EXPRESSION AND CHARACTERISATION OF THE HEPATITIS C VIRUS NON-STRUCTURAL PROTEIN 3

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ABSTRACT

The non-structural protein 3 (NS3) of hepatitis C virus (HCV) possesses three activities which are likely to be essential for virus replication; a serine protease located in the N-terminal region and helicase and NTPase activities located in the C-terminal region. Several expression vectors have been constructed which encode full length NS3 with and without an amino-terminal polyhistidine tag and these have been used to express NS3 in *E. coli*. When expressed in *E. coli*, recombinant NS3 was found to form inclusion bodies. Soluble NS3 was recovered from the inclusion bodies using a denaturation-renaturation procedure and this protein was used for characterisation of the helicase and NTPase activities of full length NS3. NS3 associated polynucleotide stimulated NTPase activity and DNA helicase activity were demonstrated and characterised with respect to optimal reaction conditions and substrate preference. NS3 is a member of the DEAD-box family of helicases which are characterised by the presence of conserved amino acid motifs. Substitution of amino acids within the conserved motifs of NS3 demonstrated that these residues were essential for optimal helicase and NTPase activity.

The presence of NS3 in hepatocytes from patients with chronic HCV infection has been demonstrated using anti-NS3 antisera and NS3 was shown to be predominately located in the cytoplasm but was also detected in the nucleus. NS3 has been shown to inhibit cAMP-dependent protein kinase (PKA) via a region of NS3 which shares homology with PKI, a potent inhibitor of PKA. This PKI-like region of NS3 was compared between HCV genotypes and amino acid sequence differences were identified that predicted that type 1a NS3 would not inhibit PKA whilst type 1b NS3 would. However, it was shown that both type 1a and 1b NS3 sequences were able to inhibit PKA, though type 1b NS3 appeared to have a greater inhibitory effect.

PUBLICATIONS

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CONTENTS

	title page	1
	abstract	2
	publications	3
	contents	4
	list of figures	10
	list of tables	12
	abbreviations	13
	CHAPTER 1: Introduction	17
1.1	Recognition of Non-A, Non-B Hepatitis	17
1.2	The Discovery of Hepatitis C Virus	19
1.3	Characteristics of Hepatitis C Infection	20
1.3.1	Pathogenesis	20
1.3.2	Epidemiology	24
1.3.3	Transmission	24
1.3.4	Diagnosis	25
1.3.5	Prevention and Treatment	27
1.4	The *Flaviviridae* Family	29
1.5	HCV Genome Organisation and Heterogeneity	30
1.6	Structural Proteins	34
1.6.1	Core	34
1.6.2	Envelope Glycoproteins E1 and E2	38
1.7	Non-structural Proteins	40
1.7.1	NS2 and the NS2/3 Protease	40
1.7.2	NS3; a Multifunctional Protein	42
1.7.3	NS4 - NS4A and NS4B	60
1.7.4	NS5 - NS5A and NS5B	61
1.8	5' and 3' Untranslated Regions	64
1.9	Replication and Cell Culture of HCV	68
1.10	Expression of Recombinant Proteins	71

1.10.1	Expression in *E. coli*; the T7-based Expression System	71
1.10.2	Expression in Insect Cells; the Baculovirus Expression System	72
1.11	Aims	74
	CHAPTER 2: Materials and Methods	76
2.1	Materials	76
2.1.1	Buffers and Solutions	76
2.1.2	*E. coli* Growth Media	81
2.1.3	Cell Culture Growth Media	82
2.1.4	*E. coli* Strains	82
2.1.5	Viruses	83
2.1.6	Oligonucleotides	83
2.1.7	Plasmids	85
2.1.8	Enzymes	85
2.1.9	Chemicals	86
2.1.10	Antibiotics	86
2.1.11	Molecular Weight Standards	87
2.1.12	Immunological Reagents	88
2.1.13	Films and Photographic Reagents	89
2.1.14	Dialysis Tubing	89
2.2	Techniques of Nucleic Acid Manipulation	89
2.2.1	Organic Extractions	90
2.2.2	Ethanol Precipitation	91
2.2.3	Restriction Enzyme Digestion of DNA	91
2.2.4	Dephosphorylation of 5' DNA Ends	92
2.2.5	End Filling of DNA Fragments	92
2.2.6	Gel Purification of DNA Fragments from Agarose	93
2.2.7	Ligation of DNA	93
2.2.8	Radiolabelling of DNA	94
2.2.9	Purification of Radiolabelled Oligonucleotides	95
2.2.10	Site Directed Mutagenesis	96
2.2.11	DNA Sequencing	98

2.2.12	Alkaline Lysis Method of DNA Preparation (Miniprep)	100
2.2.13	Boiling Miniprep Method of Plasmid DNA Preparation	100
2.2.14	Large Scale Preparation of Plasmid DNA	101
2.2.15	Extraction of DNA from Insect Cells	103
2.2.16	Determination of DNA Concentration	103
2.2.17	Polymerase Chain Reaction	104
2.2.18	Capture Cloning of PCR Products	105
2.3	Techniques of Protein Manipulations	107
2.3.1	Production of Protein Extracts	107
2.3.2	Isolation of Protein Sequestered in Inclusion Bodies	108
2.3.3	Bradford Estimation of Protein Concentration	108
2.3.4	Coomassie Blue Staining of SDS-PAGE Gels	109
2.3.5	Western Transfer of Protein Extracts	109
2.3.6	Immunological Detection of Immobilised Proteins	110
2.3.7	N-terminal Protein Sequencing	111
2.3.8	Protein Purification: Column Chromatography	111
2.4	Electrophoretic Techniques	112
2.4.1	Electrophoresis of DNA Sequencing Products	112
2.4.2	TBE Polyacrylamide Gel Electrophoresis	115
2.4.3	Agarose Gel Electrophoresis of DNA	116
2.4.4	SDS-Polyacrylamide Gel Electrophoresis (PAGE)	117
2.5	Bacterial Techniques	119
2.5.1	Maintenance of *E. coli* Strains	119
2.5.2	Production of Competent *E. coli*	119
2.5.3	Transformation of Chemically Competent *E. coli* Cells with Plasmid DNA	121
2.5.4	Transformation of Electro-competent *E. coli* Cells with Recombinant DNA	121
2.6	Handling of Insect Cells and Baculoviruses	122
2.6.1	General Handling Considerations	122
2.6.2	Freezing and Defrosting of Sf9 Cell Stocks	122
2.6.3	Routine Maintenance of SF9 Cells	123

2.6.4	Cell Counting and Viability Estimation	124
2.6.5	Infection of Sf9 Cells	125
2.6.6	Plaque Assay	127
2.6.7	Production of Recombinant Baculoviruses	129
2.6.8	Screening for Recombinant Baculoviruses	130
2.6.9	Production of Viral Stocks	130
2.7	Assays	131
2.7.1	Band Shift Assay	131
2.7.2	Helicase Assay	131
2.7.3	Malachite Green NTPase Assay	132
2.7.4	Radioactive ATPase Assay	132
2.7.5	PKA Assay	133
2.7.6	Immunofluorescence of HepG2 Cells for NS3	134
2.7.7	Immunostaining of Hepatocytes for NS3	135
	CHAPTER 3: Expression and Purification of Recombinant Proteins	137
3.1	Expression of NS3 in *E. coli*	137
3.1.1	Construction of NS3 Expression Plasmids	137
3.1.2	Construction of NS3 Mutants	138
3.1.3	Expression of NS3 Protein	140
3.1.4	Production of Soluble NS3 Protein	147
3.1.5	Purification of Insoluble NS3 Protein	168
3.1.6	Refolding of Solubilised NS3	172
3.1.7	Improving Refolding Efficiency	175
3.1.8	Refolding of NS3 Protein from Different Clones	178
3.2	Further Purification of Refolded NS3	178
3.2.1	Metal Chelate Chromatography	178
3.2.2	Heparin Sepharose Chromatography	180
3.3	Expression of NS3 in Insect Cells	180
3.3.1	Construction of NS3 Expressing Recombinant Baculovirus	180
3.3.2	Expression of NS3 Using Recombinant Baculovirus	187
3.4	Expression of NS5A/B Cleavage Site (NS5A/BΔ) in *E. coli*	197

3.4.1	Construction of NS5A/BΔ Expression Construct	197
3.4.2	Expression of Recombinant NS5A/BΔ Protein	197
3.4.3	Purification of Insoluble Protein and Subsequent Solubilisation	198
	CHAPTER 4: Characterisation of Activities of NS3	201
4.1	DNA Binding Activity of HCV NS3 Protein	201
4.2	NTPase Activity of HCV NS3 Protein	201
4.2.1	Demonstration of NTPase Activity	201
4.2.2	Optimisation of Conditions for NTPase Activity	209
4.2.3	Polynucleotide Stimulation and Kinetic Analysis of NTPase Activity	209
4.3	Helicase Activity of HCV NS3	211
4.3.1	Demonstration of Helicase Activity	211
4.3.2	Optimisation of Conditions for Helicase Activity	217
4.3.3	Nucleotide Triphosphate Utilisation as Energy Sources of Helicase Activity	224
4.4	Mutational Analysis of Helicase and NTPase Activities of HCV NS3 Protein	224
	CHAPTER 5: Non-viral Functions of NS3	232
5.1	Subcellular Localisation of NS3 in Hepatocytes from Infected Patients	232
5.2	NS3 Inhibition of cAMP-dependent Protein Kinase	238
	CHAPTER 6: Discussion	243
6.1	HCV Replication and NS3	243
6.2	Expression of NS3 in *E. coli*	244
6.3	Expression of NS3 in Insect Cells	251
6.4	NS3 has DNA Binding, Helicase and Polynucleotide Stimulated NTPase Activity	253
6.5	Mutational Analysis of NS3 Helicase and NTPase Activity	259
6.6	Subcellular Localisation of NS3	264
6.7	Inhibition of cAMP-dependent Protein Kinase by NS3	266

6.8	Future Work	271
6.9	Conclusions	274
	CHAPTER 7: References	276
	appendix I: plasmids	341
	acknowledgments	345

LIST OF FIGURES

1.1	Organisation of the HCV genome	17
1.2	Relationship between the members of the family *Flaviviridae* and members of the plant virus supergroups	31
1.3	Genome organisation and polyprotein processing of HCV	32
1.4	Alignment of conserved regions of NS3 from HCV and related viruses	43
1.5	Diagrammatic representation of the NS3 helicase domain determined by X-ray crystallography showing a proposed mechanism	52
1.6	Alignment of HCV NS3 amino acid residues 1488-1500 with PKI and the type II regulatory subunit of PKA (R^{II})	59
3.1	Amino acid substitutions made to the conserved DEAD-box helicase motifs I, II and VI of NS3	139
3.2	Restriction digest of putative $C_{1318}S$ NS3 mutants	141
3.3	Time course of expression of NS3 in JM109 (DE3)	144
3.4	Detection of NS3 with an anti-histidine tag antibody	146
3.5	Expression of pAW3(1027-1657) in *E. coli* BL21 (DE3)	148
3.6	Expression of pAW3(1027-1657) in *E. coli* BL21 (DE3) pLysS and HMS174 (DE3)	149
3.7	Time course of expression of NS3 in JM109 (DE3) at 25°C and 30°C	152
3.8	Time course of NS3 expression induced with 0.1 mM IPTG	155
3.9	Extraction of NS3 protein in the presence of detergents	157
3.10	Co-expression of NS3 with the *E. coli* chaperone complex groELS	161
3.11	Expression of NS3 in Super Broth at 37°C and 25°C	163
3.12	Expression of NS3 from pET3(1038-1657), pRS3(1027-1657) and pRP3(1027-1657)	166
3.13	Comparison of the NS3 amino acid sequence of different HCV isolates	169
3.14	Purification of inclusion bodies containing insoluble NS3	171
3.15	NS3 extraction in the presence of protease inhibitors	173
3.16	NS3 mutant proteins	174
3.17	NS3 purification by metal chelate chromatography	180

3.18	Nickel inhibition of NS3 NTPase activity	183
3.19	NS3 purification using heparin sepharose affinity chromatography	185
3.20	PCR analysis of recombinant baculoviruses	188
3.21	Expression of NS3 in insect cells	189
3.22	Effect of culture temperature on NS3 recombinant baculovirus expression	193
3.23	Use of protease inhibitors for the harvest of NS3 from insect cells	195
3.24	Expression of NS5A/BΔ in *E. coli* JM109 (DE3)	199
3.25	Purification of inclusion bodies containing insoluble NS5A/BΔ	200
4.1	Demonstration of DNA oligonucleotide binding by NS3 using a band shift assay	202
4.2	Specificity of NS3 ssDNA binding	203
4.3	Polynucleotide stimulated NS3 associated NTPase activity	204
4.4	Antibody inhibition of NS3 NTPase activity	206
4.5	Inhibition of NS3 associated NTPase activity by the presence of zinc during the refolding procedure and the effects of storage on NTPase activity	207
4.6	Optimisation of conditions for NS3 NTPase activity	210
4.7	Polynucleotide stimulation of NS3 NTPase activity	212
4.8	Calculation of the K_M and k_{cat} values for NS3 ATPase activity using a Lineweaver-Burke plot	214
4.9	Helicase activity of NS3	216
4.10	Absence of NS3 helicase activity at 25°C	218
4.11	NS3 helicase activity at 37°C	219
4.12	Time course of NS3 helicase activity and helicase activity as a function of NS3 concentration	220
4.13	Optimisation of conditions for NS3 associated helicase activity	221
4.14	Ability of various nucleotide triphosphates to provide an energy source for NS3 helicase activity	225
4.15	Mutational analysis of NS3 associated NTPase activity	227
4.16	Mutational analysis of NS3 associated helicase activity	229
5.1	Detection of HCV NS3 in transfected HepG2 cells	233

5.2	Immunostaining of HCV infected hepatocytes	235
5.3	Sequence alignment of the PKI-like NS3 sequence from different HCV genotypes	239
5.4	Inhibition of PKA activity with full length NS3	241
5.5	Inhibition of PKA activity with peptides corresponding to the NS3 PKI-like region	242

LIST OF TABLES

2.1	Solutions and volumes used for the preparation of a 10% polyacrylamide gel	116
2.2	Volumes used to make 10% SDS-PAGE stacking and running gels	118
2.3	Seeding densities for commonly used tissue culture vessels	126
3.1	Site directed mutagenesis of NS3	142
3.2	Summary of attempts to refold denatured NS3 purified as inclusion bodies	177
3.3	Amount of soluble NS3 recovered following refolding of NS3 expressed from different plasmids	179
3.4	Summary of attempts to express full length NS3 in insect cells	192
4.1	Kinetic analysis of NS3 NTPase activity	215

ABBREVIATIONS

aa	amino acid(s)
AcMNPV	*Autographa californica* mononuclear polyhedrosis virus
ADP	adenosine diphosphate
AIH	autoimmune hepatitis
ALT	alanine aminotransferase
APS	ammonium persulphate
AP	alkaline phosphatase
ApR	ampicillin resistance
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
bDNA	branched DNA
Bis	N N' methylene bisacrylamide
BOG	n-octyl β-D-glucopyranoside
bp	base pairs
BSA	bovine serum albumin
BVDV	bovine viral diarrhoea virus
C	core protein
cAMP	adenosine 3',5' cyclic monophosphate
cDNA	complementary DNA
CHAPS	(3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
CTP	cytidine triphosphate
Da	Daltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate

dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
ddNTP	dideoxynucleotide triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide 5' triphosphate
ds	double strand
DTT	dithiothreitol
E1(2)	envelope protein 1(2)
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra acetic acid
eIF	elongation initiation factor
ELISA	enzyme-linked immunosorbent assay
EMC	essential mixed cryoglobulinaemia
ER	endoplasmic reticulum
FEIA	fluorescence enzyme immunoassay
GTP	guanosine triphosphate
HAV	hepatitis A virus
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HCC	hepatocellular carcinoma
HDV	hepatitis delta virus
HGV	hepatitis G virus
HIV	human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
HRP	horseradish peroxidase
HRPL	horseradish peroxidase luminescent
HVR	hypervariable region
IFN	interferon

IPTG	isopropyl β D thiogalactopyranoside
IRES	internal ribosome entry site
ISDR	interferon sensitivity determining region
IVDU	intravenous drug user
kbp	kilobase pairs
kDa	kiloDaltons
LT-βR	lymphotoxin-β receptor
MCS	multiple cloning site
mRNA	messenger RNA
NANBH	non-A non-B hepatitis
NF-κB	nuclear factor κ B
NPH	nucleoside triphosphate phosphohydrolase
NS	non-structural protein
nt	nucleotide
NTP	nucleoside triphosphate
NTPase	nucleoside triphosphatase
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PEG	polyethylene glycol
Pi	inorganic orthophosphate
PKA	protein kinase A
PKI	protein kinase A inhibitor
PKR	protein kinase R
PMSF	phenylmethylsulphonyl fluoride
PTB	polypyrimidine tract binding protein
PTH	post transfusion hepatitis
RIBA	recombinant immunoblot assay
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase PCR
SDS	sodium dodecyl sulphate
SF	super family
SRP	signal recognition particle
ss	single strand
TAH	transfusion associated hepatitis
TCA	trichloroacetic acid
TEMED	N N N' N' Tetramethylethylenediamine
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Tris	tris[hydroxymethl]aminoethane
TTP	thymidine triphosphate
UTP	uridine triphosphate
UTR	untranslated region
UV	Ultra violet
X GAL	5 bromo 4 chloro 3 indoyl β D galactopyranoside

Chapter 1: Introduction

Hepatitis C virus (HCV) was identified as the major causative agent of non-A, non-B (NANB) hepatitis by Choo *et al.* in 1989 (1) and the genome was identified as a positive sense, single stranded RNA molecule of an estimated 9,500 nucleotides in length. The genome organisation of HCV was subsequently determined by sequence comparison with the pestivirus and flavivirus genomes (2-4) (**Figure 1.1**).

