig. 2.2). Both RNA species showed little evidence of degradation which would be characterised by smearing of the RNA bands.

3.2.3 Sucrose Density Gradient Centrifugation

Sucrose density gradient centrifugation was used to separate the mixed RNA into its two constituents: RNA 1 and RNA 2. Satisfactory separation of the two species of RNA was obtained on the sucrose gradient with two discrete, nucleic acid-containing peaks being evident on the sedimentation profile (Fig.2.3).This profile is produced as result of the absorbance of UV light by the sucrose fractions. Electrophoresis of each RNA species on a agarose-formaldehyde gel produced a discrete band with little degradation. The separated forms were found to co-migrate with the corresponding constituents of the unseparated RNA (Fig. 2.2).

3.2.4 Agarose Electrophoresis of dsRNA

The dsRNA replicative form of the genome was successfully isolated from infected larval tissue using a cellulose CF11 cellulose column on the basis of the affinity of this dsRNA for cellulose in the presence of 16 % ethanol., Following elution of the

dsRNA from the column, it was precipitated and the resuspended Electrophoresis of the extracted dsRNA, together with DNA markers of known molecular weight, on a non-denaturing agarose gel yielded two discrete bands of 4.2 MDa and 1.8 MDa. Little degradation was observed with either strand (Fig. 2.4).

3.2.5 Affinity Chromatography on poly(U) sepharose

Subsequent to the addition of mixed ssRNA to the column, maximum absorbance was observed in fractions collected during washing of the column with loading buffer, indicating that a large proportion of the RNA was eluted in these fractions. This would suggest the RNA lacks a poly(A) tract as no binding of the RNA to the column would appear to have taken place. A slight peak in absorbance was also observed in fractions collected following the addition of the elution buffer (fig.2.5).
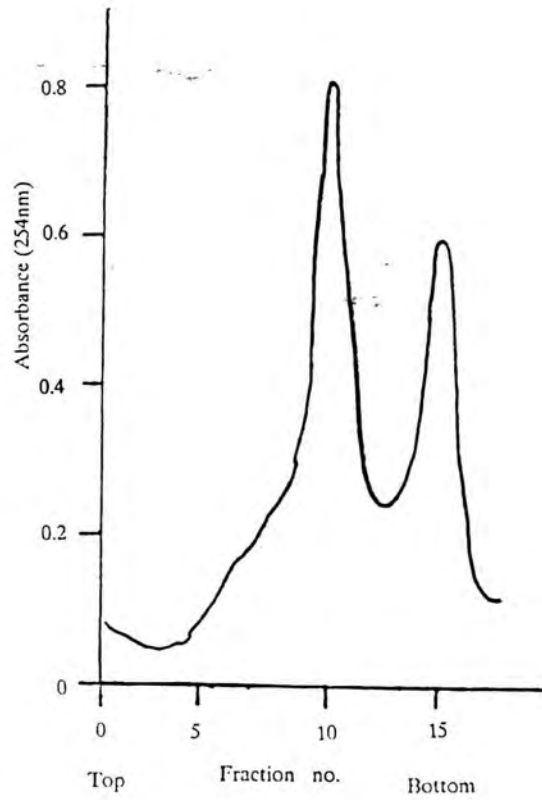
Fig. 2.3 Sedimentation profile of NωV RNA on a 5-15% sucrose density gradient.
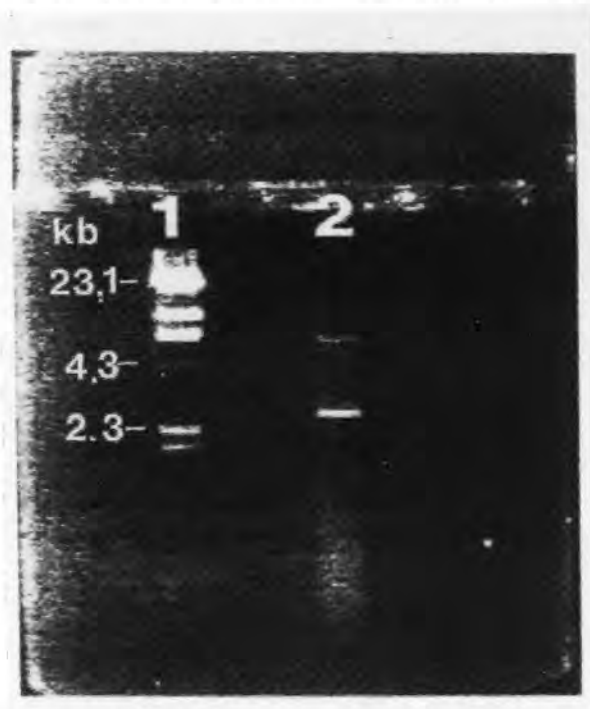
Fig 2.4 Electrophoresis of NωV dsRNA on a non-denaturing agarose gel (Lane 1 : DNA markers (see Appendix 1F); Lane 2: NωV dsRNA).
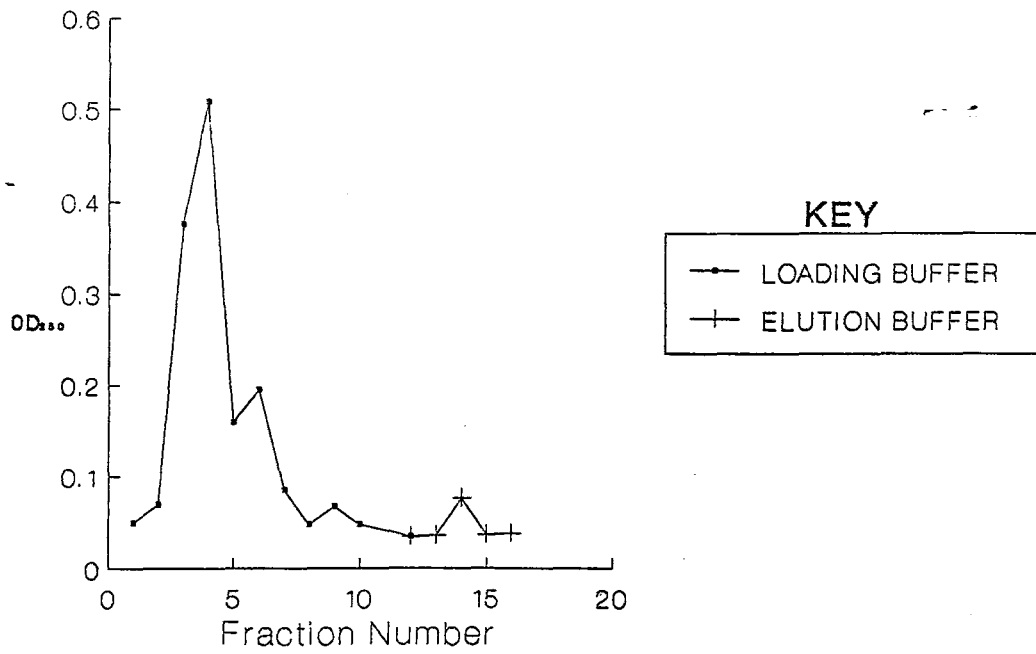
Fig. 2.5 Elution of NωV RNA from a poly(U) sepharose column.

3.3 Cell Free Translation and Immunoprecipitation

Cell-free translation allowed the synthesis of viral proteins following the addition of the viral RNA to the rabbit reticulocyte lysate system by making use of the enzymes and amino acids present in this system. The synthesis of viral protein was monitored by the incorporation of the [^{35}S]-methionine into newly synthesised proteins.

The optimal conditions for *in vitro* translation of the viral RNA were determined on the basis of maximal incorporation of [^{35}S]-methionine into newly synthesised protein.

