



<http://researchspace.auckland.ac.nz>

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.

<http://researchspace.auckland.ac.nz/feedback>

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.

GENE SEQUENCING AND *IN VITRO*
SYNTHESIS OF THE ROTAVIRUS
NON-STRUCTURAL GLYCOPROTEIN

Kevin F.H. Powell
Cell Biology Department

Thesis submitted in partial fulfilment of
the Degree of Doctor of Philosophy
of the University of Auckland
August 1986

UNIVERSITY OF AUCKLAND LIBRARY
BIOLOGY

~~THESES~~

87-19

cop. 2

TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	vi
ABSTRACT	viii
ABBREVIATIONS	xi
CHAPTER ONE INTRODUCTION	1
CHAPTER TWO MATERIALS AND METHODS	14
2.1 Materials	14
2.1.1 General chemicals	14
2.1.2 Culture media	14
(i) Cell culture media	14
(ii) Bacterial culture media	14
2.1.3 Cells, viruses, bacterial strains and vectors	16
(i) Cells and viruses	16
(ii) Bacterial strains	17
(iii) Vectors	18
2.1.4 Enzymes	19
(i) Restriction enzymes	19
(ii) Other enzymes	19
2.1.5 Radioisotopes	20
2.2 Methods	20
2.2.1 Preparation of genomic dsRNA from purified virus	20
2.2.2 Preparation of poly A polymerase	21
2.2.3 Adenylation of rotavirus RNA	22
2.2.4 Synthesis of cDNA	23
2.2.5 'Blunt ending' ds cDNA	23
2.2.6 The homopolymer tailing reaction	24
2.2.7 cDNA cloning	25
2.2.8 Preparation of competent <i>E.coli</i> cells and transformation	25
(i) Strains RR1 and C600	25
(ii) Strain JM101	26
(iii) Transformation	26

	<u>Page</u>
2.2.9 DNA purification	27
(i) Small-scale isolation of plasmid DNA	27
(ii) Large-scale isolation of plasmid DNA	28
2.2.10 Restriction enzyme digestions	28
2.2.11 Agarose gel electrophoresis	29
2.2.12 Polyacrylamide gel electrophoresis	29
(i) 'Nondenaturing' gels	29
(ii) 'Denaturing' gels	30
(iii) Autoradiography	31
(iv) Discontinuous SDS - gel electrophoresis	31
2.2.13 Screening and assignment of recombinant plasmids	31
2.2.14 Recovery of DNA from gels	32
(i) Elution of cDNA	32
(ii) Elution of DNA fragments generated by restriction enzyme digestion	32
2.2.15 Subcloning DNA fragments	33
2.2.16 DNA sequencing	33
(i) Preparation of template DNA	34
(ii) Deoxyribonucleosides	34
(iii) The sequencing reaction	35
2.2.17 RNA transcription from plasmids containing the SP6 promoter	36
2.2.18 M13-mediated <i>in vitro</i> site-directed mutagenesis	37
2.2.19 Translation of SP6-generated mRNA <i>in vitro</i>	38
2.2.20 Computing	39
CHAPTER THREE CLONING cDNA COPIES OF ROTAVIRUS GENOMIC RNA	40
3.1 Introduction	40
3.2 Results	44
3.2.1 Assay and preliminary characterisation of poly A polymerase	44
3.2.2 Adenylation of rotavirus RNA	46
3.2.3 cDNA synthesis	49
3.2.4 Annealing of ss cDNA	51
3.2.5 The homopolymer tailing reaction and production of chimeric plasmids	54

	<u>Page</u>
3.2.6 Summary of the cloning protocol adopted and NCDV clones obtained	58
CHAPTER FOUR THE NUCLEOTIDE SEQUENCE OF GENOMIC SEGMENT 10	61
4.1 Introduction	61
4.2 Results	62
4.2.1 Assignment of the cDNA clone	62
4.2.2 Size analysis of gene 10 clones and subcloning genomic segment 10 cDNA	62
4.2.3 The sequencing strategy adopted for gene 10	63
4.3 Analysis and comparison of the gene 10 sequence	65
4.3.1 Sequence analysis of NCDV gene 10	65
(i) Conservation of ends	65
(ii) Untranslated regions	66
(iii) Initiation codons	67
(iv) The Fickett testcode	68
(v) Translation and reading frames	68
(vi) Codon usage table	69
4.3.2 Comparative analysis of gene 10 sequences for four rotavirus strains	69
(i) Overall homology	69
(ii) Regional homology	71
4.4 The sequence of protein NCVP5	75
4.4.1 The amino acid sequence of the NCVP5 gene 10 product	75
4.4.2 Comparative studies of NCVP5 proteins	76
4.4.3 The hydropathic profile of NCDV NCVP5	79
4.4.4 The Chou and Fasman plot	81
4.5 Summary of the sequence information for NCDV genomic segment 10	82
CHAPTER FIVE AN INVESTIGATION OF NCVP5 FUNCTION INVOLVING SITE-DIRECTED MUTAGENESIS OF THE CLONED GENE	84
5.1 Introduction	84
5.2 Results	89
5.2.1 Strategy for the site-specific deletions of gene 10 cDNA	89

	<u>Page</u>
5.2.2 Construction strategy for the deletion mutants	91
(i) The N-terminal deletions	91
(ii) The internal deletions: oligonucleotide directed mutagenesis	94
5.2.3 Transcription of the deletion mutants	96
5.2.4 Translation of the mRNA derived from the deletion mutants	98
5.3 Summary of site-directed deletion mutants obtained and the results of translation/ glycosylation studies	105
CHAPTER SIX CONCLUSIONS AND PROPOSED FUTURE DIRECTIONS FOR RESEARCH	107
6.1 Cloning rotavirus RNA	107
6.2 The sequence of genomic segment 10	108
6.3 Membrane insertion of NCVP5	109
REFERENCES	119
APPENDIX ONE PUBLICATIONS	131
APPENDIX TWO EVOLUTIONARY CONSIDERATIONS	132
APPENDIX THREE CHOU AND FASMAN SECONDARY STRUCTURE PREDICTIONS	134

ACKNOWLEDGEMENTS

I am grateful for the patient supervision of my supervisors, Associate Professor A.R.Bellamy and Dr H.E.D.Lane, whose ideas and guidance throughout experimental work and critical appraisal during thesis preparation have proved invaluable.