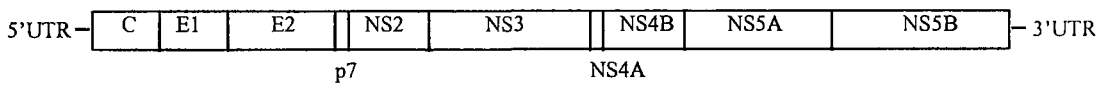


Figure 1.1 Organisation of the HCV Genome

Through the identification of amino acid motifs and by *in vivo* and *in vitro* assays, activities now have been assigned to several of the viral proteins and the replication cycle of the virus is beginning to be understood (5). Based on sequence comparison, individual HCV isolates have been determined to be one of six different genotypes (1 - 6) which are further divided into subtypes (a, b, c) (6). In this introduction the discovery and classification of HCV and the clinical and molecular biological features of the virus are reviewed.

1.1 Recognition of Non-A, Non-B Hepatitis

In the 1960's only two types of viral hepatitis were recognised; infectious hepatitis and serum hepatitis. Infectious hepatitis was shown to be spread by the faecal-oral route and have an incubation period of 2 - 6 weeks before the onset of symptoms. The agent responsible for infectious hepatitis was identified as hepatitis A virus (HAV) (7). Serum hepatitis was shown to be transmitted parenterally, primarily by contact with contaminated blood or blood products, for example blood transfusions, and had a longer incubation period of 12 - 14 weeks. The agent responsible for serum hepatitis was identified as hepatitis B virus (HBV) (7). At this time evidence also existed for the presence of further agents of hepatitis associated with blood transfusions, so-called transfusion associated hepatitis (TAH) or post transfusion hepatitis (PTH). In studies of intravenous drug users, more than two bouts of hepatitis were sometimes observed (8-10) and multiple episodes of hepatitis were also observed in haemophiliacs (11) which suggested that more than two causative agents were responsible. In addition TAH was associated with a distinct incubation period of 6 - 9 weeks which is different from that of both HAV and HBV (12) and TAH was associated with a high level of chronicity (7,13). However the possibility of the existence of an alternate agent of TAH was not pursued until the development of diagnostic tests for HAV and HBV.

When tests for HBV surface antigen (HBsAg) were applied to serum samples of patients with TAH, HBV was detected in as few as 25% of cases (14-17). Screening of blood supplies for HBV with these tests did not prevent the occurrence of TAH (18) and it was shown that presence of antibody to HBsAg, indicative of immunity to HBV, did not protect against TAH (19). These observations provided compelling evidence for the existence of a non HBV agent of TAH. HAV was unlikely to be the alternative agent since it had a shorter incubation period and secondary bouts of acute hepatitis,

associated with HAV, were not observed with TAH. This was confirmed with serological tests for HAV (15,16,20). The term non-A, non-B hepatitis (NANBH) was used to describe hepatitis that could not be attributed to HAV or HBV.

1.2 The Discovery of Hepatitis C Virus

In the late 1970's an animal model for NANBH was developed. Several groups showed the transmission of NANBH to chimpanzees after administration of various human innocula (serum, plasma, clotting factors, liver homogenates) via different routes (intravenous, intramuscular, subcutaneous, intrahepatic) (21-24). The responsible agent was shown to be transmissible from chimpanzee to chimpanzee and was able to produce a persistent viraemia and chronic liver disease which mirrored disease progression in humans (24,25). The availability of infectious sera from chimpanzees allowed further studies of the causative agent. Treatment of infectious inocula with chloroform rendered it non-infectious and filtration studies showed this agent to be of less than 80 nm in diameter (26,27). This data was consistent with the causative agent being a small enveloped virus.

Despite the development of an animal model, a marker for the disease such as virus-like particles, virus-specific antigens or antibodies remained elusive. One reason for the failure of conventional virological techniques to identify such a marker was believed to be a result of relatively low titres of NANBH virus in infected individuals (28). Recombinant DNA technologies were then applied to this problem and in 1989 Choo *et al* described the isolation of a cDNA clone derived from an NANBH viral genome (1). A cDNA library was constructed in the bacteriophage vector λgt11 from

nucleic acid isolated from NANBH infectious chimpanzee plasma. The library was then screened for clones expressing viral antigen using serum from a patient with chronic NANBH and a positive clone was identified. A larger overlapping clone was then identified which did not hybridise to human DNA or to DNA isolated from NANBH infected chimpanzees to confirm viral origin. Using this clone it was deduced that the HCV genome was a single stranded RNA molecule of approximately 10,000 nucleotides (1).

1.3 Characteristics of Hepatitis C Infection

1.3.1 Pathogenesis

The primary site of hepatitis C virus replication is the liver of the infected patient which results in inflammation of the liver i.e. hepatitis. The actual cause of liver damage is not fully understood but evidence points to a role of the host's immune response as the major cause in addition to any direct viral cytopathic effect. Chu *et al* could not find any correlation between the histologic activity index or any individual histologic parameters and the presence of HCV RNA (29) and studies have shown that though immunosuppressed liver transplant recipients exhibit high titres of circulating virus these patients may be asymptomatic (30). Also in some patients severe HCV-associated liver disease or even liver failure following abrupt cessation of immunosuppression has been observed (31). These observations indicate a role of the immune response in HCV hepatitis.

Evidence exists which suggests that lymphocytes may be a site for HCV replication although this is not conclusive. A single inoculum containing multiple HCV

quasispecies with respect to hypervariable region 1 (HVR1) of E2 glycoprotein (section 1.6.2) was used to inoculate hepatic and lymphatic cell lines (32). Different quasispecies predominated in each cell line suggesting that the original inoculum contained both hepatotropic and lymphotropic HCV species. However, a study of the HCV quasispecies of hepatocytes and PBMCs of infected patients did not show any correlation between tissue specificity and individual quasispecies (33). Therefore the apparent HCV tropism of experimental cell lines may not be applicable to HCV replication *in vivo*. HCV replication has been reported in human T-cell lines infected *in vitro*, by detection of both positive and negative strand viral RNA using strand specific PCR (34,35). However, this has not been confirmed in lymphocytes from HCV infected individuals where conflicting reports have been made (36,37) and questions over the specificity of strand specific PCR have cast doubt on these data (37,38). HCV E2 has been shown to bind CD81, a membrane spanning protein expressed on the surface of various cell types including hepatocytes and B lymphocytes (39) which may explain the presence of HCV RNA in PBMCs. But whether or not PBMCs represent a site for HCV replication remains to be confirmed.

Hepatitis C virus has an average incubation period of between 5 and 12 weeks, though this can be a short as 2 weeks and as long as 26 weeks (40,41). Acute hepatitis C infection is usually asymptomatic, the only evidence of infection being a slightly raised alanine aminotransferase (ALT) level. When symptoms do occur they are of a general hepatitis such as malaise, anorexia, jaundice, pain in the upper left abdomen and nausea, and are usually of less severity than for hepatitis A and B infections (40).

It is estimated that as many as 50% of patients with acute hepatitis will develop a chronic infection (13,42), though some studies suggest this may be an underestimate

and that as many as 80% may develop a chronic infection (43). Chronic infection is usually evident as persistently raised ALT levels, though the actual level is subject to fluctuations and in as many as a third of patients the ALT levels may be normal (13). Patients with chronic hepatitis may have few if any symptoms and those symptoms are usually mild and similar to those of acute infections although some patients complain of more aggressive symptoms including extreme fatigue, malaise and pain. The progression of chronic hepatitis does not have a typical course, it varies in severity and is generally slow. Approximately 20% of patients go on to develop cirrhosis (13,42) which will normally occur within two to three decades but can occur within 2 years. Some patients show no signs of cirrhosis after 20 years and it is unclear if these patients will go on to develop cirrhosis. Chronic hepatitis C infection has also been linked with the development of hepatocellular carcinoma (HCC) (44-47). Anti-HCV antibody was detected in 40% of European HCC patients (48) and HCC is most common in patients infected with HCV type 1b (47-49). In one long term follow up study of 131 patients with transfusion associated hepatitis disease progression 5% of patients developed HCC at 28 years post transfusion (42).

Disease progression is likely to be affected by both viral and non-viral factors. Viral factors affecting progression include virus genotype, the level of viraemia and the genetic diversity (quasispecies) of the virus in an affected individual (50-52). Non-viral factors include immune deficiency (hypogammaglobulinaemia or immunosuppression associated with organ transplant), coinfection with human immunodeficiency virus (HIV) or HBV, alcoholism and mode of infection (53-58). It has been suggested that infections associated with transfusion progress more rapidly than those associated with intravenous drug use (54) and this may be attributed to the larger viral inoculum of

transfusion, or to greater genetic diversity during infection which has been linked to a larger viral inoculum (59).

Upon development of cirrhosis, symptoms of end-stage liver disease occur such as fatigue, muscle weakness and wasting, jaundice and intestinal haemorrhage. At this stage liver transplantation is the only means of treatment and liver failure is the outcome in around 20% of patients with cirrhosis (13). As mentioned above, patients with cirrhosis may proceed to HCC. The role of HCV in the development of HCC is not fully understood. It is possible that development of HCC is a result of cirrhosis and associated hepatocyte necrosis and regeneration rather than a direct carcinogenic affect of HCV since the vast majority of patients with HCV-associated HCC have previously developed cirrhosis (60,61). However, the hepatitis C viral proteins core and NS3 have been associated with the transformation and immortalisation of cells in experimental systems (62-64) and shown to interact with the p53 tumour suppressor protein (65-67). These proteins have also been detected in cancerous areas of liver (68) thus implicating a possible role for these viral proteins in the development of cancer of the liver.

Hepatitis C infection shows extrahepatic complications including essential mixed cryoglobulinaemia (EMC) which is associated with arthritis, purpura, muscle and joint ache and glomerulonephritis (69). This condition is thought to be a result of cryoprecipitable immune complexes comprising rheumatoid factor associated with specific HCV antibodies (70). HCV has also been implicated in type 2 autoimmune hepatitis (AIH) associated with type 1 liver-kidney microsomal antibody (71), and the majority of HCV infected patients with type 2 AIH show antibodies to GOR protein (71,72). GOR protein is believed to be host derived and may represent an HCV-specific autoepitope (71).

1.3.2 Epidemiology

HCV is prevalent throughout the world with 130 countries reporting HCV infection (60). As well as being the major cause of transfusion associated NANB hepatitis, HCV is also a common cause of hepatitis in recipients of contaminated blood products, for example blood clotting factors administered to haemophiliacs. Prevalence rates vary geographically. Rates in healthy blood donors in the UK and northern Europe are between 0.01% and 0.02% (73) and much higher rates have been observed in developing countries with rates from 6.5% to 20% observed in some African populations (74-76).

HCV is present in world populations as six major genotypes (1 - 6) made up of genetically distinct sub types denoted a, b or c (6,77). Types 1a and 1b predominate in the USA and in Japan (78-82) and in Europe, types 1a, 1b, 2 and 3 are common (83-85). Type 4 is common in African countries, type 5 in South Africa and types 1b, and 2 in the Far East (85-87).

1.3.3 Transmission

HCV has been detected in the blood, ascitic fluid, semen, saliva and urine of HCV positive patients with chronic liver disease (88), though the major risk of transmission is through contact with contaminated blood or related products. The major route of transmission of HCV is by the parenteral route and the highest risk groups are recipients of contaminated blood or blood products or intravenous drug users (IVDUs)

(89,90). Since the development of diagnostic tests for HCV and routine screening of blood and blood products, the risk of transmission by this route has been greatly reduced (91). Organ transplantation, haemodialysis and tattooing represent other parenteral routes of HCV infection (89). Risk of transmission through needlestick injury has also been documented for health workers but the risk is considered to be low (92-94) possibly due to small inoculum size/titre.

Other, non-parenteral routes of transmission have been described. Sexual transmission of HCV has been suggested from the observation of increased prevalence rates in high promiscuity groups such as prostitutes, homosexuals and patients with sexually transmitted disease (95-98). Vertical transmission is rare although studies suggest that maternal coinfection with HIV or high-level viraemia may increase this risk (99,100) and vertical transmission has been observed from anti-HIV negative mothers which appears to be linked to the absence of anti-C-100 antibodies at delivery (101). Breast feeding appears not to be implicated in HCV transmission (102). Transmission through saliva has been considered following a report of HCV transmission through a human bite and a high rate of anti-HCV in New York dentists (103,104). Household contacts such as sharing razors or toothbrushes may also be a factor in HCV transmission. For many cases the transmission route is unknown which may be a result of incomplete knowledge of a patients history or transmission having occurred during mass immunisation procedures. Related viruses are transmitted via insect vectors (105) and although it has been considered, there is currently there is no evidence for transmission of HCV via insect vectors.

1.3.4 Diagnosis

Symptoms of hepatitis C are similar for other causative agents of hepatitis and so prior to the discovery of HCV and the development of specific markers, diagnosis was made by discounting other possible causes. NANBH could be diagnosed if there were no markers for HAV or HBV, no other causes of hepatitis such as Epstien-Barr virus (EBV) or cytomegalovirus (CMV) infection or a history of drug or alcohol abuse, and no underlying metabolic disorders such as Wilson's disease (106).