3.3.1 General Translation

In the rabbit reticulocyte lysate system, the translation efficiency of the tested messenger RNAs was determined on the basis of their ability to direct the incorporation of [^{35}S]-methionine into TCA precipitable proteins (Fig.3.1). TMV RNA was found to incorporate approximately 30 times more label into its proteins than the RNA free lysate (blank). 1 µg NωV RNA / 30 µl lysate gave approximately an 8-fold higher incorporation of [^{35}S]-methionine than the blank, while the addition of 1 µg t-RNA to this reaction mix brought a further doubling in the incorporation of the label.

3.3.2 Optimisation of RNA concentration

Determination of the optimal concentration for NωV RNA in the rabbit reticulocyte lysate system, showed that an increase in RNA concentration up to 0.2 µg RNA /

µl lysate produced a corresponding increase in protein synthesis. With a further increase in concentration however, the incorporation of label was found to decrease considerably (Fig.3.2).

3.3.3 Optimisation of K^+ concentration

The endogenous concentration of the K^+ in the lysate was 142.2 mM. Within the range tested, the effect of an increase in the K^+ concentration was found to bring about a dramatic decrease in the synthesis of proteins by the NωV messenger up to a concentration of 150mM at which point the incorporation of label was reduced to the level found with the blank lacking any additional RNA (Fig. 3.3).

3.3.4 Optimisation of Mg^{2+} concentration

The endogenous Mg^{2+} concentration of rabbit reticulocyte lysate was 2.95 mM. A slight increase in incorporation of label was observed within the range tested up to a concentration of 2.15 mM Mg^{2+} after which the synthesis of proteins was found to decrease gradually (Fig.3.4).

3.3.5 Optimisation of Radiolabel Concentration

An increase in the concentration of $[^{35}S]$-methionine gave a corresponding increase in the amount of label incorporated into proteins synthesised by NωV.
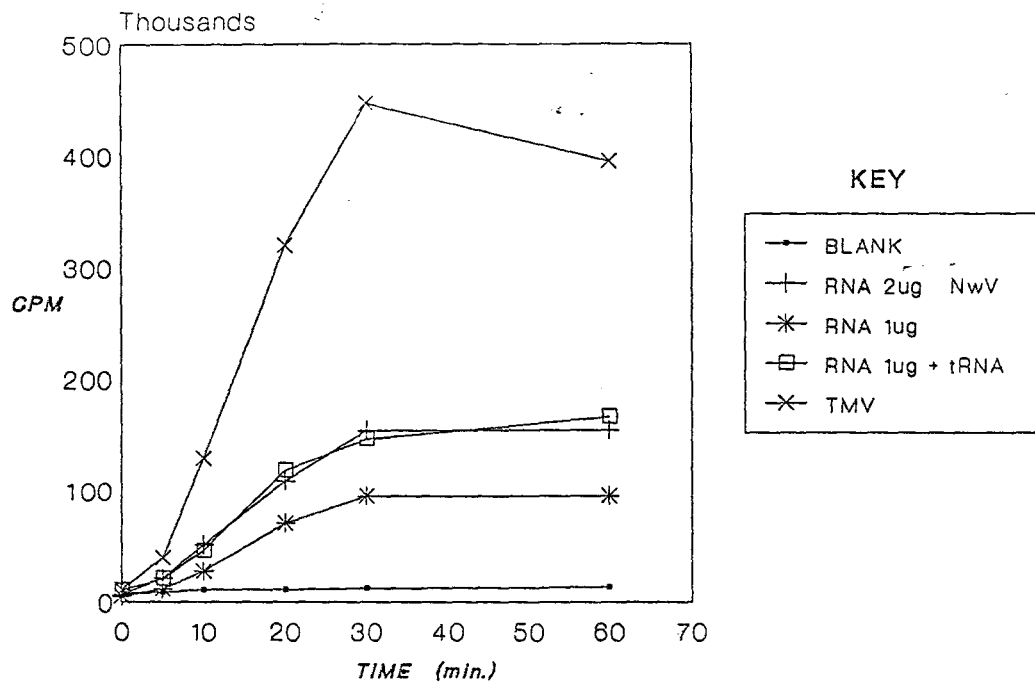
Fig. 3.1 Time course of incorporation of [^{35}S]-methionine into TCA precipitable proteins by the cell-free translation of NωV and TMV RNA's.
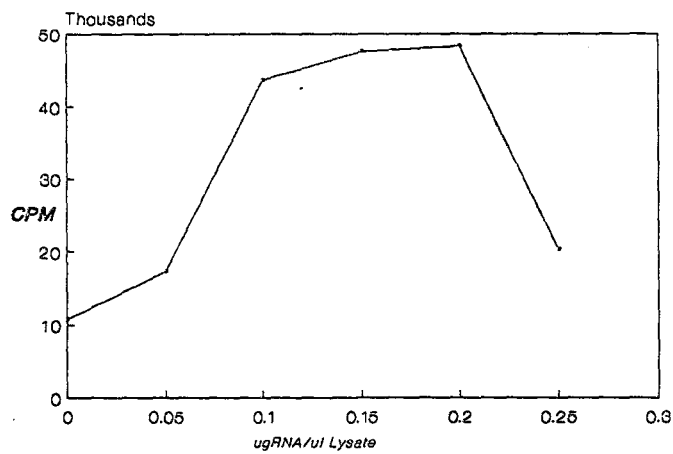
Fig. 3.2 Synthesis of TCA precipitable proteins over a range of NωV RNA concentrations.
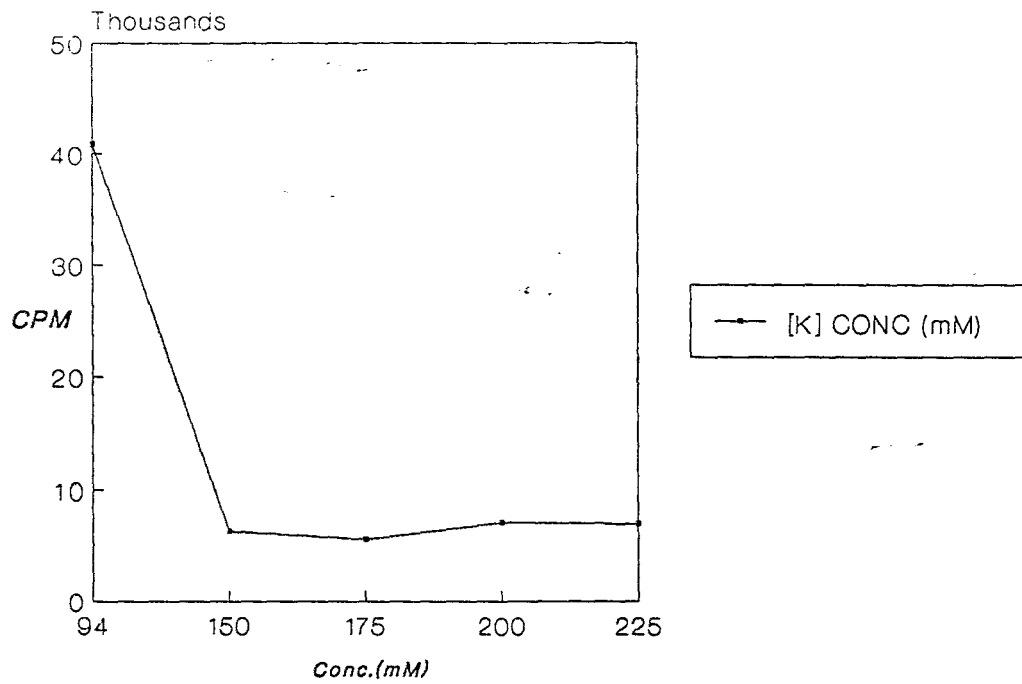
Fig. 3.3 Effect of the increase of potassium concentration on the NωV directed cell-free synthesis of TCA precipitable proteins.
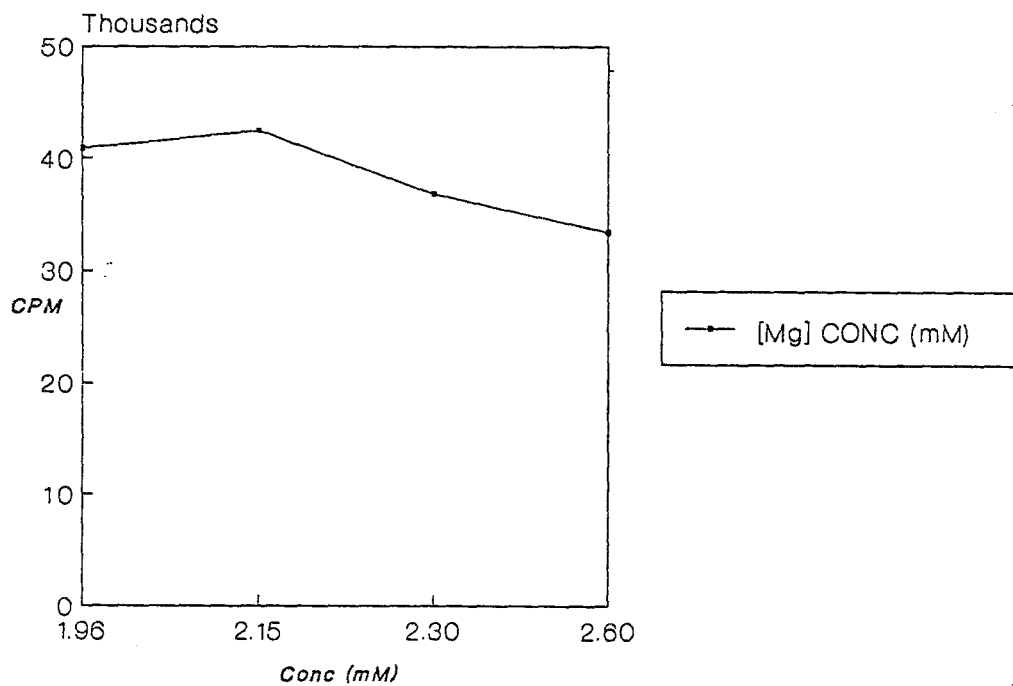
Fig. 3.4 Effect of the increase of magnesium concentration on the NωV directed cell-free synthesis of TCA precipitable proteins.