I gratefully acknowledge research materials provided by Professor P.H.Atkinson, Dr. G.W.Both, Dr. R Forster and Mr. W.F.Chadderton. My thanks also are due to Dr. P.R.Gunn for initial guidance concerning the sequencing techniques used in this work. I have greatly appreciated the friendly and helpful atmosphere created by the staff and students of the Cell Biology Department during the course of my work. In particular I wish to thank Mr. T.Gruitjers and Mr. K.Ashbridge who provided valuable help with photography and computing respectively.

I extend my thanks to Mrs.M.Dickson for the cheerful and dedicated manner in which the sometimes daunting task of typing this thesis has been carried out. My sincere thanks also are due to my friends who have lent generous support. To my parents I wish to record my lasting gratitude for their unstinting care and support. Their continuing interest in my work has played a major role in the final completion of this thesis.

Finally the work presented was carried out during the tenure of a Post Graduate Scholarship provided by the Medical Research Council of New Zealand. I should like to thank the Council for the financial support which enabled this work to be carried out.

- oOo -

ABSTRACT

1. Recombinant DNA techniques have been applied to the dsRNA genome of the bovine rotavirus Nebraska Calf Scours Diarrhoea virus (NCDV). The sequence of a full-length cloned copy of genomic segment 10 of NCDV has been determined using the Sanger dideoxynucleoside sequencing technique by subcloning cDNA into M13 vectors.
2. Genomic segment 10 codes for the non-structural protein NCPV5, a protein which appears to be involved in virus maturation (Estes *et al.*, 1983). Determination of the nucleic acid sequence of the gene has enabled the amino acid sequence of the bovine NCPV5 protein to be inferred. Comparison of the inferred amino acid sequence with homologous sequences derived from other virus strains (Both *et al.*, 1983c; Baybutt and McCrae, 1984; Okada *et al.*, 1984; Ward *et al.*, 1985) has enabled conserved regions of the molecule to be identified. A small region of the NCPV5 protein has been identified (residues 131-161) which exhibits considerable variability between rotavirus strains.
3. A computer-based algorithm has been utilised to predict the folding pattern of NCDV gene 10 mRNA. This reveals a 'panhandle' structure which differs from that proposed for the related gene of strain Wa rotavirus (Okada *et al.*, 1984)

but both molecules possess a common feature in that the initiation codon falls within a potentially-stable duplex formed with a portion of the 3' untranslated region.

4. A series of four site-directed deletion mutants of the cloned gene were constructed in order to investigate the functional significance of the three N-terminal hydrophobic regions of the NCVP5 protein. Two mutants were constructed using conveniently-located *Hind*III and *Bam*HI restriction enzyme sites. The other two mutants were generated using M13 vectors and synthetic oligonucleotides. These modifications yielded genes coding for proteins in which portions of the first and second hydrophobic regions had been deleted.
5. DNA corresponding to the 'wild-type' coding region was inserted into an SP6 transcription vector to enable mRNA to be produced *in vitro*. This mRNA, when incubated in a reticulocyte lysate, directed the synthesis of a protein of the correct size (20 K). The addition of dog pancreatic microsomes to the reaction yielded a protein product (29 K) of a size consistent with the glycosylated ('wild-type') form of the NCVP5 protein.
6. The four variant forms of the NCVP5 gene were also inserted into SP6 transcription vectors and the protein products synthesised by the resulting mRNAs studied. All four mRNAs

directed synthesis of variant protein products of the anticipated size.

7. The ability of the four variant proteins to become glycosylated and to associate with membranes was investigated. The topology of the proteins in the membrane was examined by digestion with proteolytic enzymes. Variant proteins altered in the first or second hydrophobic regions retained their ability to associate with membranes, suggesting that the third hydrophobic region, which was not altered, might play a role in membrane association.
8. A model for the disposition of NCVP5 in the endoplasmic reticulum is proposed in which the first hydrophobic region is located within the lumen of the endoplasmic reticulum, the second hydrophobic region spans the membrane and the third hydrophobic sequence associates independently with the membrane from the cytoplasmic side leaving the C-terminus of the molecule exposed to the cytoplasm. The model proposed accounts for the experimental observations but is in conflict with current mechanisms proposed for the insertion of proteins into membranes. (Wickner and Lodish, 1985).

ABBREVIATIONS

AMV	avian myeloblastosis virus
BCIG	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside
bp	base pairs
Ci	curie (3.7×10^{10} disintegrations per second)
cDNA	complementary DNA produced by reverse transcription of an RNA template
CF	complement fixation
DBM	diazobenzyloxymethyl
DEPC	diethylpyrocarbonate
ds	double stranded
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
ELISA	enzyme-linked immunosorbent assays
IPTG	isopropyl- β -D-thiogalactopyranoside
K	kilodaltons
Kb	kilobase (1000 bp)
psi	pounds per square inch
RF	replicative form
SDS	sodium dodecyl sulphate
ss	single stranded
SSC	saline sodium citrate (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0)
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
tris	2-amino-2-hydroxy-methyl-propane-1,3-diol
Tm	melting temperature
w/v	weight per volume
v/v	volume per volume