Since the discovery of HCV, specific markers of infection have been developed and hepatitis C can be accurately diagnosed. Initially, the presence of circulating antibodies to HCV are identified using commercial ELISA (enzyme linked immunosorbent assay) kits which employ recombinant viral proteins such as core, NS3, NS4 and NS5 as the capture antigens. Positive ELISAs are confirmed using a RIBA (recombinant immunoblot assay) which consist of individual viral antigens on a paper strip (107,108). More recently a fluorescence enzyme immunoassay (FEIA) for the measurement of HCV core protein has been reported for the early diagnosis of acute hepatitis C (109).

Due to the low levels of circulating virus it is not possible to directly detect viral antigens in serum samples for confirmation of viraemia. However, the presence of circulating virus can be determined by the detection of viral RNA. Two methods are used, RT-PCR (110) and branched DNA analysis (111). RT-PCR uses primers specific to the 5' untranslated region (UTR) of the HCV genome to reverse transcribe viral RNA and the cDNA product is amplified by PCR. 5' UTR sequence is used since this is conserved across different isolates (112). This method is highly sensitive and can detect levels as low as 100 genome equivalents per ml as well as detecting viraemia soon

after infection. The second method, branched DNA analysis, is not as sensitive as RT-PCR but is a simple and rapid method of detection and quantification. This technique uses branched oligonucleotides consisting of a unique HCV specific sequence which hybridises to the target sequence. Multiple copies of small oligonucleotides labelled with alkaline phosphatase then bind the branched oligonucleotide enabling detection (111).

1.3.5 Prevention and Treatment

Since the development of serological tests for HCV and their application to blood and organ donors, infection by blood transfusion and organ transplant is now very low (91,113,114). In addition, testing has allowed the separation of infected renal dialysis patients from uninfected patients reducing transmission in this way. Anti-HCV vaccine development is currently underway and the envelope glycoproteins E1 and E2 are candidate antigens. Choo *et al* demonstrated protection of chimpanzees from HCV infection by prior challenge with recombinant E1 and E2 viral glycoproteins of the same virus genotype, which had been purified from HeLa cells infected with a recombinant vaccinia virus expression vector (115). Kobayashi *et al* demonstrated that high levels of anti-E2 was associated with rapid resolution of acute hepatitis (116). The antigenic regions of E2 encompass the hypervariable region (HVR-1) which is thought to provide a mechanism for evading the host immune response (117). The challenge in vaccine design is therefore to produce immunity against antigens of different HCV strains, isolates and even quasispecies.

Currently there are no universally effective treatments for HCV positive individuals. The only treatment approved in Europe and North America is alpha-interferon (α-IFN). Approximately 50% of patients respond to this treatment as demonstrated by the return of ALT levels to normal; however, only 20 - 25% maintain a response upon cessation of treatment (118). Administration of α-IFN is associated with side effects which include flu-like symptoms, irritability, fatigue, depression, anorexia, nausea and migraine headaches (119). In addition, patient response to this treatment is dependent on virological factors such as virus strain, serum HCV RNA levels prior to treatment, anti-core antibody and genetic variation within the quasispecies population (59,120).

Ribavirin is emerging as another potential treatment for HCV infection. This purine nucleoside inhibits the replication of a variety of RNA viruses though its mode of action remains unknown. However, ribavirin and interferon-alpha combination therapy appears to stimulate the hosts immune response and in particular the type 1 T helper cell activity in HCV infected patients (121). Ribavirin treatment alone results in the normalisation of ALT levels but is unable to significantly reduce viraemia (122,123) and is not considered an effective anti-HCV treatment. However, when used in combination with α-IFN this treatment is more effective and it is estimated that up to 50% of patients will show a sustained response (HCV RNA negative by PCR 6 months after cessation of treatment) to the combination therapy (118). Sostegni *et al* showed that concomitant administration of ribavirin and α-IFN was able to induce a sustained response in patients with chronic hepatitis who had not responded to α-IFN alone (124). New anti-HCV strategies include the application of hairpin ribozyme gene therapy (125,126), antisense technology (127,128) and the development of selective antiviral agents against viral proteins, most notably the NS3 protease and NS3 helicase and NS5B RNA-dependent RNA polymerase (129-131).

1.4 The *Flaviviridae* Family

The *Flaviviridae* family is made up of the flaviviruses, pestiviruses and hepaciviruses and HCV belongs to the genus hepacivirus. The HCV polyprotein shares many features with the polyproteins of pestiviruses and flaviviruses. The polyproteins are of similar size and amino acid sequence comparisons have identified similarities between HCV, pestivirus and flavivirus polyproteins and also with the polyproteins encoded by members of the picornavirus-like (potyvirus) and alphavirus-like (carmovirus) plant virus supergroups (3). Regions of similarity centre around conserved motifs of protease, helicase and RNA-dependent RNA polymerase motifs which are colinear among HCV, flavivirus and pestivirus polyproteins (3,4,132). Whilst the overall amino acid sequence identity is not high between HCV, pesti- and flaviviruses their hydrophobicity profiles are very similar (4,132,133). At the nucleotide level, the 5' UTR of HCV has substantial similarity to the 5' terminus of pestiviruses (132,134) suggesting that HCV is more closely related to pestiviruses than flaviviruses.

The identification of three novel flavi-like viruses GBV-A, GBV-B and GBV-C (HGV) has been described (135-137). These viruses share a similar genomic organisation to HCV and as much as 55% amino acid homology to regions of the HCV polyprotein (136,137). The serine protease of GBV-B NS3 shares similarities with HCV NS3 at the active site and has been demonstrated to cleave the corresponding cleavage sites of HCV (138).

Sequence comparison of the helicase domain of HCV, GB viruses (GBVs), flaviviruses, pestiviruses and plant viruses enabled the construction of a phylogenetic

tree which showed that HCV and GBVs are closely related and clustered with pestiviruses, flaviviruses and plant viruses (139) (**Figure 1.2**). GBV-C has been included in this phylogenetic tree between GBV-A and GBV-B based on sequence comparisons of the NS3 and NS5B regions of HCV, and GBV-A, B and C (140). The GB viruses have been grouped with HCV to form a new genus, the hepatitis C viruses or hepaciviruses within the *Flaviviridae* family.

1.5 HCV Genome Organisation and Heterogeneity

HCV has a positive strand RNA genome of approximately 9500 nucleotides consisting of 5' and 3' untranslated regions (UTRs) flanking an open reading frame (ORF) encoding a polyprotein of between 3010 and 3033 amino acids (2,132,133,141). The exact genome and polyprotein length varies depending on the HCV genotype. The basic organisation of the HCV genome was elucidated by comparison of the hydrophobicity of the HCV polyprotein with flavivirus and pestivirus polyproteins and alignment of conserved protease, helicase and RNA-dependant RNA polymerase motifs (2-4,132,133). A 5' untranslated region (UTR) of 341 nt precedes the ORF which is arranged NH_2 - core (C) - envelope glycoprotein 1 (E1) - E2 - p7 - non-structural protein 2 (NS2) - NS3 - NS4A - NS4B - NS5A - NS5B - COOH (142-145) (**Figure 1.3**). A 3' UTR is located downstream of the ORF. C, E1 and E2 comprise the structural proteins and the remainder comprise the non-structural proteins. The encoded polyprotein is processed co- and post-translationally by host cell and viral proteases to yield the mature structural and non-structural viral proteins (**Figure 1.3**). Processing of the structural proteins to release C, E1, E2 and p7 was observed to be dependent on

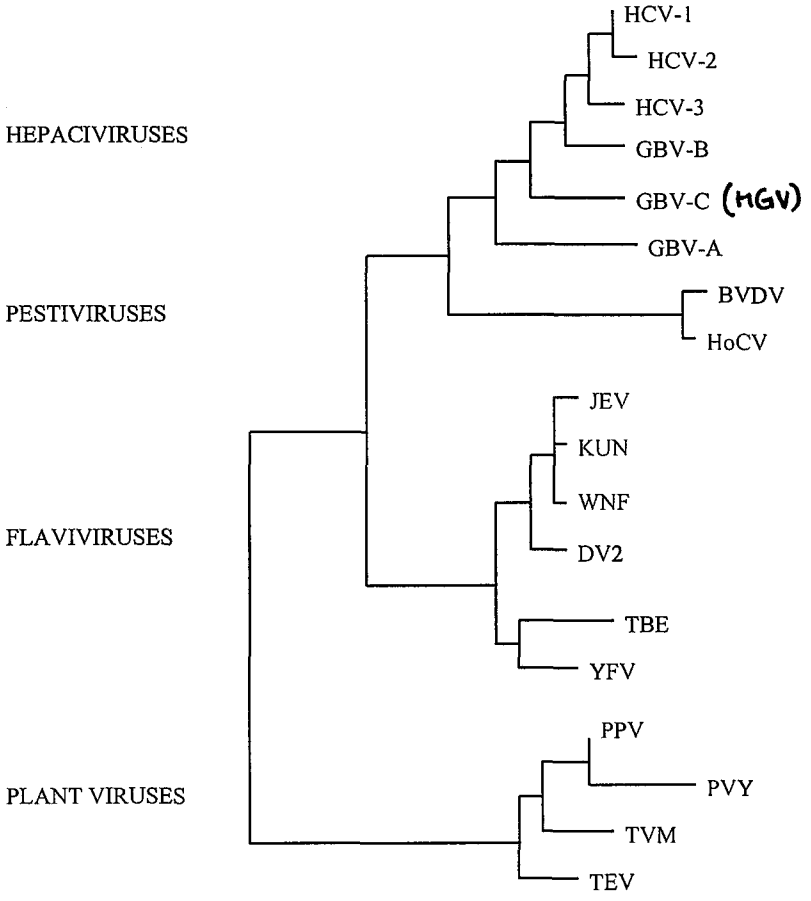


Figure 1.2 Relationship between the members of the family Flaviviridae and members of the plant virus supergroups. HCV type 1 (HCV-1), HCV type 2 (HCV-2), HCV type 3 (HCV-3), GB virus-B (GBV-B), GB virus-C (GBV-C), GB virus-A (GBV-A), bovine viral diarrhoea virus (BVDV), hog cholera virus (HoCV), Japanese encephalitis virus (JEV), Kunjin virus (KUN), West Nile fever virus (WNF), dengue virus type 2 (DV2), tick-borne encephalitis virus (TBE), yellow fever virus (YFV), plum pox virus (PPV), potato virus Y (PVY), tobacco vein mottling virus (TVM), tobacco etch virus (TEV) Diagram adapted from a phylogenetic tree constructed from a comparison of the helicase protein amino acid sequences (139).

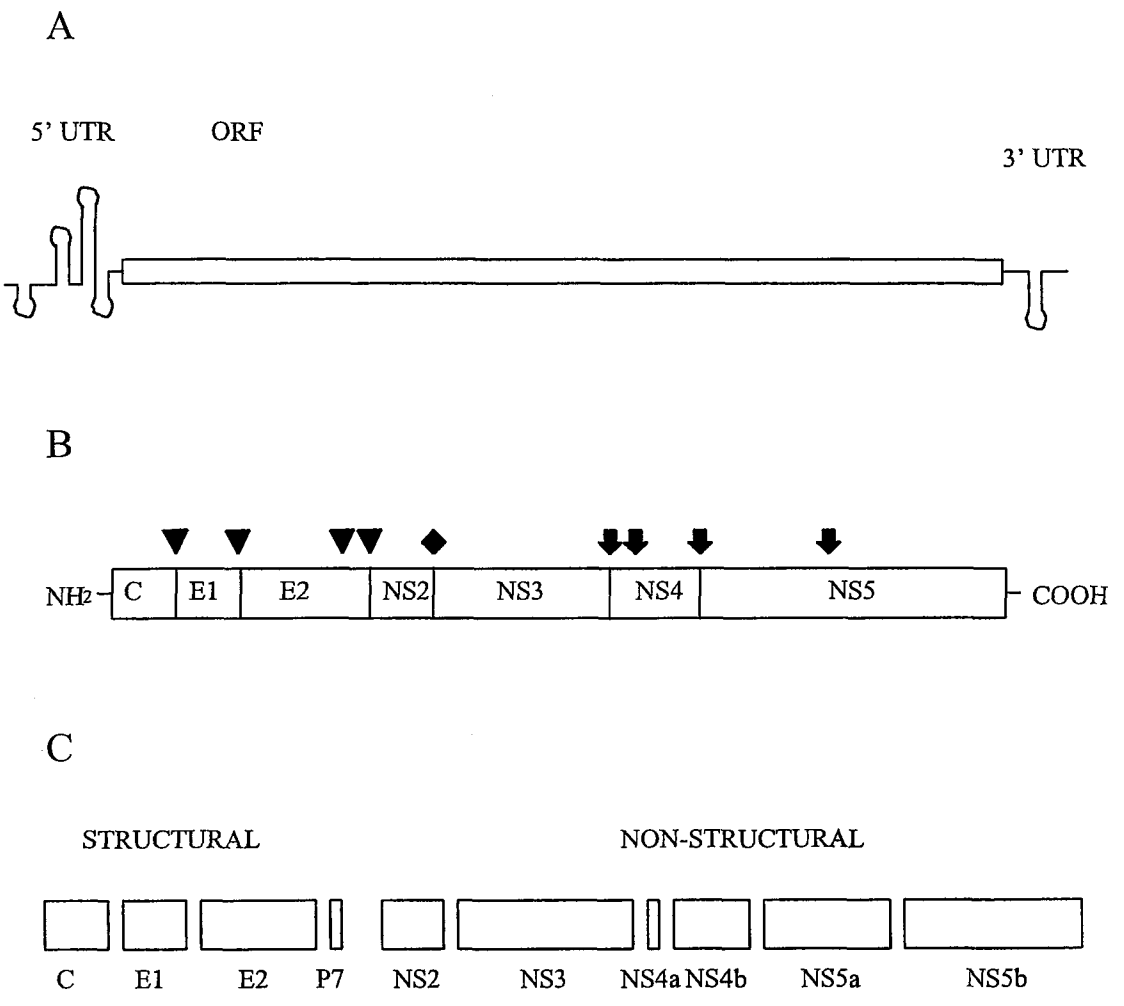


Figure 1.3 Genome organisation and polyprotein processing of HCV. A; HCV genome showing 5' UTR and IRES element, open reading frame (ORF) and 3' UTR putative stem loop. B; Organisation of the HCV polyprotein showing signal peptidase (▼) NS2/3 protease (◆) and NS3/4A protease (⬇) cleavage sites. C; Mature HCV proteins.

microsomal membranes in cell free translation experiments (145). This indicates a requirement for host cell signalases and putative cleavage sites for signalases were observed at the expected positions. (145). Release of NS2 from the polyprotein is completed by cis-cleavage of the NS2/3 junction by a putative zinc dependent metalloprotease contained in the C-terminal region of NS2 and N-terminal region of NS3 (146-148). Processing of the remaining viral proteins at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions is by the NS3/4A serine protease (149-152).

The HCV genome is highly variable at nucleotide and amino acid level and this is likely to be the result of errors introduced during replication by the viral RNA-dependent RNA polymerase. Even in a single infected individual a number of closely related, distinct genomes exist, referred to as quasispecies (153). Regions of variability are unevenly distributed throughout the HCV genome (154) and two clusters of variation at amino acid level within E2 and NS5A have been implicated in contributing to viral persistence during chronic infection. A higher than expected rate of change was observed at the 5' end of E2 glycoprotein (155-157). This region of E2, comprising amino acids 386 - 414 is the most variable of the HCV genome and is referred to as hypervariable region 1 (HVR1) (158,159). Distinct antibody-binding epitopes have been mapped to this region and the emergence of E2 HVR1 variants in a chronically infected patient has been documented (160). Amino acid replacement in this region alters the predicted secondary structure (161) and sequence specific epitopes induce the production of isolate specific antibodies (162). These data are consistent with the hypothesis that escape mutants are selected by immune pressure. Heterogeneity in the E2 HVR1 also correlates with reduced response to α-IFN treatment (163). Type 1 and especially 1b HCV have been associated with reduced sensitivity to α-IFN treatment

compared with other types and this is associated with a higher pre-treatment viral titre (164) possibly attributed to escape mutants. Also, changes in quasispecies population in response to α-IFN treatment were observed implying differing sensitivity of HCV species to α-IFN (165-168). This lends itself to the suggestion that viral genetic heterogeneity is a response to the host immune response. The second cluster of sequence variation is at a C-terminal region of NS5a and is also implicated in response to α-IFN (169). This region (aa 2209-2248), which has been named the interferon sensitivity determining region (ISDR), has been associated with the outcome of α-IFN treatment in Japanese studies (169,170).