3.3.6 PAGE and Autoradiography

Translation products directed by mixed NωV RNA (i.e. RNA 1 and RNA 2) were resolved on a polyacrylamide gel. In order to differentiate these proteins from the endogenous rabbit reticulocyte lysate proteins it was necessary to carry out autoradiography, as all of the newly synthesised viral proteins carried [^{35}S]-methionine and could therefore be detected in this manner. Autoradiography indicated the presence of five dominant proteins with molecular weights of 124 kDa, 104 kDa, 72 kDa, 69 kDa and 68 kDa (Fig.3.5). Other minor proteins of molecular weights, 48 kDa, 44 kDa and 43 kDa were also evident. TMV encoded proteins were electrophoresed together with the NωV proteins as a control.

When the individual RNA species were translated separately and their products separated by electrophoresis, the large RNA (RNA 1), was found to encode three major proteins of 124 kDa, 104 kDa and 69 kDa. The small RNA (RNA 2) encoded two major proteins of 72 kDa and 68 kDa (Fig. 3.6).

3.3.7 Immunoprecipitation

Immunoprecipitaion was used in order to attempt to identify the putative coat protein precursor on the basis of the recognition of antiserum raised against the virus for this protein. Although no immunoprecipitation product was obtained for proteins encoded by RNA 1 (large RNA), RNA 2 (small RNA) was shown to encode two immunoprecipitatable proteins of molecular weights, 72 kDa and 42 kDa (Fig. 3.7). Control reactions containing anti-NβV antiserum and no antiserum to give produce visible bands.
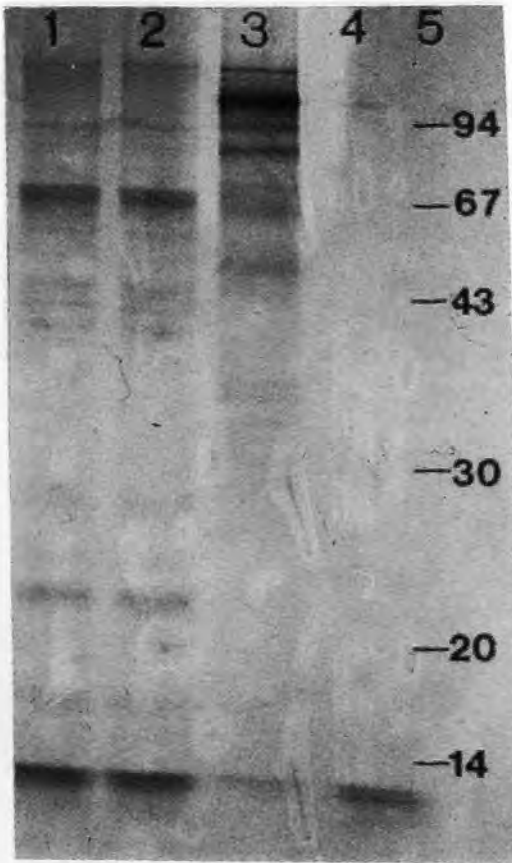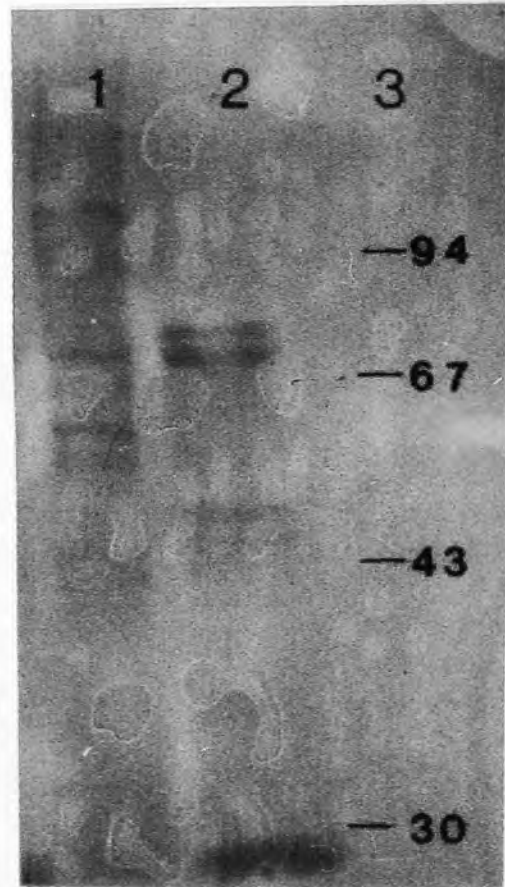
Fig. 3.5   Fig. 3.6

Fig 3.5 Autoradiograph of proteins encoded in a rabbit reticulocyte lysate by viral RNA (Lane 1 and 2 : NωV mixed RNA; Lane 3 : TMV RNA; Lane 4 : RNA-free control; Lane 5: Markers).

Fig. 3.6 Autoradiograph of proteins encoded in a rabbit reticulocyte lysate by NωV RNA 1 (Lane 1) and RNA 2 (Lane2) compared with molecular weight markers (Lane 3).
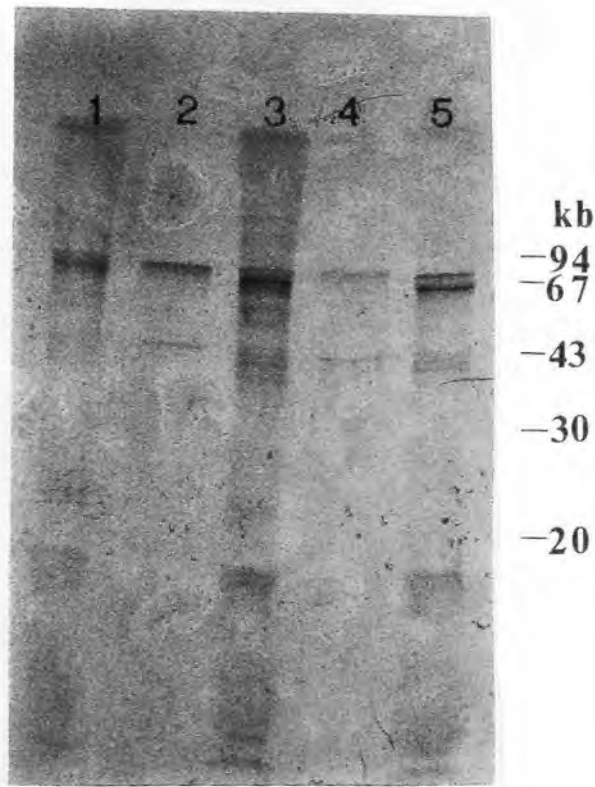
Fig. 3.7 Autoradiograph of immunoprecipitable viral encoded proteins (Lanes 1, 3, and 5 : RNA 2 encoded proteins (not immunoprecipitated); Lanes 2 and 4: immunoprecipitated RNA 2 encoded proteins).

3.4 cDNA Synthesis, PCR Amplification and Cloning

3.4.1 cDNA Synthesis

Using the cDNA synthesis kit, synthesis of cDNA from mixed viral RNA was attempted. The success of this synthesis was monitored by the incorporation of $[^{32}P]$-dCTP into DNA. This incorporation was determined through Cerenkov counting in a scintillation counter.

Incorporation of the label into the newly synthesised cDNA was found to be :-

$$\frac{47\ 168\ cpm}{405\ 506\ cpm} \times 100 = 11.63\ \%$$

Elution of the cDNA through a Sephadex G75 column yielded a peak containing in excess of 400 000 cpm. This peak was taken to be the region containing the cDNA. The constitutive fractions of this peak were pooled.

Electrophoresis of the pooled cDNA sample on an alkaline agarose gel and subsequent autoradiography produced a diffuse smear representing cDNA fragments of between 200 and 300 base pairs.

3.4.2 PCR of cDNA

Little success was achieved with attempts to amplify the cDNA using PCR. However when the PCR was carried out directly from the RNA, incorporating reverse transcription (RT-PCR), cDNA was both synthesised and amplified.

Incorporation of the label into the cDNA was found to be at a level of approximately 45%. Electrophoresis of the cDNA following PCR amplification revealed a smear indicating the synthesis of fragments of approximately 350 base pairs in size (Fig. 4.1). It is also possible to see the position of the unincorporated primers on the gel. It would appear that the annealing temperatures were adequate to allow priming.

Fig. 4.1 Electrophoresis of RT-PCR generated cDNA fragment (Lane1 and 4 : marker; Lane : 2 primers; Lane 3: cDNA).

3.4.3 Cloning

No white colonies were obtained when cells transformed with the pUC-cDNA ligation mix were plated. Approximately 3.6×10^3 blue colonies were obtained after transforming competent cells with 1 μg of the control pUC DNA. Similarly 3.0×10^3 blue colonies were obtained for cells transformed with 1 μg of cleaved pUC, ligated in the absence of the cDNA insert. This indicated that although the cells were sufficiently competent and the ligase reaction was working efficiently, no success was attained in attempting to clone the cDNA. As no white colonies were obtained mini-preps were not carried out.

3.5 Hybridisation Studies

3.5.1 Hybridisation to Viral RNA

Following hybridisation with the [^{32}P]-labelled probe synthesied by RT-PCR of the mixed RNA, three exposed areas were observed on the autoradiograph of the Dot Blot corresponding to the positions of the viral RNAs (Fig. 5.1). The dots were of increasing intensity from the 1/10 dilution sample to the undiluted RNA (1 μg / μl). No dot was obtained for the hybridisation of the probe to the control RNA.

3.5.2 Squash Blot

The same probe was used as for the hybridisartion to the viral RNA. A clean autoradiograph with no dark patches was produced following exposure to the Squash Blot probed with radiolabelled NωV cDNA, indicating that no binding of the probe to membrane bound nucleic acid had occurred.
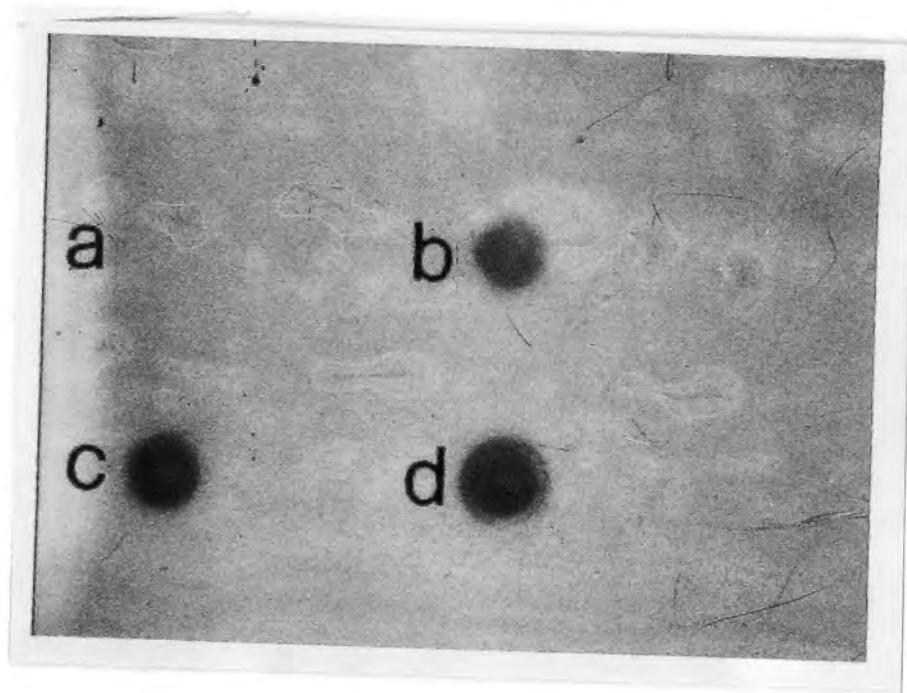
Fig. 5.1 Autoradiograph of Dot Blot (a : control; b : 1/10 dilution NωV RNA: 1/5 dilution NωV RNA; c : undiluted NωV RNA at 1 μg/ μl).

CHAPTER 4: DISCUSSION

4.1 Extraction and Purification of NωV

The virus could be extracted in relatively large concentrations from the thawed larvae, with little apparent degradation having occurred during either storage or purification as reflected in the spectrophotometric traces, no estimate of residual viability could however be obtained. Centrifugation on a CsCl gradient resulted in a reduction in the yield of the virus obtained, therefore virus purified on a sucrose gradient was used for extraction of the genome, as these preparations appeared sufficiently pure for this purpose.

An estimated RNA content of approximately 8%, determined spectrophotometrically, is slightly lower than that observed for other members of the Tetraviridae, which range from 8.5% for *Thosea asigna* (Reinganum *et al.*, 1978) and 11% for NβV (Struthers and Hendry, 1974). However, the combined size of the bipartite genome of the NωV (~ 3.0 MDa), is considerably larger than that of other members of this family (*Dp*V = 1.8 MDa; *Tni* = 1.9 (King *et al.*, 1984); NβV = 1.8 MDa (Hendry *et al.*, 1985), and thus the virus has a greater coding capacity. This suggests that additional, uniquely required proteins are encoded by

the NωV genome, which are not necessary for the infectivity and replication of other members of this family.

A figure of approximately 65 kDa for the molecular weight of the capsid protein is in agreement with that found by Hendry *et al.* (1985) but is slightly larger than the figure of 62 kDa obtained by Agrawal and Johnson (1992). The appearance of a 6 kDa protein on gels fixed prior to staining concurs with the results obtained by Hanzlik (1991, unpublished). Although this band was faint and could not be observed on a photograph it could be seen by the naked eye . Hanzlik used a rapid colloidal stain and suggested that the rapidity of this method of staining allowed visualisation of the 6 kDa protein before it diffused out of the gel as might happen with "slower" staining procedures. Hanzlik further proposed that this protein was distinct, and not a degradation product of the 65 kDa protein, as a clear N-terminal structure was obtained from the blotted protein which was immunogenically distinct from the larger protein. Agrawal and Johnson (1992) demonstrated, on the basis of densitometry of negatives of Coomassie-blue stained gels, that both the large and small capsid proteins were in equimolar concentrations.