HCV genotypes can be distinguished according to sequence similarity and studies of the 5' UTR identified three main HCV types: 1, 2 and 3 (171). Sequence analysis of NS5 further divided the HCV types into six major genotypes comprising 11 genetically distinct viral populations (77) whilst a similar study of E1 gene sequences suggested at least 12 genotypes (172). HCV nomenclature was standardised by comparison of NS5 sequence of variants from a world-wide panel to distinguish six genotypes (1, 2, 3, 4, 5 and 6) each divided into up to three subtypes (a, b and c) (6). Novel HCV isolates from Thailand, Burma, Indonesia and Vietnam originally classified as types 7, 8, 9, 10 and 11 have been reclassified so that types 7, 8, 9 and 11 are subtypes of genotype 6 and type 10 a subtype of genotype 3 (173,174).

1.6 Structural Proteins

1.6.1 Core

The 21 kDa viral capsid protein is comprised of the N-terminal 181 amino acids of the HCV polyprotein (145). This protein, named core (C) is highly basic in nature and is associated with the cytoplasmic side of the ER anchored by the C- terminal domain (145,175). Interaction with E1 may also be involved in the association of core with the ER and this may be an important step in the formation of virus particles. Immunoprecipitation studies and deletion mapping have demonstrated that interactions occur between the C-termini of both proteins and these interactions are predicted to occur in the ER membrane (175). Also in cell transfection studies, a core-tissue plasminogen activator (tpa) hybrid protein localised to the nucleus when expressed alone, but to the perinuclear region when expressed with E1 (175). Though the mechanism of assembly of the core protein monomers into the viral nucleocapsid is not understood, multimerisation of core is likely to occur by protein-protein interactions between a domain mapped to amino acids 36 - 91 of core (176). This region overlaps with the RNA binding domain located between aa 1 - 75, which has been shown to bind ribosomes and is proposed to bind viral genomic RNA during nucleocapsid formation (177). Formation of virus-like particles from structural genes expressed in insect cells has also been observed. Using a recombinant baculovirus containing nt 259 - 2819 of the HCV genome which contains part of the 5'UTR and the coding region for core, E1, E2, p7 and 21 amino acids of NS2, Baumert *et al* demonstrated the production of enveloped particles of core E1 and E2 which specifically incorporated HCV RNA in a manner independent of p7 (178).

In addition to full length core (p21) species of 19 kDa (p19) and 16 kDa (p16) have also been identified by both *in vitro* and cell culture expression studies (177,179,180). The relative expression of p16 appears to vary with HCV strain (181)

and may be dependent on the presence of E1 (179). Cleavage at residue 173 to produce p19 and production of the 151 aa p16 is presumably mediated by host signalase cleavage within two hydrophobic regions of core (residues 121 - 151 and 170 - 191) which are proposed to be located in the ER membrane (179,182). These core species show differing subcellular localisation; both p21 and p19 are located at the ER when expressed together however, p19 expressed alone and p16 have been shown to locate to the nucleus (179,182). Substitution of the C-terminal 66 amino acids of core for β-galactosidase (β-gal) results in nuclear localisation of the core-β-gal fusion protein and putative nuclear localisation signals have been identified in the N-terminus of core (183,184). It appears that in the absence of a C-terminal ER anchoring domain, one, or other of these putative signals is sufficient for transport of core to the nucleus.

Barba *et al* showed that core affected cellular lipid metabolism in hepatic and non-hepatic cell lines suggesting that core may play a role in liver steatosis, a characteristic of HCV infection (185). This study demonstrated that core was located entirely in the cytoplasm, associated either with the ER or lipid droplets contradicting reports showing a nuclear localisation of core. However, it should be noted that this work was done using core from HCV type 1b which is not associated with production of p16, the core species most associated with nuclear localisation (181).

Cytoplasmic p21 associated with the ER, is probably the site of RNA packaging and virion assembly, however the implications of nuclear located p19 and p16 are not fully understood.

Core has been shown to interact with a variety of cellular proteins and has been implicated in the development of HCC. Ray *et al* have shown that core may inhibit apoptosis (64,186) and transform primary rat embryo fibroblasts (63) and transgenic

mice expressing core are associated with the development of hepatocellular carcinoma (187). The exact mechanism by which core modulates these activities is unknown though core has been shown to down-regulate transcription of p53 (66) and p21 (188) which are involved in regulation of cell cycle progression. Core has also been shown to regulate the activity of transcription factors such as NF-κB and activating protein-1 (189,190). To identify other potential cellular targets of core, Hsieh *et al* screened a human liver cDNA library for core-interacting proteins using the yeast two hybrid system (191). The heterogeneous nuclear ribonucleoprotein K (hnRNP K), a transcriptional regulator was shown to interact with core. In addition core was able relieve the suppression of the human thymidine kinase gene promoter by hnRNP K. Core has also been shown to interact with and modulate the action of the lymphotoxin-β receptor (LT-βR) (192,193). The function of LT-βR is not yet understood however, it is believed to be involved in the development of peripheral lymphoid organs (194). LT-βR also has a high level of amino acid homology with tumour necrosis factor receptor (TNFR) which is involved in the control of cell growth and antiviral responses (195,196). Modulation of LT-βR function by core suggests a mechanism for evasion of the hosts immune defence and viral persistence. Finally, the N-terminal region of core has been shown to interact with the human DEAD-box proteins CAP-Rf (197) DBX (198) and DDX3 (199). Core was demonstrated to alter the transactivation and ATPase activities of CAP-Rf (197) and to co-localised with DDX3 and DBX in core expressing mammalian cell lines (198,199). Interaction with core was via the C-terminal regions of both DEAD-box proteins which contain a region rich in arginine and serine residues (197,199). This arginine/serine rich region is common in proteins involved in mRNA

splicing and interaction of core with these proteins suggests a mechanism for the disruption of gene regulation and cell biochemistry by core.

These observations suggest possible roles for the different species of core protein in viral persistence and disease pathogenesis. However, this work has been conducted in experimental model systems and may not reflect the effects of core expression *in vivo*. For example, Zhu *et al* demonstrated binding of core to the tumour necrosis factor receptor (TNFR) and the enhancement of tumour necrosis factor (TNF) mediated apoptosis using HepG2, HeLa and BC10ME cell lines (200). Conversely, core has also been shown inhibit TNF-mediated apoptosis in a different cell line, MCF7 (186) and the expression of core in transgenic mice at levels comparable to those found in naturally infected patients showed little correlation with the development of liver disease or HCC (201).

1.6.2 Envelope Glycoproteins E1 and E2

The envelope glycoproteins E1 and E2 are the major structural proteins, E1 (35 kDa) is released from the polyprotein by signal peptidase cleavage at amino acid 383 and E2 (70 kDa) by cleavage at amino acid 746 (142,145,202). E2 is sometimes evident as an extended protein including p7 (aa 747 - 809) which is removed by signal peptidase cleavage (142,202). E1 and E2 possess signal sequences at their respective N-termini which direct the proteins to the ER where translocation to the lumen occurs and signal peptidase cleavage at the core/E1, E1/E2 and E2/p7 junctions takes place (145,202). A hydrophobic sequence at the N-terminus of p7 may facilitate translocation into the ER allowing cleavage at the E2/p7 junction (202). Cleavage at the E2/p7 junction is often

incomplete and multiple species of E2 exist (202-204). Baumert *et al* demonstrated the production of enveloped particles of core E1 and E2 which specifically incorporated HCV RNA in a manner independent of p7 (178). It is not known if E2/p7 appears in the mature virion and the roles of E2/p7 and p7 are unknown.

The translocated E1 and E2 glycoproteins remain in the ER anchored to the inside of the lumen (145,205) where formation of the E1/E2 heterodimer and glycosylation occurs. E1 and E2 associate slowly to form a mature heterodimer through non-covalent interactions which require the N-terminal regions of both proteins (206-210). E2 is essential for correct folding of E1 (211) and assembly of the E1/E2 heterodimer is likely to require the chaperone calnexin which has been shown to interact with both E1 and E2 (212,213). The monoclonal antibody H2 recognises the E1/E2 after it has dissociated from calnexin and this is likely to represent the E1/E2 heterodimer prior to budding of the virus (214). E1 and E2 each contain a number of potential N-linked glycosylation sites (2,172,215) which are glycosylated with high-mannose carbohydrate chains (145,205,207). The absence of complex glycans, which are acquired in the trans-Golgi, suggests that the E1/E2 complex does not go further than the cis-Golgi (208,214) and association with calnexin may aid this retention. Retention of the E1/E2 complex in the ER suggests that HCV may bud from the ER and exit the infected cell via exocytosis in a manner resembling that of the flaviviruses (105). The absence of envelope glycoproteins on the cell surface will result in a reduced immune response to HCV infected cells and this may aid the development of chronicity observed with most HCV infections.

The envelope glycoproteins are likely to bind cell surface ligands prior to virus internalisation and as a result they may determine cell tropism. E2 has been shown to

bind CD81, a tetraspanin found on the surface of hepatocytes (39). Binding of CD81 by E2 is blocked *in vitro* with antibodies which neutralise HCV infection *in vivo*, suggesting that virus entry may be mediated by CD81 binding (39).

E2 contains the most variable region of the HCV genome. Hypervariable region-1 (HVR-1) is a region of extreme hypervariability in E2 and is composed of the 25-28 N-terminal amino acids (158,216). This variation has been proposed to be a result of selection of random mutations that occur during genome replication, which are able to evade the host's immune system and thus enabling viral to persistence (217). Several lines of evidence support this theory, for example variability of this region was not observed in a patient with agammaglobulinaemia (53,218). In addition antibodies to HVR-1 are able to neutralise E2 binding and block HCV infection in tissue culture (219,220) and are effective in chimpanzee challenge experiments (221). These data suggest that E2 and HVR-1 in particular is important in the immune response to HCV.

1.7 Non-structural Proteins

1.7.1 NS2 and the NS2/3 Protease

NS2 is a 21 - 23 kDa protein located at aa 810 - 1026 of the HCV polyprotein (142,143,147) and is released from the polyprotein by signal peptidase cleavage at the N-terminus (142,145,222) and autoproteolysis at the C-terminus (147,148). This autoproteolysis is catalysed by aa 827 - 1207 of the HCV polyprotein (147,148) which encompasses most of NS2 and the N-terminal one third of NS3. This region is referred to as the NS2/3 protease. From inhibition studies and point mutation analysis, the NS2/3 protease was proposed to be a zinc dependent metalloprotease (148). Two amino acids

located in the NS2 region, H_{952} and C_{993} were identified as possible catalytic residues (147,148) which may be involved in the co-ordination of a zinc ion. However, it is not certain whether the zinc ion is bound at this site, at a zinc binding site located in NS3 (223) or both. Mutations which altered the amino acids of the catalytic triad of the NS3 serine protease activity located in the N-terminus of NS3 had no effect on NS2/3 processing (147,148,224). However, NS3 C-terminal truncations inhibited this cleavage (147,148,224) indicating that the N-terminal region of NS3 was required for cleavage. Analysis of the substrate requirements of the NS2/3 protease showed it was tolerant to amino acid changes in the P5 to P3' region with the exception of proline substitutions which have the ability to dramatically alter conformation and a preference for hydrophobic residues at P1 and P1' (225). This suggested that recognition of secondary structure rather than primary amino acid sequence was more important for recognition of the NS2/3 cleavage site. Therefore, the NS3 portion of the NS2/3 protease may not contribute a catalytic subunit but be required for correct protein folding around the cleavage site. However, Reed and co-workers have demonstrated bi-molecular cleavage of the NS2/3 site (225). It was shown that an NS2/3 region with a mutated NS2 region could be cleaved by a functional NS2 region supplied in *trans* and that NS2/3 with inactive NS3 truncations could be complemented by supplying active NS3 in *trans*. This latter observation is consistent with NS3 supplying a catalytic subunit for NS2/3 cleavage.

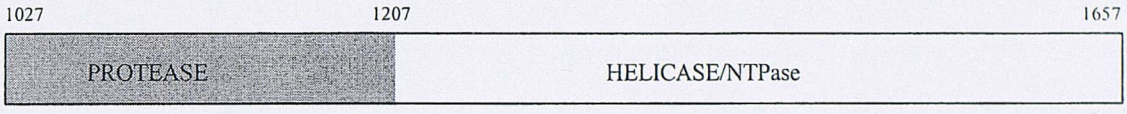
Deletion of the analogous protein of pestiviruses appears to have no effect on viral replication, cleavage at the NS2/3 junction is not essential for virus replication and does not occur in non cytopathic isolates of BVDV (226,227). Whilst NS2/3 protease

activity appears to be required for full activity of the HCV NS3 serine protease (228) no other roles for NS2 have yet been identified.

NS2 is a transmembrane protein with the C-terminus translocated in the lumen of the ER and the N-terminus located in the cytosol (229) and membrane association may aid folding and processing of the nascent polypeptide (229,230). Immunoprecipitation studies have shown that NS2 may also be associated with the structural proteins (203,206) and NS3 has been shown to immunoprecipitate with anti-E1 and anti-E2 antibodies in the presence of NS2 (206). These data suggest that NS2 may help locate NS3 and the viral replication machinery to the ER membrane.

1.7.2 NS3: A Multifunctional Protein

NS3 (amino acids 1027 - 1657 of the HCV polyprotein) has a molecular mass of approximately 70 kDa and is released from the HCV polyprotein following NS2/3 protease mediated cleavage at the N-terminus and self cleavage at the C-terminus (148-150). Sequence comparison with the corresponding region of the flavivirus and pestivirus polyproteins identified conserved motifs indicative of two activities. A serine protease activity is located in the N-terminal third, which overlaps with the NS2/3 protease and a helicase and associated NTPase is located in the C-terminal two thirds of the protein (**Figure 1.4**) (3,4,132). The catalytic domains of the NS3 protease and helicase/NTPase activities do not overlap and these can be cloned and expressed separately as recombinant proteins which retain the respective activities. These separate domains have been used in the majority of research to characterise the associated activities and have allowed the crystal structure of these domains to be resolved.