Antiserum which was obtained from a rabbit following two intramuscular injections with NωV, was determined as having a titre of 1/256 using an Ouchterlony Test. This titer was deemed sufficient for subsequent use in immunoprecipitation studies.

4.2 Extraction and Electrophoresis of NωV genome

Intact ss RNA was isolated from the purified virus. Two species of RNA were

found with molecular weights of 2.1 MDa and 0.9 MDa. The large RNA (RNA 1) was slightly larger than the 1.8 MDa suggested by Hendry *et al.*(1985), while the small RNA (RNA 2) was found to be the same size as suggested previously.

RNA 1 is larger than the RNA observed in any of the Tetravirdae, all of which have RNA's in the region of 1.8 to 1.9 MDa (Hendry *et al*, 1985; Morris *et al.*, 1979; Chao *et al.*, 1983). The bipartite nature of the genome is also unique amongst the Tetraviridae and more closely resembles the Nodaviridae, all of which contain a bipartite genome encapsidated within a single virion (Matthews, 1982). The size of each of the NωV RNA's is however, approximately double that found for the corresponding nodaviral RNA's (Hendry *et al.*, 1991).

The dsRNA was also successfully extracted with minimal degradation. Two species of the dsRNA with molecular weights of 1.9 and 4.2 MDa were found. The size of these RNA's is consistent with that expected for the replicative form (RF) of each of the genomic RNA's. The lack of any smaller molecules of dsRNA would suggest the absence of any sub-genomic messengers, as although these might be degraded in the single stranded form and thus not isolated, one would expect to find them in the replicative form which is more resistant to degradation by ribonucleases. Similarly, Du Plessis *et al.* (1991) found no sub-genomic messengers in the RF of NβV , with only a single molecule of 3.6 MDa representing the RF of the viral genome.

Chromatography of the genomic RNA on the poly(U) sepharose shows that it lacks a poly(A) tract, as minimal binding of the RNA to the column occurred. The small

peak of UV-absorbing material which eluted following the addition of the elution buffer represented a minor proportion of the original RNA. Studies of *Tni*V and Nε V have shown that the genomes of both of these viruses lack a poly (A) tract (King *et al.*, 1985), suggesting that this sequence, observed in many of the small, single-stranded RNA viruses (Moore, 1991) is absent in the Tetraviridae.

4.3 Cell-free translations and Immunoprecipitations

It is evident from the results that the NωV RNA is capable of directing the synthesis of proteins *in vitro* in the rabbit reticulocyte lysate system. The levels of incorporation of the label into the translated proteins were found to be relatively low, with a level approximately 8-fold greater than the RNA-free blank. This however could be stimulated to a level of approximately 15-fold greater than the messenger free lysate by the addition of t-RNA to the system. Although these levels are not comparable to those found with the RNA's of other insect viruses such as Cricket Paralysis Virus (Reavy and Moore, 1981), they are on par with those found with other Tetravirus RNA including *Tni*V RNA and NεV RNA which could both only stimulate a maximum synthesis of protein that was 10-fold above background (King *et al.*, 1984). These levels are however below those found for NβV RNA, which gave a 30-fold greater incorporation of label than the messenger-free lysate (Du Plessis *et al.*, 1991).

It would therefore appear that NωV RNA is a relatively poor messenger on the basis of its incorporation of [^{35}S] methionine into the viral proteins synthesised *in vitro*. The lysate system itself might be responsible for the low efficiency of translation, as

the system is optimised for the *in vitro* translation of the RNA of TMV. Therefore other lysate systems including those derived from insect cells might be tested in an attempt to resemble conditions *in vivo* thus improving the levels of translation.

The first of the dose response experiments was aimed at determining optimal RNA concentration for translation in this system. Similar *in vitro* translation experiments conducted on *Tni*V and NɛV RNA (King *et al,*. 1984) revealed optimal RNA concentration of 3 µg RNA/ assay and 2 µg RNA/ assay respectively. The optimal RNA concentration for NꞷV was found to be 0.2 µg RNA/ µl lysate, which with a total lysate volume of 30 µl corresponds to approximately 6 µg RNA/ assay. This is therefore considerable higher than that of the other Tetraviruses.

In the optimisation of the lysate with respect to Mg^{2+} and K^+ concentrations, the endogenous concentration of these ions played a limiting factor on the range which could be tested. This was because in order to obtain lower concentrations than those tested, excessive dilution of the lysate through addition of water would have been necessary, while to achieve a higher concentration would have required excessive dilution of the lysate through the addition of the salt solution. The results would suggest that, although over the range tested, a K^+ concentration of 94 mM would appear to be optimal, a lower concentration of K^+ ions than tested might have yielded greater protein synthesis. The optimal concentration for Mg^{2+} was found to be 2.15 mM. The inhibitory effect displayed by excessive Mg^{2+} and K^+ concentrations would suggest that in excess, these ions have an antagonistic effect on translation, possibly through interference with the responsible enzymes. No

similar experiments have been carried out with other members of the Tetraviridae, however Guarino *et al.* (1981) found the optimal Mg^{2+} and K^+ concentrations for *in vitro* translation of the genome of the Nodavirus BBV to be 1.5 mM and 70 mM respectively. These levels are somewhat lower than for those of NωV and might reflect some differences in the ionic composition of their respective host cells.

As would be expected, the [^{35}S] methionine dose response experiment showed that an increase in the concentration of [^{35}S] methionine gave a corresponding increase in incorporated label. As no plateauing effect was achieved with an increase in radiolabel concentration, a point at which the system became saturated with [^{35}S]-methionine was not reached. 15 µCi [^{35}S] methionine gave sufficient incorporation of label, for subsequent use in PAGE and autoradiography experiments.

Of the five predominant proteins encoded by the NωV genome, it would appear that the large RNA (RNA 1) is responsible for encoding the 124 kDa, 104 kDa and 69 kDa proteins while RNA 2 is responsible for encoding the 72 kDa and 68 kDa proteins. RNA 1 with a molecular weight of 2.1 MDa and RNA 2 with a molecular weight of 0.9 MDa have the capacity to encode proteins with an aggregate molecular weight of 200 kDa and 90 kDa respectively. Through sequence studies of the RNA 2, Agrawal and Johnson (1992) showed that this component of the genome contained one long open reading frame (ORF) with the capacity to encode a 70 kDa protein. They further showed that the RNA 2 contained a 366-base untranslated leader, and a 150-base non-coding region at the 3' end. This would therefore confirm that the smaller proteins observed are spurious, and arose as a

result of early termination during translation. This might be due to the secondary structure of the RNA which prevents efficient translation. This may also be true for the proteins encoded by RNA 1. Alternatively these proteins could arise through a post-translational event in the form of cleavage of the polypeptide. Should this be the case, it is likely that the protease responsible is viral encoded for the cleavage to occur in a cell-free system.

Due to the structural and genomic features of the Tetraviridae, one would expect the functional proteins which they encode to include a capsid protein, a RNA-dependent RNA-polymerase (ie. a replicase) and possibly a protease (Reavy and Moore, 1984). No protein corresponding to the capsid protein (65 kDa) was observed on the autoradiographs suggesting that this protein might arise as a result of a post-translation event such as the cleavage of a larger protein. Results from the immunoprecipitation showed that a 72 kDa protein and a 43 kDa protein synthesised by RNA 2 were recognised by antiserum raised against the virus. This would indicate that the 72 kDa protein might represent the putative capsid precursor, as in order for it to be recognised, it would have to contain the same epitopes as the capsid protein. This concurs with the sequencing results obtained by Agrawal and Johnson (1992). It is most likely that the 43 kDa protein is a degradative or early termination product of the 72 kDa protein. It is possible that the cleavage of the 72 kDa protein to its capsid form is host cell mediated or as seen with Nodaviridae a consequence of virion assembly (Hendry, 1991), thus explaining the absence of a 65 kDa protein in the cell-free translated proteins. The presence of a 6 kDa protein in the virus, is in compliance with this suggestion, as this would

represent the portion remaining following cleavage from the 72 kDa protein.