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1079            1104          1161                1229              1311
   WTVYHGAGSK --- VDQDLVG --- LKGSSGGPLLC            HAPTGSGKSTKVP --- DIIICDECHSTD       HCV-1b
   WTVYHGAGTR --- VDQDLVG --- LKGSSGGPLLC            HAPTGSGKSTKVP --- DIIICDECHSTD       HCV-1a

   SSVDHVTAGK --- VKTDSGC --- LKGWSGLPIFE            TLATGAGKTTE-L --- SYIFLDEYHCAT       BVDV
   SSVDHVTCGK --- VKTDSGC --- LKGWSGLPIFE            TLATGAGKTTE-L --- SYIFLDEYHCAT       HoCV

   HTMWHVTRGA --- VKEDRLA --- PSGTSGSPIVN            LFHPGAGKTRRFL --- EVIIMDEAHFLD       YFV
   HTLWHTTKGA --- VKEDRLC --- PTGTSGSPIVD            LLHPGAGKTRKIL --- NLFIMDEAHFTD       WNV
   HTMWHVTRGA --- VKKDLVS --- SPGTSGSPIID            DLHPGAGKTKRYL --- NLIIMDEAHFTD       DV2
   HTLWHTTRGA --- VKEDRIA --- PRGTSGSPILD            DLHPGSGKTRKIL --- NLFVMDEAHFTD       JEV
       H             D            S                    G GK              DE H
```

Figure 1.4 Alignment of conserved regions of NS3 from HCV and related viruses. NS3 represented as a bar with the relative position of the protease (shaded) and helicase shown. Conserved residues are shown in bold and the residues of the serine protease catalytic triad **H D S**, helicase motif I (Walker A) **G** (X) **G K** and helicase motif II (Walker B) **D E** (X) **H** are indicated. Sequences shown are; HCV type 1b (HCV-1b), HCV type 1a (HCV-1a), bovine viral diarrhoea virus (BVDV), hog cholera virus (HoCV), yellow fever virus (YFV), West Nile virus (WNV), dengue virus 2 (DV2), Japanese encephalitis virus (JEV).

NS3/4A Protease

By sequence comparison with related viruses the conserved residues His-1083, Asp-1107 and Ser-1165 were identified and these correspond to the catalytic triad of serine proteases (3,133). Amino acid mutations to these residues demonstrated that the NS3 serine protease was required for processing at the NS3/4A, 4A/4B, 4B/5A and 5A/5B using *in vitro* transcription and translation and transient mammalian expression systems (150-152,224). The 3/4A junction was shown to be processed rapidly by *cis* cleavage and the remaining sites cleaved more slowly in *trans* (150,231,232), with NS5A/B cleavage the most efficient (228,233).

N-terminal sequence analysis of NS3 processed polyprotein products identified the NS3 cleavage sites (224) and comparison with other HCV isolates identified highly conserved residues. For NS3 dependent *cis* cleavage, threonine was always found in the P1 position and serine in the P1' position. At *trans* cleavage sites cysteine was always present at the P1 site, and either serine or alanine at P1'. Using this data Grakoui *et al* proposed the consensus sequence D/E X X X X C_{cis}/T_{trans} - S/A for serine protease cleavage and this was confirmed by more extensive analysis of residues required for NS3 dependent cleavage (234-236). Residues at position P1 were shown to be the most critical substrate determinants and substrate requirements were more stringent at *trans* sites than the *cis* cleavage site. This suggested that *cis* cleavage is determined by polyprotein folding and *trans* cleavage by specific interactions at the scissile bond. In addition Pizzi *et al* built a model of the specificity pocket of NS3 based on known structures of serine proteases and predicted a preference for cysteine at position P1 for *trans* cleavage.

NS4a was shown to form a stable complex with the NS3 N-terminus (237-239) and was demonstrated to be essential for efficient NS3 mediated processing (231,232,240,241). This resembles the requirement of flavivirus and pestivirus NS3 for the viral cofactors NS2B and p10 respectively (227,242,243). Cleavage of the HCV polyprotein at NS4A/4B and NS4B/5A junctions was dependent on NS4A but cleavage at the NS5A/5B junction was efficient even in the absence of NS4A. An identical requirement for NS4A was observed for GBV-C (HGV) polyprotein processing (244). The minimum region of NS4A required for activity was amino acids 22 - 31 (of NS4A) encompassing a region of hydrophobic residues, in particular Ile-25 and Ile-29 (237,245-247); amino acids 15 - 22 of NS3 were necessary for NS4A binding (238,248). The NS3/4A protease was shown to be inhibited by chymotrypsin specific inhibitors but not trypsin-like protease inhibitors, suggesting that the NS3/4A protease is a chymotrypsin-like protease (249).

The minimal region of NS3 required for full protease activity was mapped to amino acids 1038 - 1206 (250). Use of truncated NS3 proteins encompassing this region has enabled the expression of the protease domain using prokaryotic and eukaryotic systems and development of *in vitro* cleavage assays which in turn made further analysis of NS3 protease activity possible (251-254). NS3/4A protease was shown to require glycerol, detergent (CHAPS), DTT, pH 7.5 - 8.5 and low ionic strength for optimal activity (255). The relative cleavage efficiency in terms of k_{cat}/K_m for the different cleavage sites was shown to be NS5A/B > NS4A/B > 4B/5A using peptides corresponding to the respective cleavage sites (255). Recombinant NS3 and peptides corresponding to the cleavage site and NS4A activating region were used to determine the kinetic parameters K_m and k_{cat}. In the absence of the NS4A cofactor, K_m varied

from 100 μM to 3900 μM and k_{cat} from 0.05 min^{-1} to 16 min^{-1} (255-258) depending on the cleavage substrate and region of NS3 protease assayed. Addition of NS4A peptide increased the cleavage efficiency by up to 100 fold by increasing substrate affinity and turnover (255-258). Similar results were observed using peptides corresponding to the active region of NS4A with full length NS3 (247,259) and with NS3 proteins expressed with full length NS4A fused at the C-terminus (260-262). With the latter constructs, processing of the NS3/4A junction was observed with full length NS3 (260,261) but not protease domain (262) indicating the structural importance of the C-terminal domain of NS3 for cleavage at this site. An NS3 protease construct with NS4A (aa 21 - 32) fused at the N-terminus of NS3 was expressed in *E. coli* and this protein showed a similar cleavage activity of the NS5A/B cleavage site to that of full length NS3/4A protein (263). The protease activities of full length NS3 and the NS3 protease domain expressed as a distinct protein were similar but NS4A stimulation of full length NS3 was greater than that of the protease domain (259).

Alignments of HCV NS3 amino acid sequence with NS3 of the related viruses GBV-A, GBV-B, and GBV-C and with 2A protease of picornavirus identified strictly conserved residues at C_{97}, C_{99}, C_{145} and H_{149} of HCV NS3 (223). Interestingly, mutation of these cysteine residues in HCV NS3 had previously been shown to reduce serine protease activity (148). These residues were proposed to compose a metal binding domain (223,264). Elemental analysis of purified NS3 protease domain revealed the presence of zinc, in an equimolar ratio to the protease, (264) which was shown to be tightly bound to NS3 (223). Zinc was demonstrated to be essential for correct folding of NS3 protease (223,264) and a structural model was proposed where the zinc ion is bound by the three cysteine residues and a histidine residue via a water molecule

(223,265). This binding domain defines a novel class of zinc binding chymotrypsin-like proteases which includes the NS3 proteases of HCV, GBV-A, GBV-B and GBV-C and the 2A protease of picornaviruses.

The crystal structure of the NS3 protease domain of HCV BK and H strains has been resolved in the presence and absence of NS4A (266-269). Kim *et al* demonstrated that NS3/4A adopts a chymotrypsin like fold, and that the structure of the catalytic triad was similar to other serine proteases with chymotrypsin like folds (267). This is also consistent with the observed inhibition of protease activity by chymotrypsin-like protease inhibitors (249). The specificity pocket is shallow and non-polar, formed from the side chains of invariant residues F_{154}, A_{157} and L_{135} as predicted by Pizzi *et al* (270). This phenylalanine residue is one of the main determinants of the shape and physiochemical characteristics of the S_1 pocket and is conserved in GBV-B NS3 which shares HCV NS3 cleavage site specificity (138). A zinc binding site, requiring residues C_{97}, C_{99}, C_{145} and H_{149} of NS3 was observed (266,267). The location of this site, away from the active site implies a structural role, possibly stabilising the active site (266,267). The N-terminal 30 amino acids extends away from the protease domain in the NS3 crystal structure (266) and provides the binding site for NS4A (267). Binding of NS4A improves stability of the active site, bringing the residues of the catalytic triad closer together and introduces a new substrate binding surface potentially affecting substrate residues immediately downstream of the cleavage site (269). The identification of NS4A dependent inhibitor peptides supported this observation. Landro *et al* proposed that NS4A was close to, or in direct contact with the P4' substrate position since removal of NS4A resulted in a loss of binding of inhibitors which extended to the P4'

position (271). This provides a structural basis for the improved substrate affinity and turnover observed in the presence of NS4A (255-258).

The NS2B/3 protease of flaviviruses, analogous to HCV NS3/4A protease is required for processing of the non-structural proteins but is also involved in the processing and maturation of the structural proteins (272-274). NS2B/3 cleaves at the C-terminus of core protein to produce the mature protein observed in the nucleocapsid and also upregulates signalase processing of the structural proteins. However there is currently no evidence of a similar role for HCV NS3/4A or the more closely related p80/p10 protease of pestiviruses.

NS3 Helicase/NTPase

DNA and RNA helicases are divided into three superfamilies (SF1, SF2 and SF3) based on sequence comparison and identification of conserved motifs (275,276). Viral RNA helicases are found in all three; SF1 contains the alphavirus-like proteins, SF2 includes the NS3-like proteins of flaviviruses and pestiviruses and SF3 includes the picornavirus-like proteins (275).

The C-terminal two thirds of NS3 contains the RNA helicase/NTPase domain (277-282) and the minimal domain required for functional HCV NS3 NTPase and helicase activities has been mapped to amino acids 1209 - 1608 (282). Amino acid motifs in this region relating to these activities indicate that NS3 helicase is a member of the DEXH subfamily of the DEAD box NTPase/RNA helicases (132,283,284). The DEAD box family of proteins belong to SF2 and are characterised by seven conserved motifs (275,285). Motifs I (GXGKS/T) and II (DEXX), also referred to as the A and B

motifs of the Walker box (286) are required for NTP binding and hydrolysis (287,288). Motif III (S/TAT) is thought to be important for dsRNA unwinding (288,289) and Motif VI (QRXGRXGR) is required for NTP hydrolysis and RNA unwinding (289,290). The functions of the other motifs (Ia, IV and V) have not been elucidated, but it is likely they contribute to the overall secondary protein structure. The DEAD-box family is further divided into three subfamilies of proteins that contain either a DEAD, DEAH or DEXH amino acid sequence at motif II (283,284). This family of proteins are found in prokaryotes and eukaryotes and perform a wide range of functions including mRNA stabilisation (291), RNA splicing (292), rRNA maturation and ribosome biogenesis (293,294) and transcription termination (295).

Several groups have demonstrated the RNA helicase (277,279,280) and NTPase (233,278,279,281) activities of NS3, which is active as a monomer (296) and has also been shown to catalyse the unwinding of dsDNA substrates (280,297). The ability to unwind both dsRNA and dsDNA substrates has been described for only three other helicases, simian virus 40 large T antigen (298) nuclear DNA helicase II (299) and vaccinia virus nucleoside triphosphate phosphohydrolase II (NPH II) (300).

An *E. coli* expressed protein corresponding to amino acids 1193 - 1657 of NS3 was used to characterise the helicase associated NTPase activity (278). NTPase activity was shown to be stimulated by polynucleotides. Both DNA and RNA polynucleotides were able to stimulate activity with poly(U) and poly(dU) showing greatest stimulation. Gwack *et al* showed that ssRNA > ssDNA > dsDNA for NTPase stimulation (297) and this is consistent with NS3 requiring a single strand region for binding nucleic acids (280). A variety of nucleoside triphosphates (NTPs) were hydrolysed by NS3 with ATP the preferred and GTP and dGTP the least preferred substrate (278). NTPase activity

was shown to be dependent on magnesium and optimal conditions were shown to be pH 6.5, 2.5 mM $MgCl_2$ and low ionic strength (278). HCV NS3 NTPase activity was remarkably similar to the homologous proteins of flaviviruses (301) and pestiviruses (302) except with respect to polynucleotide stimulation.

Using a strand displacement assay RNA (280) and DNA (297) helicase activity has been characterised. Tai *et al* demonstrated unwinding with RNA/RNA, RNA/DNA and DNA/DNA substrates (280). NS3 was able to bind 5' or 3' single stranded regions but was only able to unwind substrates containing a 3' single stranded region implying 3' to 5' directionality. ATP and divalent metal cations (Mg^{2+} or Mn^{2+}) were required for activity which was optimal at pH 6.5 - 7.0 and was drastically reduced above pH 7.5 and in the presence of monovalent cation. The characteristics of NS3 DNA helicase activity were similar to RNA helicase activity (297). In accordance with NTPase data (278) all nucleotides were able to provide energy for helicase activity with GTP and dGTP the poorest substrates (297). Kinetic studies of helicase activity demonstrated that the enzyme was shown to unwind with low processivity and the rate limiting step was release of product, consistent with a passive mechanism for strand separation (296).

The nucleic acid binding activity has been investigated by several groups. Consistent with the observed maximal NTPase stimulation with poly(U), NS3 shows the greatest affinity for poly(U) homoriboploymers (303,304), requiring between 7 and 20 nucleotides for binding (304). The serine protease domain was not required for binding activity, although it was shown that full length NS3 has a higher affinity for RNA than the helicase domain alone (303). A stable complex of NS3 with poly(U) was observed in the absence of ATP however, in the presence of excess ATP, poly(U) binding was not

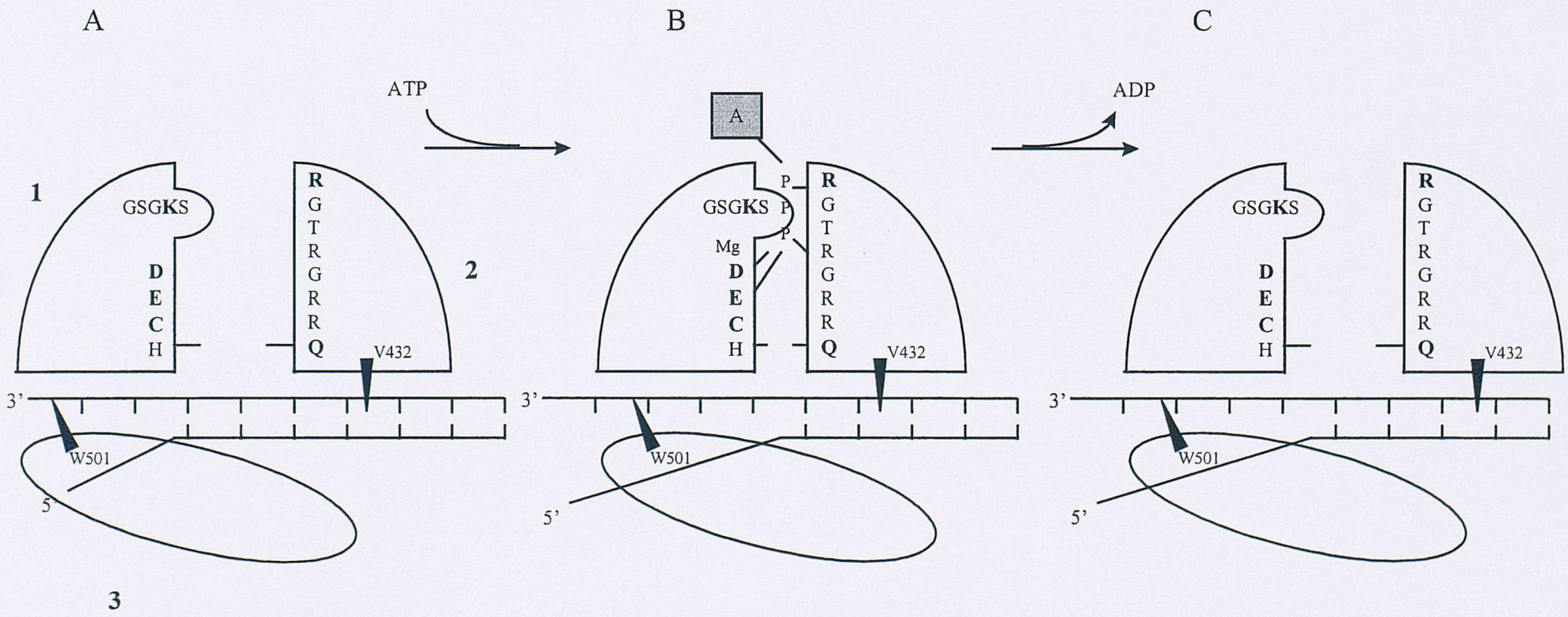
observed, suggesting that ATP is required for the release of nucleic acid from NS3 (259).

The structure of the NS3 helicase domain has been resolved by X-ray crystallography (305,306) and provides an insight into the mechanism of unwinding. Both studies identified a similar overall structure, with the helicase domain forming a triangular structure consisting of domains 1, 2 and 3 where domain 2 is flexible and able to rotate in relation to 1 and 3. However, Kim *et al* derived the structure of NS3 helicase bound to an oligonucleotide (oilgo(dU) 8'mer) (306) which was shown to be bound in a different orientation compared to that predicted by Yao *et al* (305). The former structure is consistent with proposed structure of Rep helicase (307) and with mutational analysis of conserved amino acid residues of HCV NS3 reported in this thesis and by Kim *et al* (289) and shall be discussed here.