Immunoprecipitation studies of *Tni*V RNA and NεV RNA translation products (Reavy and Moore, 1984; King *et al.*, 1984) yielded a number of precipitated proteins in the size range of the 70 kDa capsid protein. Du Plessis *et al.* (1991), however, were unable to immunoprecipitate the single 70 kDa protein encoded by the genome of NβV . If the 72 kDa protein of NωV RNA 2 does represent the putative coat protein precursor, this arrangement of genes therefore resembles that of the Nodaviridae where the coat protein gene is also found on the small RNA (Hendry, 1991).

Reavy and Moore (1984) suggested that the 104 kDa protein of *Tni*V might represent the replicase enzyme which is derived by cleavage from a 140 kDa polypeptide. This conclusion was arrived at on the basis that similar proteins of approximately 104 kDa involved in replication were observed in the translation products of other insect viruses (Guarino *et al.*, 1978). This might therefore suggest that the replicase of NωV is synthesised as a 124 kDa precursor which undergoes cleavage to give the active 104 kDa RNA-dependent RNA-polymerase. If this were the case, the arrangement of the genes on RNA 1 would again appear to resemble the system seen in the Nodaviridae, where the replicase gene is located on the large RNA (Hendry, 1991).

Based on their amino acid sequence for the putative coat protein precursor encoded by RNA 2, Agrawal and Johnson (1992) postulate that cleavage of this protein

occurs between the N570 and F571 residues and would thus require a viral protease of novel specificity. It is possible that this protein is encoded by RNA 1 and represents the 68 kDa polypeptide observed in the cell-free translation experiments.

4.4 cDNA Synthesis, PCR Amplification and Cloning

An incorporation of radiolabel into the newly synthesised cDNA of less than 10% using the cDNA synthesis kit indicates that there was very poor reverse transcription of the template RNA. Using this kit one would expect a transcription efficiency in the region of 80-90% for first strand synthesis.

When the quality of the reverse transcription products were analysed on an alkaline agarose gel, from which an autoradiograph was produced, the cDNA was found to be relatively small, in the region of 200 to 300 base pairs. Similar sized cDNA fragments (of less than 300 base pairs) were synthesised by Agrawal and Johnson (1992), using a degenerate primer based on the capsid protein amino acid sequence, with the exception of a single clone containing an insert of 780 base pairs which they were able to synthesise. With template RNA of approximately 2.1 MDa and 1.8 MDa "full length" cDNA of approximately 6.5 and 3.0 kb could be produced; however, using random priming to initiate reverse transcription, full length transcripts would not be expected. As this work was completed prior to the publishing of the sequence of the small RNA strand by Agrawal and Johnson (1992), it was not possible to design primers based on this sequence which may have allowed synthesis of a full length cDNA of the small RNA.

Attempts to polyadenylate the RNA using a poly(A) polymerase were unsuccessful, possibly due to a blockage of the 3' end of the RNA. However had this polyadenylation been successful, it might have been possible to use an oligo (dT) primer to initiate reverse transcription, so as to generate full size fragments. Relying on random hexanucleotide primers meant however, that primer binding occurred at arbitrary points on the genome resulting in fragments of varying length being synthesised.

The small size of the fragments in relation to the template might suggest that the action of the reverse transcriptase is being mildly inhibited or prematurely terminated. This might be due to some secondary stucture present in the RNA which prevents the reverse transcriptase from reading along the entire length of the template RNA. Similarly the action of the *Taq* polymerase during the RT-PCR would appear to be in some way inhibited in the amplification of the cDNA as again only fragments of a very small size were produced. It is most probable that during the first few cycles of PCR these smaller fragments were synthesised preferentially, and it would therefore be these small fragments which would be synthesised almost exclusively in the subsequent cycles, as they would be the predominant template for amplification. It is unlikely that these fragments represent "primer-dimers" produced as a result of self amplification, as these fragments are considerably larger than double the size of the primers (they are ten times larger), the size of which can b seen on the gel. Furthermore it is unlikely that these fragments arise as result of self-amplification as a control lacking the RNA did not give amplification and neither did prior use of these primers (T.Downing, personal communication).

Again, had the sequence been available for the small RNA it would have been possible to design primers specific to the template RNA, thus ensuring more efficient amplification.

It would appear that the annealing temperatures used were sufficient to allow for nonspecific priming of the template. Had specific primers been designed it would have been necessary to take into account the Tm of the primers.

The RT-PCR however gave more favourable results. During the synthesis of cDNA approximately 45% of the label provided was incorporated. This was considerably higher than that achieved during first strand synthesis using the cDNA synthesis kit. Again however specific primers would have given a better amplification and possibly improved the incorporation of the label due to the synthesis of longer cDNA fragments.

Following 40 cycles of the PCR, electrophoresis of the PCR products on an agarose gel indicated that cDNA in the region of 400 bp had been amplified. [^{32}P]-dCTP which had not been incorporated during the first strand synthesis of the cDNA was incorporated into the amplified cDNA during PCR. The advantage of these radiolabelled cDNA products is that they can be used directly as probes for hybridisation experiments without having to carry out an additional labeling step which not be as efficient. When synthesising probes however, it might be preferable to label the cDNA following amplification, to avoid contamination of the thermal cycler with a radiolabel.

Repeated attempts at cloning of the cDNA, synthesised using the cDNA synthesis kit, into a pUC vector were unsuccessful. Control experiments indicate that the competency of the *E.coli* cells was sufficient and the reaction mixtures necessary for both transformation and ligation adequate. The lack of success might be attributed to the small quantities of cDNA synthesised. This would have meant that during the ligation step, the blunt ends of the plasmid were re-ligated without any NωV cDNA.

In order to produce a probe, this step may be bypassed as sufficient probe might be synthesised using RT-PCR. Although the radiolabelled fragments synthesised through RT-PCR were of a very small size, they were sufficient for the recognition of homologous RNA in hybridisation experiments.

If the cDNA was to be sequenced it would be necessary to insert the cDNA into a suitable vector such as pUC or M13. Better results might be achieved using cDNA which has been amplified using RT-PCR however time constraints prevented this. Cloning of the amplified cDNA with a "T-tailed" vector may have circumvented the problems experienced with the self-annealing of the vector and the lack of cDNA in the ligation reaction. This might have improved the number of transformants produced. Sequencing the genome of this virus and other Tetraviruses will be important in establishing the phylogenetic relationships between these viruses of which so little is known. As yet the only genomic sequence known for a member of the Tetraviridae is that of the RNA 2 of NωV (Agrawal and Johnson, 1992).

Comparison of the amino sequence of the ORF on NωV RNA 2 with those of the nodavirus coat protein α shows that the sequence homology is very low (Agrawal and Johnson, 1992), suggesting that the evolution of these viruses might be very diverse.

4.5 Hybridisation studies

From the results obtained from the autoradiograph for the hybridisation to the viral RNA, it would appear that the probe produced through RT-PCR is capable of recognising the viral RNA. As the control RNA was not recognised one can also assume that the probe is sufficiently specific. It might however be necessary to test different conditions of stringency in order to determine which are optimal for hybridisation using this specific probe. It is unlikely that the hybridisation was due to binding of the primer used during PCR to the viral RNA, as firstly if only unincorporated primer was bound, the primer was unlabelled and would not appear on the autoradiograph. Secondly if hybridisation was due to binding of incorporated primer, the strength of binding in such a short region of cDNA fragment of 200-300bp at the washing conditions used, would be insufficient for the probe to remain bound and therefore no dot would be seen on the autoradiograph.

The increase in intensity of the dots in relation to the concentration of RNA present shows that it might be possible to quantitate RNA concentrations using standardised conditions of blotting and hybridisation. The lack of results obtained for the squash blots has a number of possible explanations. Firstly, it may be possible that the "infected" larvae from which the gut material was obtained did not in fact contain

virus, as diagnosis was made purely on the basis of symptoms. The "infected" appearance of the larvae may have in fact been due to another environmental factor which had not been considered. The larvae may have been infected with a different virus, the genome of which would not be detected by the probe. This is however an unlikely explanation, as virus had been previously extracted from the same batch of larvae which had been stored at -20°C. A second possible explanation is that the gut tissue does not actually contain virus or viral RNA. Again this is an unsatisfactory explanation as it is known that the gut is the site of viral replication (Matthews, 1982) and one would therefore expect to find viral RNA in abundance.

Two final explanations, a combination of which is the most likely, are that either the blotting and hybridisation conditions were not suitable or the probe was not sufficiently sensitive to detect the concentration of RNA present. It is a possibility that the hybridisation conditions were not optimum to cause denaturation of the dsRNA present in the gut. Without this denaturation, hybridisation would not be possible as there would be no single stranded template for the probe to bind to and thus during following washing no signal would be detected. It would also be necessary to optimise the stringency of the washing stages so as to achieve sufficient binding of the probe without compromising the specificity of detection.

It would have been a more conclusive result to have attempted to extract total DNA from viral infected tissue and use this as a target for the probe. This would have eliminated problems experienced with the squash blot method.

CHAPTER 5: CONCLUSION

Purified NωV was successfully isolated from infected larvae producing a source from which the ssRNA genome could be isolated. The purified virus also permitted a determination of the sizes of the capsid proteins which constitute the virus coat. From this purified virus it was possible to produce an antiserum of a relatively high titre.

The genome of the virus was extracted and separated on a sucrose gradient. Electrophoresis of the RNA on a formaldehyde agarose gel revealed the accurate molecular weights of the two strands. The replicative form of the genome was also isolated from infected tissue. As only two species of dsRNA were isolated it is unlikely that any sub-genomic messengers exist. Affinity chromatography on a poly(U) sepharose gel confirmed that the viral genome is not polyadenylated.

Conditions for the optimal cell-free translation of the NωV genome in a rabbit reticulocyte lysate system were determined. Electrophoresis and immunoprecipitation of the translated proteins showed both RNA 1 and RNA 2 to be capable of directing protein synthesis. It was possible to determine approximate sizes for these proteins and, on the basis of these sizes and of the reactivity of these proteins with anti-sera, to postulate that RNA 1 encodes a RNA-dependent RNA polymerase of approximately 104 kDa and a protease of 68 kDa, while the RNA 2 encodes a capsid protein precursor. This agrees with the findings of Agrawal and Johnson (1992) who, on the basis of sequence analysis, showed that RNA 2 was

responsible for direction of the synthesis of the capsid protein precursor.

As this work was carried out prior to the publication by Agrawal and Johnson (1992) of sequence data for RNA 2 it was not possible to design specific primers which might now be possible. This would have eliminated many of the problems experienced in cDNA synthesis and RT-PCR. Hence reverse transcription of the RNA and the subsequent cloning of the product was not particularly successful. However with the use of RT-PCR, some success was achieved and it was possible to generate a small cDNA fragment which was radiolabelled for use as a probe in hybridisation experiments. Synthesis of a full length probe derived from RNA 2 using RT-PCR might be achieved by design of primers to the 5' and 3' ends of the RNA based on the published sequence by Agrawal and Johnson (1992). This probe could then be used for homology studies with the genomes of other members of the Tetravirdae.

It is still of great importance to be able to clone cDNA to RNA 1 so as to derive nucleotide sequence from this component of the genome and to assist in assigning functions to the proteins encoded by it. Using a similar protocol as that followed by Agrawal and Johnson (1992) for the elucidation of the sequence of RNA 2, it might be possible to achieve this. Development of a probe from this cDNA would also be important for homology studies with other Tetravirus genomes.

On the basis of the results obtained at present, it is apparent that NωV is similar to the Nodaviridae on the basis of its divided genome and the postulated arrangement

of its putative genes on this divided genome. However the very low sequence homology observed at the amino acid level between the NωV putative coat protein precursor and the nodavirus coat protein α (Agrawal and Johnson, 1992), would infer that an evolutionary relationship between the two is relatively distant. NωV might however represent an evolutionary intermediate between the Nodaviridae and the Tetraviridae. The difference of NωV from the other known members of the Tetraviridae in respect of its divided genome might warrant it being placed in a distinct sub-group within this family.

Dot-blots showed that the probe, synthesised from the genomic NωV RNA through RT-PCR using arbitrary primers, recognised NωV ssRNA. This same probe however was unable to detect the presence of this RNA in squash blots made from the gut of apparently infected larvae. This technique therefore requires considerable modification of the blotting and hybridisation conditions to allow recognition of viral RNA present in the larval gut. Should it be possible to routinely use squash-blotting as a method of diagnosis of infection, the presence or absence of the virus in field-collected larvae may be determined on an annual basis. Alternatively, other blotting methods making use of total RNA from larvae might prove more efficient as a diagnostic tool. This could be an important consideration when deciding to spray pine plantations with artificial insecticide or allowing the virus to act as a natural biological control agent.

APPENDIX 1

Buffers and Solutions for Gel Electrophoresis:

A. SDS - Polyacrylamide Gels

The buffers and solutions which were used for PAGE were modifications of those devised by Laemmli (1970).

1. Resolving Buffer solution (1 M Tris-HCl pH 8.8)

Tris	60.6 g
HCl conc.	7.3 ml
Distilled H_2O	500.0 ml

2. Stacking Gel Buffer Stock Solution (1M Tris-HCl pH 6.8)

Tris	60.6 g
HCl (conc.)	41.0 ml
Distilled H_2O	500.0 ml

3. Bath Buffer Stock Solution (10x)

Tris	30.3 g
Glycine	144.1 g
SDS	10.0 g

The solution was made up to 1 litre with distilled H_2O. The buffer stock solution was diluted 10x in distilled water before use.

4. Acrylamide Stock Solution

Acrylamide	150.0 g
bis-Acrylamide	4.0 g
Distilled H_2O	500.0 ml

5. Dissociation Buffer

SDS	5.0 g
2-Mercaptoethanol	5.0 ml
Glycerol	7.5 ml
Bromophenol blue (0.2% - wt/vol)	2.5 ml
1M Tris-HCl pH 6.8	6.3 ml
Distilled H_2O	28.7 ml

6. Slab Gel Formulations

SOLUTION	4% Stacking gel	10% Resolving gel
Acylamide:Bis stock	2.0 ml	13.35 ml
1 M Tris - HCl pH 6.8	1.9 ml	-
1 M Tris - HCl pH 8.8	-	15.0 ml
Distilled H_2O	9.25 ml	9.25 ml
SDS (wt/vol)	0.15 ml	0.4 ml
80%Glycerol (vol/vol)	1.0 ml	-
1.5% $(NH_4)_2S_2O_8$	0.7 ml	2.0 ml
TEMED	0.02 ml	0.02 ml
Total	15.02 ml	40.02 ml

7. Staining Solution

Methanol	45.0 ml
Glacial acetic acid	10.0 ml
Coomassie brilliant blue	0.2 g
Distilled H_2O	45.0 ml

8. Destain Solution

Methanol	450 ml
Glacial Acetic Acid	70 ml
Distilled H_2O	480 ml

B. Protein markers:

These were obtained from Pharmacia Fine Chemicals.