All conserved residues of helicase superfamily SF2 are contained in domains 1 and 2 which are arranged in an adenylate kinase like fold, with a phosphate binding loop composed of helicase motif 1 in domain 1. Residues of motif I (G_{1233}SGKS) and II (D_{1316}ECH) reside in domain 1 and of motif VI (Q_{1486}RRGRTGR) in domain 2. These are arranged with the side chains of conserved residues facing into an interdomain cleft between domains 1 and 2 which is capable of opening and closing to bind ATP (**Figure 1.5**). Mutational analysis of HCV NS3 and related helicases suggest that the opening and closing of the domains may be controlled in part by interactions between His-1319 of motif II and Glu-1486 of motif VI which are sited at opposite sides of the interdomain cleft (288-290). A channel is formed between domains 1 and 2 and domain 3 which binds the oligonucleotide. Binding is via interactions between the phosphate



Figure 1.5. Diagrammatic representation of the NS3 helicase domain determined by X-ray crystallography showing proposed mechanism. A; RNA bound in a cleft formed between domains I and II and domain III with an interdomain cleft between I and II. B; Binding of ATP via the β-phosphate by a phosphate binding loop formed from helicase motif I (GSGKS) and the γ-phosphate with Mg^{2+} by motif II (DECH) closes the cleft and the ATP phosphates are bound by arginine residues of motif VI (QRRGRTGR). Domain II translocates along the bound RNA displacing ssRNA in a 3' to 5' direction with respect to the template (top) strand. C; ATP hydrolysis facilitates reopening of the cleft and translocation of domain II. Valine (V432) and tryptophan (W501) disrupt base stacking and favour 3' - 5' movement of the helicase. (Adapted from reference 304).


A
B
C
ATP
ADP
A
1
2
3
GSGKS
DECH
RGTRGRRQ
V432
P
Mg
3'
5'
W501

backbone and symmetrically equivalent residues formed from secondary structure elements of domains 1 and 2.

Displacement of RNA is likely to be a passive event as proposed by kinetic studies (296) caused by the opening and closing of the interdomain cleft driven by ATP hydrolysis and aided by the disruption of base stacking by residues V432 and W501 (**Figure 1.5**). The orientation of W501 favours movement of the polynucleotide in the 5' to 3' direction thus conferring 3' to 5' directionality for translocation of NS3. In this proposed mechanism translocation of NS3 and separation of RNA strands resembles a ratchet and a general ratchet-like mechanism has been proposed previously for the RecB helicase (308).

Further insight into the mechanism of NS3 action was provided by mutational analysis of the conserved motifs of NS3 (289,309). Mutations to motifs I and II, involved in NTP binding and hydrolysis, abolished or reduced ATPase activity and were associated with a concomitant loss of helicase activity, (289,309) indicating that NTP hydrolysis is essential for helicase activity. Mutations to motif VI, thought to be important for NTP hydrolysis and helicase activity, were also investigated and affected all activities of the NS3 helicase domain. Glu-1486 was shown to be required for NTP hydrolysis and helicase activity but mutation of this residue actually increased RNA binding (289). It was concluded that this residue was not involved in RNA binding and this confirms the observation of Gallinari *et al* that ATP hydrolysis is required for dissociation of bound nucleic acid (259). Arginine residues at positions 1490 and 1493 were shown to be required for NTPase and helicase activity but not RNA binding, and mutation to the other residues of this motif also had no or little effect on RNA binding (289).

Full Length NS3

Several groups have reported the isolation of full length NS3 (233,259,310) and full length NS3/4A complex (261,311,312) and the three activities of NS3 have been characterised using full length protein recovered from mammalian (311,312), insect (261) and bacterial (259) cells. Biochemical characteristics of the activities of full length NS3 are essentially the same as for the respective N- and C-terminal domains, though full length NS3 has a higher affinity for the NS4A protease activating domain (259). In addition Morgenstern *et al* (312) showed that full length NS3 required approximately ten fold less poly(U) for maximal ATPase stimulation compared with helicase domain alone (313) and it was proposed that this may be a consequence of an increased in affinity for poly(U) of full length NS3 over helicase domain (303).

Cleavage of dengue virus 2 NS3 has been observed in infected cells (314) and Shoji *et al* (315) have presented data showing internal cleavage of HCV NS3 when expressed in insect and mammalian cells. These cleavages were in conserved helicase motifs which would be expected to abrogate helicase and NTPase activity and therefore cleavage of these proteins in this way may reflect regulation of viral replication. However, HCV NS3 cleavage has not been confirmed in infected hepatocytes and so may not be an event in the normal virus life cycle.

Despite the apparent independence of the two enzymatic activities, there is no evidence to suggest that the corresponding domains of NS3 are cleaved during the natural life cycle of the virus. The multifunctional nature of NS3 may reflect economical packaging of essential viral proteins but may imply a functional interdependence

between the two domains. Expression and isolation of full length NS3 has enabled investigation of this. Using active full length NS3/4A isolated as a non-covalently associated complex from mammalian cells Morgenstern *et al* were able to demonstrate stimulation of protease activity up to five-fold by polynucleotides (312). Stimulation of protease activity by different polynucleotides showed similarities to the polynucleotide binding and NTPase stimulation described above, with poly(U) showing greatest stimulation. This suggests that polynucleotide binding by the NS3 helicase domain influences protease activity and shows that the two domains are not entirely distinct. However, Gallinari *et al* (259) showed polynucleotide inhibition of protease activity as a result of polynucleotide binding of a positively charged region at the protease substrate binding pocket. The contradicting observations of these two groups may be a result of the NS4A cofactor used, Morgenstern *et al* used full length NS4A isolated in association with NS3 whereas Gallinari *et al* used a peptide corresponding to amino acids 21 - 34 of NS4A aa supplied in *trans*. Interdependency between the helicase and protease domains was further indicated by inhibition of helicase activity by the NS4A peptide (aa 21 - 34) (259). This interdependency between the two domains of NS3 indicates a possible mechanism for switching between different conformations to allow control of viral replication.

NS3: A Role in Host Cell Control?

For successful virus replication it may be necessary for the virus to control host cell metabolism to ensure the production of viral proteins and genome replication and to avoid virus clearance by the host. Apoptosis of infected cells is a common pathway of

host clearance of viruses. Many viruses have evolved mechanisms to suppress apoptosis in an attempt to combat this method of viral clearance, for example the cowpox virus gene product CrmA which has been reported to inhibit the activity of interleukin-1β converting enzyme to inhibit Fas-, tumour necrosis factor (TNF)- and CTL-induced apoptosis (316,317). Suppression of apoptosis is also believed to be a prerequisite for tumour formation as demonstrated by human papillomavirus E6 protein (318). HCV NS3 has also been implicated in suppression of apoptosis which may play a role in the development of HCV associated HCC. Sakamuro *et al* (62) demonstrated transformation of NIH3T3 cells transfected with cDNA corresponding to the NS3 protease domain (aa 1020 - 1295) and showed that these cells were able to form tumours in nude mice. Also NIH3T3 cells constitutively expressing the N-terminal two thirds of NS3 (aa 1027 - 1459) encompassing the protease domain and part of the helicase domain showed resistance to actinomycin D-induced apoptosis and reduced expression of p53 in response to actinomycin D treatment (319). These data suggest that the N-terminal region of NS3 may suppress apoptosis and promote tumour formation in a p53 dependent pathway. Studies of the nuclear localisation of NS3 in the presence and absence of p53 supported this. Using the vaccinia virus-T7 hybrid expression system, wild type p53 was shown to enhance the nuclear localisation of full length NS3 and a C-terminally truncated NS3 (aa 1027 - 1459), but to have no effect on the localisation of an N-terminally truncated NS3 (aa 1202 - 1459) which had the protease domain deleted (65). Mutant p53 co-localised with all forms of NS3 in the cytoplasm and NS4A, which is proposed to localise NS3 to the ER membrane was only able to partially inhibit this nuclear accumulation (65). A similar study by Muramatsu *et al* (320) showed the same results.

NS3 has also been implicated in the inhibition of protein phosphorylation by cAMP-dependent protein kinase (PKA) (321,322). A number of viral proteins have been shown to interfere with intracellular protein phosphorylation, for example gp120 and gp160 of HIV and the HBV transactivator protein HBx (323-325), which may affect normal host cell biochemical processes. PKA is a serine/threonine kinase which is activated by the second messenger cAMP (326-328) and exists in the cell cytoplasm as a tetramer (R_2C_2) consisting of two catalytic subunits (C) and two regulatory subunits (R). cAMP released in response to ligand bound cell surface receptors, binds to the R subunits resulting in the dissociation of the holoenzyme into an R dimer and two active C subunits. The C subunits are then able to migrate to the nucleus for phosphorylation of chromatin associated proteins to regulate gene expression and cell growth (329). In addition to nuclear functions, PKA also regulates other cellular processes such as cytoskeletal motility and secretion (330,331). The regulatory subunit inactivates the catalytic subunit by binding to the active site of the catalytic subunit via an amino acid sequence similar to the substrate recognition sequence of the catalytic subunit. Two types of R subunit exist, R^I and R^{II} which differ at this recognition sequence (327) and Walsh *et al* identified a heat stable PKA inhibitor (PKI) which contains a sequence resembling the R^{II} recognition sequence (332). Analysis of the NS3 amino acid sequence identified a stretch of 14 amino acids which was similar to the recognition site of R^{II} and PKI (**Figure 1.6**) (321). A 43.5 kDa fragment of NS3 (aa 1189-1525) encompassing this region and short synthetic polypeptides corresponding to the 14 amino acid sequence were able to inhibit PKA activity (321). In addition, NS3 (aa 1189 - 1525) introduced into Hep-2 cells blocked forskolin induced translocation of PKA to the nucleus and was associated with a reduction of histone phosphorylation (322). This

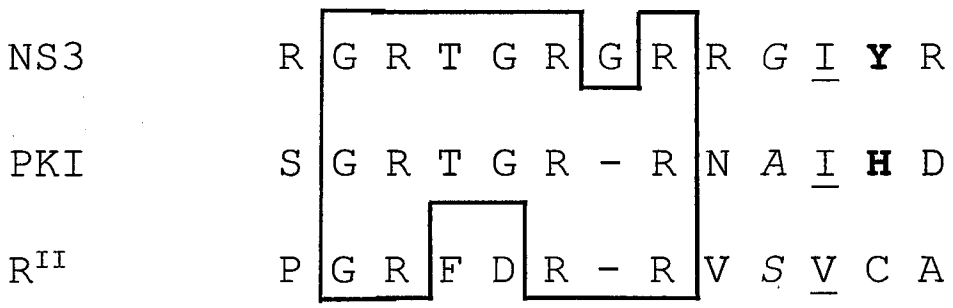


Figure 1.6 Alignment of HCV NS3 amino acid residues 1488-1500 with PKI and the type II regulatory subunit of PKA (R^{II}). Identical residues are shown in a box, residues in italics are small neutral amino acids, underlined residues represent hydrophobic amino acids and ring containing amino acids are shown in bold. Diagram adapted from reference (321).

suggests that NS3 or NS3 derived peptides may influence an important signal transduction pathway in hepatocytes, which may have implications for the mechanisms of viral persistence and the pathogenesis of HCV infections. Interference of cellular signalling pathways has been described for other viral proteins, for example bovine papillomavirus type 1 E5 oncoprotein permanently activates the platelet-derived growth factor receptor (333) and vaccinia virus growth factor activates the epidermal growth factor receptor (334). It is therefore conceivable that HCV viral proteins, including NS3 may affect cellular signalling.

1.7.3 NS4 - NS4A and NS4B

NS4 is released from the viral polyprotein by NS3 protease to yield NS4A (6 kDa) and NS4B (27 kDa). As mentioned above (section 1.7.3), NS4A forms a stable complex with NS3 and is essential for full activity of NS3 serine protease. In this respect NS4A is a functional analogue of flavivirus NS2B (242,243), and pestivirus p10 (227) proteins. NS4A of GBV-C (HGV) also performs the same function (335). The minimal region of NS4A required for NS3 association and protease activity has been mapped to amino acids 22 - 31 (237,245-247). However, a higher catalytic efficiency was observed for full length NS3/4A - with respect to protease activity - than for the NS3 protease domain or full length NS3 with an NS4A peptide (247,312). This suggests that full length NS4A is required for optimal protease activity.

NS4A also acts to stabilise NS3 and anchor the protein to the ER membrane (143,336). In addition NS4A has been shown to complex with NS5B (337) and so may be responsible for localising the entire viral replication machinery to the ER membrane.

NS4A appears to influence the phosphorylation state of NS5A (338) possibly playing a role in controlling the activity of NS5A, though the significance of this is as yet unclear. There is currently no ascribed function for the NS4B protein.

1.7.4 NS5 - NS5A and NS5B

HCV is more like pestiviruses than flaviviruses with respect to NS5. HCV NS5 is cleaved by NS3 to produce NS5A and NS5B (142,144) in a manner analogous to pestiviruses (339) and in contrast to flaviviruses which possess a single NS5 protein (105). In addition flavivirus NS5 is proposed to possess methyl transferase activity (340) which is absent in HCV and pestivirus NS5 proteins.

NS5A has been located to the cytoplasmic membrane around the nucleus suggestive of a role in the viral replication complex (341) and is observed as a 56 kDa or a 58 kDa protein (143). This size difference was originally thought to be a result of a further cleavage event but was subsequently shown to be due to a difference in levels of phosphorylation (338). Phosphorylation of NS5A is at serine and threonine residues (338,342,343) between amino acids 2200 - 2250 and at a C-terminal region for basal level phosphorylation (56 kDa NS5A) (341). Hyperphosphorylation (58 kDa NS5A) is dependent on serine residues at positions 2197, 2201 and 2204 (341) and is enhanced by NS4A (336,338). Enhancement of phosphorylation by NS4A requires association of NS4A with NS5A at amino acids 2135 - 2139 located at the N-terminal region of NS5A (344). NS5A is phosphorylated by an uncharacterised serine/threonine kinase which associates with NS5A *in vivo* (NS5A-associated kinase) (342,343,345) and also *in vitro* by PKA (342). The later observation is interesting since HCV NS3 has been shown to

inhibit PKA action (321). Inhibition studies of the NS5A associated kinase were consistent with a kinase activity belonging to the CMGC group of serine/threonine kinases (343). This pattern of phosphorylation exists in diverse HCV isolates (338,343) and flavi- and pestiviruses (345) suggesting that phosphorylation of NS5A or association of NS5A with kinases may be important in the life cycle these viruses. The significance of the differently phosphorylated forms of NS5A is not clear but may affect subcellular localisation. For example, hyperphosphorylated NS5 of the flavivirus Dengue type 2 has been shown to localise in the nucleus instead whereas hypophosphorylated NS5 remains in the cytoplasm and associates with NS3 (346). Phosphorylation may alter the subcellular localisation of HCV NS5A and regulate the role of NS5A in viral replication. This is not without precedent since transcriptional activation and thus regulation of viral replication of the rhabdoviruses and paramyxoviruses, both RNA viruses, by phosphorylation has been demonstrated (347,348).