Protein	Mol. Weight
Phosphorylase b	94 000 Da
Bovine Serum Albumin	64 000 Da
Ovalbumin	43 000 Da
Carbonic Anhydrase	30 000 Da
Soybean Trypsin Inhibitor	20 000 Da
Lactalbumin	14 400 Da

C. Denaturing Agarose Gel

The method used was according to Davis *et al.* (1986)

1. Gel

To a 150 ml conical flask containing 0.5 g Sea Kem LE Agarose, 29.3 ml distilled water was added and the solution microwaved for 1.5 min.. The solution was allowed to cool to 60°C and 10ml 5 x Running Buffer and 10.8 ml 37%

formaldehyde solution added. The gel was cast and allowed to set.

2. <u>10x Running Buffer</u>

0.2 M MOPS (Morpholinopropanesulphonic acid)(pH 7.0)

50 mM Sodium acetate

5 mM EDTA

3. <u>Sample Solution</u>

5 x Running Buffer	20 µl
Formaldehyde	35 µl
Formamide (deionised)	100 µl

This provided sufficient sample solution for 10 samples. 5 µl RNA was added to
15 µl sample solution. The solution was stored at -20°C.

4. <u>Loading Buffer</u>

50%   Glycerol

1mM   EDTA

0.4%   Bromophenol Blue

0.4%   Xylene cyanol

D. RNA Molecular Weight Markers

These were obtained from Boehringer-Mannheim.

Marker	Size
1	7.4 kb
2	5.3 kb
3	2.8 kb
4	1.9 kb
5	1.6 kb

E. Non-denaturing Agarose Gel

1. Gel

To a 150ml conical flask containing 0.5g Sea Kem LE Agarose, was added 50ml 1 x TAE buffer. The solution was mixed and microwaved for 1.5 min. and allowed to cool to approximately $50^{o}C$. The gel was cast and allowed to set.

2. TAE Bath Buffer (10 x stock solution)

0.4 M Tris

0.2 M Sodium acetate

0.01 M EDTA

The buffer was diluted 1:10 before use.

3. Loading Buffer

20   % Glycerol

0.02 % Bromophenol Blue

1 x TAE

The sample was diluted with an equal volume of loading buffer and layered onto the gel.

F. DNA Molecular Weight Markers

These were obtained from Boehringer-Mannheim.

Marker	Molecular Weight
1	23.130 kb
2	9.416 kb
3	6.557 kb
4	4.361 kb
5	2.322 kb
6	2.027 kb
7.	0.564 kb

G Alkaline Agarose gels

1. Gel

To a 150 ml conical flask containing 0.5 g Sea Kem LE Agarose, 40 ml distilled water was added. The solution was mixed and microwaved for 1.5 min. When the solution had cooled to approximately $60^{\circ}C$, 5ml 0.5 M NaOH and 5ml 0.01 M EDTA was added. The gel was cast and allowed to set.

2. Alkaline loading buffer

0.25 % Xylene cyanol

0.3 N NaOH

6 mM EDTA

18 % Glycerol

3. Electrophoresis buffer

10 N NaOH   5ml

0.5M EDTA   2ml

Made up to 1l with distilled water.

APPENDIX 2:

A. Buffer Saturated Phenol

100 g phenol was melted at 65°C and 0.1 g 8-hydroxyquinoline added. 100 ml 50 mM Tris-HCl (pH 8.0) was added and the solution stirred for 10 min. at room temperature. The phases were allowed to separate and the aqueous layer decanted. A further 100 ml 50 mM Tris-HCl (pH 8.0) was added and the solution stirred, allowed to stand and the aqueous layer decanted. The pH of the solution was checked. If lower than pH 7.5 the phenol was washed with a further 100ml buffer. 50ml 50mM Tris-HCl (ph 8.0) was added and the solution stored in a covered bottle at 4°C.

B. Buffers for Extraction of ds RNA:

1. <u>10 x STE Buffer (pH 8.0)</u>

1.0   M NaCl

0.5   M Tris

0.01 M EDTA

2. Extraction Buffer

Bentonite	1.0 g
SDS	1.0 g
PVP(polyvinylpyrrolidone) 10 or 40	1.0 g
10 x STE	20.0 ml
DIECA (Diethyldithiocarbamate)	0.2 g
2-mercaptoethanol	0.2 ml

The solution was made up to 100 ml with distilled water.

3. Chloroform : Isoamylalcohol

Chloroform   240.0 ml

Isoamylalcohol 10.0 ml

4. Washing Buffer:

10 x STE Buffer 100.0 ml

95% alcohol   170.0 ml

Distilled H_2O 1000.0 ml

5. Elution Buffer:

1 x STE (no alcohol is added)

C. Affinity Chromatography on poly(U) Sepharose

1. Elution buffer

10 mM EDTA

0.2 % SDS

90 % Formamide

10 mM potassium Phosphate

2. Loading buffer

0.5 M NaCl

10 mM EDTA

25 % Formamide

50 mM Tris HCl (pH 7.5)

APPENDIX 3:

A. Paper Disc Method for Measuring TCA-Precipitable Counts

1. Pretreatment of Discs:

A volume of 100 µl 1 % SDS; 3 % Casamino Acids (CAA) was pipetted onto Whatman 2.5 cm paper discs. The discs were dried under an Infra-Red (IR) lamp and stored in a dessicator over silica gel.

2. Treatment of discs containing lysate mixture sample:

A 2 µl sample of lysate mixture was pipetted onto each disc. The discs were soaked in 10 % trichloroacetic acid (TCA), 1 % Sodium Dodecyl Sulphate (SDS), 3 % CAA for 10 min. at room temperature, and then drained. The discs were then boiled for 10 min. in 5% TCA, 1.5% CAA, rinsed twice in 5% TCA, once in 95% ethanol and once in ether. The discs were again dried under an IR lamp.

B. Non-Aqueous Scintillation Cocktail

A total of 4 g PPO (2,5-Diphenyloxazole) and 0.4 g bis-MSB (p-bis-methylstyryl Benzene) (Scintillation Grade) were added to 1.0 l toluene.

C. RIPA Buffer

15 mM NaCl

1 %   Triton X-100 (vol/vol)

1 %   Sodium Deoxycholate (wt/vol)

1 %   SDS (wt/vol)

The total volume was made up to 50 ml with 10 mM Tris-HCl buffer (pH 7.2).

D. Protein A Solution

A total of 350 µl 10 % Protein A solution was microfuged for 1 min. to produce a pellet which was drained and resuspended in 800 µl RIPA buffer and 16 µl 5% Bovine Serum Albumin. 40 µl of the solution was added to each sample.

APPENDIX 4

A. Solutions for Dot-Blot Hybridisation

1. Church Hybridisation Buffer (CHB)

1.0 % Bovine Serum Albumin

7.0 % SDS

0.5 M Na_2PO_4 (pH 7.0)

1 mM EDTA

Denatured Herring sperm was added to the pre-hybridisation buffer (CHB) at a concentration of 100 µg / ml.

2. Wash Buffer A (WBA)

0.5 % Bovine Serum Albumin

5.0 % SDS

40.0 mM Na_2PO_4 (pH 7.0)

1.0 mM EDTA

3. Wash Buffer B (WBB)

1 %   SDS

40 mM Na_2PO_4 (pH 7.0)

1 mM EDTA

4. 20 x SSC

3.0 M NaCl

0.3 M Sodium Citrate (pH 7.0)

B. Solutions and Media for Cloning

1. TE buffer

10 mM Tris-HCl (pH 8.0)

 1 mM EDTA

2. LB liquid medium

10 g Bacto tryptone

 5 g Bacto yeast extract

 5 g NaCl

The medium was made up to 1 l with water and autoclaved.

3. LB plates

15 g Bacto agar

 1 l LB Medium

Medium was autoclaved and 25ml poured into each petri dish.

4. LB/Amp/X-gal/IPTG plates

A volume of 500 ml LB agar was autoclaved and cooled down to approximately 50°C and the following added under sterile conditions:

100 μg / ml Ampicillin

   0.004 % X-gal solution

   0.05 mM IPTG solution

25 ml was poured into each petri dish.

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