NS5A may also interfere with the host interferon response. Enomoto *et al* suggested that interferon (IFN) sensitivity correlated with mutations within a discrete region of NS5A (2209 - 2248) which was termed the IFN sensitivity determining region (ISDR) (169,170). However, this observation has been restricted to Japanese HCV isolates (349-352) and it has been suggested that regions other than the ISDR may be involved in IFN sensitivity (349). Subsequent studies have demonstrated that NS5A inhibits the action of the IFN-induced double stranded RNA-activated protein kinase (PKR) (353-355) thus providing a mechanism for the disruption of the IFN response. NS5A binds to PKR via aa 2209 - 2274 which consists of the ISDR and an additional 26 amino acids at the carboxyl side of the ISDR (353,355). Gale *et al* proposed a

mechanism for NS5A inhibition of PKR function where NS5A binds PKR at amino acids 244 - 296 of PKR, disrupting dimerisation and repressing PKR function (355). This would prevent PKR from phosphorylating eIF-2α and thereby result in the removal of the PKR imposed block on mRNA translation and viral replication. In addition to its antiviral properties PKR also plays a role in signal transduction pathways leading to apoptosis (356). Several studies have implicated PKR as a tumour suppressor (357) and inhibitors of PKR can induce malignant transformation (358). Therefore NS5A may be involved in suppression of apoptosis and the development of HCC.

Several groups have demonstrated regions of NS5A to be transcriptional activators using yeast and mammalian cell systems (359-361), and this activity is enhanced by mutations to the ISDR that are related to increased interferon sensitivity (361). This suggests another role by which NS5A may modulate cellular responses to interferon and NS5A may have other effects on host cell metabolism or viral replication.

NS5B is a 65 kDa protein which has been demonstrated to be phosphorylated *in vivo* and located predominantly in the perinuclear region associated with the nuclear membrane, ER or Golgi (362). The association of NS5B with membranes may be affected by its phosphorylation state (362). NS5B has primary sequence motifs, for example the GDD motif, which are conserved among all viral RNA-dependent polymerases (3,132) and RNA-dependent RNA polymerase activity of NS5B has been demonstrated using recombinant protein produced in *E. coli* and insect cells (363-365). An associated terminal transferase activity was also demonstrated, though this was believed to be due to a contaminating cellular protein (364,366). Initiation of polymerisation is likely to proceed by a copy-back mechanism which may be facilitated by terminal transferase activity, though can proceed using exogenous RNA or DNA

primers *in vitro* (364,366). The specificity of NS5B for different RNAs was studied by binding assays (366). Preference of binding to RNA homopolymers was in the order poly(U), (G), (A), (C), however the inverse was noted with respect to polymerase activity implying that tight binding inhibits processivity. NS5B showed no preference for binding HCV 3' UTR sequences. The NS5B-mediated reaction was not dependent on RNAs containing HCV specific sequences (364,366) suggesting other proteins of virus or host origin may be required to direct RNA synthesis to viral templates. The presence of HCV non-structural proteins has been shown to increase the relative activity of NS5B (364) and so may be required for efficient and targeted polymerase activity. In support of this Ishido *et al* demonstrated the formation of an NS5B/NS3/NS4A complex using the vaccinia virus T7 hybrid expression system in HeLa cells and a non T7 based expression system in COS-7 cells (337). Moreover, complexes of NS3, NS4A and NS4B (367), and NS4A and NS5A (344) have been observed suggesting the formation of a replication complex which may be anchored to the ER membrane via interactions with NS2 and the structural proteins (203,206). The non-structural proteins of the flavivirus, Japanese encephalitis virus, have also been proposed to form a similar replication complex (368).

1.8 5' and 3' Untranslated Regions

The HCV ORF is preceded by an untranslated region (5' UTR) of 341 nucleotides (2,132-134,335,369,370) though Trowbridge *et al* recently reported the presence of 8 additional nucleotides at the 5' end (371). The 5' UTR is the most highly conserved region of the genome (132,134,155) sharing 90% sequence identity between

81 HCV isolates (112) and this has enabled the development of protocols for the detection of HCV RNA (110,112) and HCV genotyping (372,373) using PCR and restriction analysis. This region also shares substantial homology with corresponding regions of other viral genomes and has 50% homology with that of pestiviruses (112,132,134). This conservation suggests an important role for the 5' UTR and it has been demonstrated to form extensive secondary structure comprising an internal ribosome entry site (IRES) which is essential for translation of the HCV polyprotein (374-376). A similar element is present in the 5' UTR of pestiviruses (377,378), has been predicted for HGV (379) and has been described previously for other positive strand RNA viruses such as picornaviruses (380). This element is absent in flavivirus 5' UTRs where translation of the flaviviral polyprotein is by a cap-dependent mechanism (381,382). The ability of the IRES to translate the ORF varies between HCV strains (374,383,384), and IRES directed translation efficiency is dependent on both IRES nucleotide sequence and secondary structure (384,385). The 5' UTR forms four stem-loop structures (I, II, III and IV) which comprise the IRES (386). Mapping studies have shown the region required for active IRES function to extend from nucleotides 30 to 370, incorporating at least 29 nucleotides from the core protein coding region (386-389), and that the 5' stem loop structure (nt 5 - 20) is not essential for activity (387,388). Fukushi *et al* showed a reduction in translation efficiency following deletion of nt 1 - 20 which removed the 5' stem loop of the UTR (386) whilst deletion of nt 5 - 20 actually increased translation efficiency (387). The authors suggested that nucleotides 1 - 4 may stabilise IRES structure or the HCV transcripts. The 29 nucleotides of core coding region are believed to contribute to the structure of stem loop IV. Sequence analysis has indicated a high level of sequence conservation at the 5' end of the coding sequence of

core protein (112,372,390) and the involvement of the region in the secondary structure of the IRES may provide an explanation for this. Mutation studies of the 5' UTR indicate the presence of a conserved helical element and pseudoknot structure required for IRES activity and that maintenance of these structures, rather than the conservation of the precise nucleotide sequence is more important for activity (391,392).

The initiator AUG is located within stem loop IV of the UTR (393) downstream of the proposed pseudoknot structure (392). Mutation of the initiator AUG at position 341 to AUU or CUG had little effect on translation initiation (388) and the insertion of an additional AUG 8 nucleotides either side of the authentic AUG did not affect correct recognition by ribosomes (394). The IRES therefore is able to accurately direct ribosome binding to the authentic AUG for initiation of translation. Other factors are likely to be required for this activity and the HCV IRES has been shown to interact with host factors including polypyrimidine tract-binding protein (PTB) (395), eukaryotic translation initiation factor 3 (eIF3) (385,396), La antigen (397) and a 25 kDa protein of HeLa cells (398). PTB and PTB-associated factors (395) and La antigen (399) were shown to be required for translation initiation. Das *et al* (399) demonstrated that binding of the UTR by La antigen was inhibited by a small yeast RNA - which had been shown previously to inhibit poliovirus IRES-mediated translation - with a concomitant inhibition of HCV IRES-mediated translation. Binding of eIF3 has been proposed to enhance binding of the 5' UTR to 40S ribosomal subunits in an orientation that promotes entry of the initiation codon into the ribosomal P site (396).

The 3' UTR is located down stream of the stop codon (2,133,335,369). It is composed of four sequence elements comprising, from 5' to 3', a highly variable region of approximately 40 nucleotides, a poly(U) tract and polypyrimidine tract composed of

mainly U residues interspersed with C residues which varies in length from 30 - 90 nucleotides and a 98 residue sequence designated the X tail (141,400,401). The X tail is highly conserved among different HCV genotypes and is not found in any other viral or human sequences (141,401).

The function of the 3' UTR is unclear but in a study of chimpanzees infected with infectious HCV cDNA clones, the poly(U) tract, polypyrimidine tract and X-tail appeared to be critical for *in vivo* infectivity of HCV (402). The 3' UTR may play a role in genome stability in the absence of a poly(A) tail or may be involved in genome replication and packaging. In some plant viral positive strand RNA genomes that do not have a poly(A) tail the 3' UTR has been shown to provide RNA stability and be important for replication and translation (403). The 3' UTR of HCV is predicted to form secondary structure elements including three stem loops (404). In the 3' UTR of picornaviruses, interaction between similar structures are required for initiation of negative strand synthesis (405,406). Both NS3 and NS5B have been shown to bind preferentially to the poly(U) homopolymers (303,304,366) and the analogous proteins of flaviviruses have been shown to interact with the 3' UTR (368). It is therefore possible that the poly(U) region of the 3' UTR provides a landing pad for the formation of a complex for initiation of RNA replication. HCV RNAs containing an X tail were translated with up to 5 times greater efficiency than corresponding RNAs lacking an X tail (407) and so the 3' UTR may also influence translation efficiency.

The 3' UTR has been shown to interact with cellular proteins including heterogeneous nuclear ribonucleoprotein L (hnRNP L) (408), proteins of 87 and 130 kDa found in a variety of human cell lines and in human hepatocellular carcinoma tissues (409) and polypyrimidine tract-binding protein (PTB) (404,407) which also

binds the 5' UTR (395). The significance of binding by these proteins is unclear, however, interactions between the 5' and 3' UTRs of vesticular stomatis virus (VSV) is important for encapsidation and replication of genomes (410). It is possible that PTB binding may bring the 5' and 3' UTRs of HCV together and have a similar effect. Binding of the 3' UTR by other cellular proteins may have a further influence on HCV genome replication and translation.

1.9 Replication and Cell Culture of HCV

HCV replication, demonstrated by the presence of the replicative form of viral RNA (negative strand) has been shown to occur in the liver of infected individuals (411,412). Several groups have also detected viral RNA in peripheral blood mononuclear cells (PBMCs) and proposed that this could be a potential extrahepatic site for HCV replication (413,414). Detection of negative strand RNA by strand specific PCR and therefore viral replication in PBMCs apparently confirmed this (414). However, doubts have been raised over the specificity of this technique (38) and reports which used protocols with a greater degree of strand specificity have contradicted these results (36,37,411). Using a thermostable reverse transcriptase (rTth) in RT-PCR, negative strand HCV RNA was detected in hepatocytes but not in PBMCs or other extrahepatic site (411). Similar results were observed by Gunji *et al* (37) who employed a strategy of RT-PCR combined with RNA modification at the 3' end to aid strand specific detection and by Mellor *et al* (36). These results indicate that the liver is the sole site of HCV replication and that viral RNA detected in PBMCs may be a result of phagocytic uptake of HCV by these cells.

Infection of primary chimpanzee hepatocytes maintained in culture has been reported and negative strand HCV RNA was detected at 4 days post infection and up to 25 days after infection, indicative of virus replication (415). This was not observed in baboon hepatocytes where residual positive strand RNA was detectable for only 11 days post infection. HCV replication has also been demonstrated in primary hepatocytes from chronically infected HCV patients by the presence of negative strand RNA (412), in primary hepatocyte culture from uninfected liver (416) and in human foetal liver cells (417).

HCV replication has been reported in a number of hepatic and non-hepatic cell culture systems. Yoo *et al* claimed replication and production of infectious virus in the hepatoma cell line Huh-7 using RNA transcripts derived from a HCV cDNA clone (418). However this lacked the X-tail component of the 3' UTR which is likely to be essential for virus replication. Seipp *et al* reported the establishment of persistent hepatitis C virus infection and replication also using Huh-7 cells enhanced by stimulation of LDL-receptor expression (419). Ikeda *et al* reported the production of a clonal cell line derived from PH5CH hepatocyte cell line (420), replication was maintained for up to 100 days at 32°C and alpha-IFN had antiviral effects on the HCV infected cells. Virus-like particles were detected by electron microscopy in cytoplasmic vesicles and the culture supernatant of HCV infected HepG2 cells (421). This culture supernatant was demonstrated to be infectious for Daudi cells (a human B-cell line). Long term culture of HCV has been reported in human lymphocyte cell lines. Viral RNA was detected in Daudi cells and HPBMa10-2 (a human T-cell line) up to one year after infection (422), in the human T cell leukaemia virus type 1 infected cell clone MT-2C (423,424) and in the MOLT-4 cell line, another human T-cell line (34).

Sequence analysis of HCV clones isolated from culture supernatants by RT-PCR suggest that particular HCV quasispecies adapt to different cell types and culture conditions (32,425,426). Therefore the demonstration of long term culture of HCV may be with virus species different to those found in an infected individual and these may not provide an authentic model for HCV infection. However, as mentioned previously (this section) evidence of HCV replication based on the detection of negative strand RNA should be interpreted with care.

Experimental infection of chimpanzees using *in vitro* transcribed full length HCV RNA has been reported by two groups (427,428). This was done using a consensus sequence derived from HCV H strain (type 1a), constructed to overcome the highly variable quasispecies nature of the virus and potential errors arising as a result of the RT-PCR cloning strategy employed in the generation of HCV clones (429). In addition these experiments provided conclusive proof that HCV is the sole causative agent of the observed disease, that no additional 5' or 3' sequences to those already characterised are required for infectivity and that variations in the poly(U/UC) tract of the 3' UTR are tolerated (427).

The absence of reliable cell culture systems will retard development of suitable assays for testing antiviral therapeutics. To overcome this chimeric viruses dependent on HCV NS3 protease activity for virus replication have been developed (430,431). The NS3 protease coding region upstream of an NS3 specific cleavage site was inserted into full length poliovirus (431) or sindbis virus (430) genomes upstream of the ORF such that NS3 protease action was required to release NS3 from the viral polyprotein. Removal of NS3 from the polyprotein was required for viral replication, therefore only chimeras containing active NS3 were viable. These systems provide novel assays for

testing putative NS3 serine protease inhibitors in cell culture, but does not remove the need for a cell culture system since it lacks other HCV proteins which interact with HCV NS3 such as NS4A.

1.10 Expression of Recombinant Proteins

1.10.1 Expression in *E. coli* - the T7-based expression system

The techniques for the expression of foreign genes in *E. coli* have in recent years become highly efficient as the underlying processes behind the system have been elucidated. Several promoters have been used to overexpress foreign proteins in *E. coli* and one of the most successful polymerase-promoter coupling systems is that of the highly active RNA polymerase of bacteriophage T7. A series of gene expression vectors have been developed, termed the pET system (Novagen) (432), which contain the T7 RNA polymerase promoter upstream of an artificial multiple cloning site (MCS) containing unique sites for several restriction enzymes. A second system based on the pET system, the pRSET system (Invitrogen) (433) has also been developed and both systems were used in the work presented here. The DNA sequence which encodes the protein of interest is cloned into the MCS of the expression vector and is transcribed in the presence of T7 polymerase. For expression in *E. coli*, the polymerase may be supplied using a second plasmid containing the T7 polymerase gene or the expression vector may be used to transform an *E. coli* strain which is lysogenic for a λ phage bearing the T7 polymerase gene. In each case the T7 polymerase gene is under the control of an IPTG inducible promoter (e.g. *lacUV5*) and in the presence of IPTG the foreign DNA is transcribed (432,433).

Expression vectors have been adapted in many ways to facilitate expression and purification of recombinant proteins. In the pET system expression vectors, the target DNA is inserted under the control of the T7*lac* hybrid promoter (434). The vectors also carry a copy of the *lacI* gene which provides enough *lac* repressor to repress both the T7*lac* promoter and transcription of the T7 polymerase under the control of the *lac*UV5 promoter. This allows tight regulation of target DNA expression which is especially useful when expressing proteins which may be toxic. Addition of T7 lysozyme can repress expression even further and the presence of lysozyme has the additional benefit of facilitating cell lysis during protein extraction (435). The pET vectors also enable N- or C- terminal fusion of peptide tags such as a ten histidine peptide tag to allow rapid purification of the target protein under native or denatured conditions and identification of target proteins with anti-histidine tag antibodies. The pRSET vectors have similar features (433).

1.10.2 Expression in Insect Cells - the Baculovirus Expression System

The baculovirus expression system is a eukaryotic alternative to *E. coli* for expression of recombinant proteins and is reviewed in (436). The baculovirus *Autographa californica* mononuclear polyhedrosis virus (AcMNPV) maintained in a *Spodoptera frugiperda* cell line Sf9 was used in the work presented here.

AcMNPV has a genome of approximately 133 kbp and this large size makes standard DNA manipulation using restriction endonucleases very difficult. To introduce target DNA into the virus genome it must first be cloned into a transfer vector. This vector contains sequences which are homologous to a region of the virus' polyhedrin

gene flanking the multiple cloning site. After cotransfection of insect cells with both the virus genome and transfer vector the target DNA is introduced into the genome by homologous recombination with the viral polyhedrin coding sequences. Polyhedrin is a non-essential protein produced by AcMNPV late in infection at very high levels and the introduction of target DNA in place of polyhedrin under the control of its strong promoter allows high levels of expression of the target protein.

In wild type AcMNPV polyhedrin surrounds the nucleocapsid forming occlusion bodies (Occ^+ phenotype) which are visible under light microscopy, recombinant virus does not produce these occlusion bodies (Occ^-), enabling identification. However, identification of recombinant viruses in this way is time consuming and so commercial kits are available where the AcMNPV genome has been manipulated to aid the recovery of recombinant viruses. For example, addition of the *lacZ* gene to the viral genome at the site of recombination allows identification of recombinants. In the presence of X-gal, non-recombinant baculoviruses produce blue plaques, whilst white plaques are produced by recombinant baculoviruses where the target DNA sequence has replaced *lacZ* by recombination. Another significant advance has been in the use of lethal deletion virus mutants which can only be rescued by recombination with the transfer vector and rarely result in false recombinant viruses.

The baculovirus system has many advantages over bacterial expression. The strong promoters used (polyhedrin and p10) allow expression of high levels of protein, the AcMNPV genome will tolerate large insertions without affecting packaging and proteins are glycosylated to an extent. In addition the virus can only replicate in invertebrates and so is safe for the user. However, the lytic nature of the virus results in host cell death and therefore expression is only transient and glycosylation of proteins is

often incorrect, since the sugar residues added often form short unbranched side chains with a high mannose content.

This system has been used to express a variety of proteins, including proteins of hepatitis C virus such as NS3 (241,253,281).

1.11 Aims

At the beginning of this research project and until recently the majority of NS3 studies were performed using proteins corresponding to either the N-terminal protease domain or the C-terminal helicase domain and not full length protein. However, there is no evidence to suggest that these NS3 domains are separated into active domains in HCV infected cells. The presence of two distinct catalytic activities in a single protein may reflect economical packaging of essential viral replicative components or may suggest that there is a functional interdependence between the two domains. Recent studies of full length NS3 have suggested that the latter may be true.

The aims of this study are twofold; to characterise the NTPase and helicase activities of full length NS3 protein and to investigate putative interactions of this protein with host cell proteins. The NTPase and helicase activities of recombinant NS3 protein will be studied *in vitro* and compared to the published characteristics of the NS3 helicase domain. The effects of amino acid substitutions at the conserved motifs of the DEAD-box family of proteins will be investigated. NS3 interactions with host cell proteins will be studied by analysing the ability of NS3 to inhibit PKA, and the ability of different HCV genotypes to inhibit PKA will also be evaluated. This work will

contribute to the understanding of the role of NS3 in the life cycle of the virus and in host cell control and disease pathogenesis.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Buffers and Solutions

The reagents used for the preparation of buffers and solutions were purchased from MERCK or Sigma except where stated. All solutions were prepared with Milli Q water unless otherwise stated.

30% Acrylamide bis (37.5:1)	29.2% (w/v) acrylamide, 0.8% (w/v) N-N'-methylene bisacrylamide, stored at 4°C. Severn Biotech
40% Acrylamide bis (19:1)	38% (w/v) acrylamide, 2% (w/v) N-N'-methylene bisacrylamide, stored at 4°C. Severn Biotech
Agarose sample buffer (10X)	0.4% (w/v) bromophenol blue, 0.4% (w/v xylene cyanol FF, 40% (w/v) sucrose in Milli Q water
AM/MG reagent	3 volumes of malachite green stock mixed with 1 volume ammonium molybdate stock, stirred for 30 minutes then filtered through 4 layers Whatman no. 1 paper. Stable for two weeks at room temperature. 100 µl 2% (v/v) Tween 20

	added per 5 ml immediately prior to use
Amido black stain	0.1% (w/v) amido black, 25% (v/v) isopropanol, 1 % (v/v) acetic acid
Ammonium molybdate stock	4.2% (w/v) ammonium molybdate in 4M HCl
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system, Sigma
Block buffer	5% (w/v) dried skimmed milk (Marvel) in PBS plus 0.05% (v/v) Tween 20
Complete™ Protease Inhibitor Cocktail	1 tablet dissolved in 2 ml Milli Q water to give a 25 X stock. Boehringer Mannheim.
Coomassie Blue stain	10% (v/v) acetic acid, 10% (v/v) isopropanol, 0.1% (w/v) Coomassie Brilliant Blue R
Denaturing solution	1.5 M NaCl, 0.5 M NaOH
Destain 1	20% (v/v) isopropanol, 10% (v/v) acetic acid
Destain 2	10% (v/v) isopropanol, 10% (v/v) acetic acid
DNA bandshift buffer (1X)	25 mM Hepes, pH 7.5, 10% glycerol, 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 0.2 mM EDTA, 0.5 mg/ml BSA
Ethidium bromide stock	15 mg/ml ethidium bromide in sterile

	Milli Q water
Fixer (sequencing gels)	10% (v/v) ethanol, 10% (v/v) acetic acid
Glycogen stock	20 mg/ml (Boehringer Mannheim)
HRPL substrate	Chemiluminescent substrates A and B (National Diagnostics) mixed 1:1 immediately prior to use
IPTG stock	1 M IPTG, filter sterilised prior to storage in 1 ml aliquots at -20°C
Lysis buffer; DNA extraction	0.1 M Tris-HCl pH 7.5, 0.1M EDTA, 0.2 M KCl
Lysis buffer; protein extraction	25 mM Tris-HCl pH 7.2, 50 mM NaCl, 0.1 % (v/v) Triton X-100, 1mM DTT, 1 mM EDTA
Lysozyme stock solution	50 mM Tris-HCl, pH 8.0, 10 mg/ml lysozyme
Lytic mix	50 mM Tris-HCl pH 8.0, 60 mM EDTA, 1% (w/v) Brij-58, 0.4% (w/v) sodium deoxycholate. Autoclave to sterilise
Malachite green stock	0.045% (w/v) malachite green hydrochloride in Milli Q water
Milli Q water	Purified and de-ionised water prepared using an Elga Option 3 water purifier and Milli Q Organex-Q system.
Miniprep solution I	25 mM Tris-HCl pH 8.0, 50 mM glucose,

	10 mM EDTA. Autoclaved prior to use and stored at 4°C
Miniprep solution II	0.2 M NaOH, 1% SDS. Prepared fresh immediately prior to use
Miniprep solution III	3 M sodium acetate (pH 4.8)
PBS	Prepared by dissolving 1 PBS tablet (Sigma) per 200 ml Milli Q water. Autoclave to sterilise
PCR dNTPs (10X stock)	5 mM each of dATP, dTTP, dCTP, dGTP, stored at -20°C
Phenol	Purchased ready saturated with water from Appligene and stored at +4°C
PMSF (1000X)	100 mM PMSF prepared in isopropanol
Protogel	30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide (37.5:1) (National Diagnostics).
Protogel buffer	1.5 M Tris-HCl, pH 8.8, 0.384% SDS (National Diagnostics).
Protogel stacking buffer	125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS (National Diagnostics).
SDS-PAGE running buffer (10X)	250 mM Tris, 1.92 M glycine, 1% (w/v) SDS (National Diagnostics)
SDS-PAGE sample buffer (non reducing, 4X)	250 mM Tris-HCl, pH 6.8, 40% (v/v) glycerol, 8% (w/v) SDS, 0.01%

	bromophenol blue
SDS-PAGE sample buffer (reducing, 4X)	As above but with 20% (v/v) β-mercaptoethanol
SequaGel buffer	1 M Tris, 1 M boric acid, 20 mM EDTA 8.3 M urea (National Diagnostics).
SequaGel concentrate	23.75% (w/v) acrylamide, 1.25% (w/v) bisacrylamide, 8.3 M urea (National Diagnostics).
SequaGel diluent	8.3 M urea (National Diagnostics)
STETL buffer	50 mM Tris-HCl, pH 8.0, 50 mM EDTA, 8% (w/v) sucrose, 5% (v/v) Triton-X-100. Autoclaved to sterilise. Lysozyme added to 1 mg/ml immediately prior to use
TAE buffer (50X)	2 M Tris, 50 mM EDTA, 5.71% (v/v) glacial acetic acid
TBE buffer (10X)	0.9 M Tris, 0.9 M boric acid, 25 mM EDTA
TE buffer	10 mM Tris-HCl pH 7.5, 1 mM EDTA
Tris sucrose	50 mM Tris-HCl, pH 8.0, 25% (w/v) sucrose sterilised by autoclaving.
Western blot transfer buffer	25 mM Tris, 192 mM glycine,20% (v/v) methanol
Western blot wash buffer	0.05% Tween 20 in PBS

2.1.2 *E. coli* Growth Media

Agar	Bacto-agar added to 2% (w/v) to required media
Luria broth (LB)	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl. Autoclaved to sterilise
Super Broth (SB)	2.2% (w/v) bacto-tryptone, 1.5% (w/v) yeast extract, 0.8% NaCl, 0.2% (w/v) Na_2HPO_4, 0.1% (w/v) NaH_2PO_4. Autoclaved to sterilise. 0.2% (w/v) glucose (sterile filtered) added after autoclaving.
SOB broth	2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 2.5 mM KCl. Autoclaved to sterilise
SOC broth	Sterile filtered glucose added to SOB broth to 20 mM
2YT	1.6% (w/v) bacto-tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl. Autoclaved to sterilise

2.1.3 Cell Culture Growth Media

TC100, SFM900II, RPMI 1640 and media supplements (FCS, glutamine, pluronic F68, penicillin and streptomycin) were supplied by Life Technologies. Sf900 SFM is a complete serum free media for insect cell culture. TC100 insect cell culture media was supplemented with 10% (v/v) foetal calf serum (FCS) 0.1 mM L-glutamine and 0.01% (w/v) Pluronic F68. HepG2 cells were maintained in RPMI 1640 growth media supplemented with 2 mM L-glutamine, 10% FCS, 100 International Units/ml penicillin and 100 μg/ml streptomycin. Inactivation of FCS was achieved by incubation at 56°C for 60 minutes.

2.1.4 *E. coli* Strains

XL-1 Blue MRF'[1]	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F' *proAB* lacI^{q}ZΔM15 Tn10 (Tet^r)]
BL21(DE3)[2]	B F$^-$ *dcm ompT hsdS* (r_B- m_B-) *gal* λ(DE3)
BL21(DE3) pLysS[2]	B F$^-$ *dcm ompT hsdS* (r_B- m_B-) *gal* λ(DE3) [pLysS Cam^r]
HMS174(DE3)[2]	F$^-$ *recA1 hsdR*(rK12$^-$ mK12$^+$) Rif R λ(DE3)
JM109(DE3)[3]	*endA1 rec*A1 *gyr*A96 *thi hsd*R17 (r_k^- m_k^+) *relA1 supE44 λ- Δ(lac-proAB)* [F' *tra*D36 *pro*AB *lac*I^{q}ZΔM15] λDE3

TOP10F'[4]	F'[*lac*I^q *Tn*10(Tet^R)] *mcr*A *Δ(mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G.

Source; [1]Stratagene, [2]Novagen, [3]Promega, [4]Invitrogen.

2.1.5 Viruses

The recombinant T7 vaccinia virus (vT7-3) (437) was obtained from Dr B. Moss (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA). Baculogold AcMNPV genome was from Pharmingen. The recombinant baculovirus NS3-2 was obtained from Dr M. McGarvey (Imperial College, Institute of Science Technology and Medicine, London). This virus contains the coding sequence for HCV, amino acids 1007 - 1630 which encompasses most of HCV NS3 (amino acids 1027 - 1657) under the control of the polyhedrin promoter.

2.1.6 Oligonucleotides and Peptides

All oligonucleotides were purchased from Oswell, peptides were purchased from Genosys.

2.1.6a PCR Primers

NS5B-HEX (P4837)	5'GCCGAGATCTTCATGAATGTGGGGGCGTCAG 3'
NS5A-HEXII (P5601)	5'GCCGGAATTCCGGAAAAAGCGTACGGTG 3'
NS316b-1 (P8198)	5'GCGCCATATGGCGCCCATCACGGCG 3'
NS316b-2 (P8199)	5'GCGCGGATCCTTACGTGACGACCTCCAG3'

2.1.6b Band Shift Oligonucleotide

W4133	5'AGGGGACTTTCCGCTGGGGACTTTCCATGGCTTC GTACCCCGGC 3'

2.1.6c Helicase Substrates

A9937	5'GAATACAGACTTGACGTGCAGGTCGACTCTAGA GGATCATCGAGCGAGCTCGTATTCCGGTCTCGCTA TAGTGAGTCGTATTAATGTCGATAAGCCAG 3'
A9938	5'TATCTGTCGAGCTCGCTCGATGATCCTCTAGAGT CGAC 3'

2.1.6d Mutagenesis primers

A6302	5' CCAGCCTCGCGTCCCGAACGCCAGCAAGACG 3'
A0849	5' ATAATTTGTGACGAGTCCCACTCCACGGATG 3'
B0534	5' CACCGGCAGTGGTGCGAGCACCAAGGTC 3'
B0535	5' CATAATAATTTGTGCCGAGTGCCACTCCAC 3'
B0536	5' CATAATAATTTGTGACGCGTGCCACTCCAC 3'
B0537	5' GTCTCCAGGACTCACCGCCGGGGCAGG 3'
B0538	5' GGCAGGACTGGCAAGGGGAAGCCAGGC 3'
B4913	5' CTGGCAGGGGGAGGCGAGGCATCTACAG 3'

2.1.6e Peptides

PEP1a	RGKPGIYR

PEP1b	RGRRGIYR
PEPc	AIRQALVCWDELTKL

2.1.7 Plasmids

Plasmid	Source
pCRII	Invitrogen
pCRNS3-F	This laboratory (see Appendix I)
pCRScript	Stratagene
pCDNA3.1	Invitrogen
pCDNS3	This laboratory (see Appendix I)
pET16b	Novagen
pET16b-βgal	Novagen
pHEX	This laboratory (see Appendix I)
pMON-15903	P.O. Olins & S.C. Lee Monsanto Corporate Research, St. Louis, USA (438).
PRSETB	Invitrogen
pVL1393	PharMingen

2.1.8 Enzymes

All enzymes from a commercial source were purchased from New England Biolabs (NEB) except where indicated. Virginia Helicase represented the NS3 helicase domain and was from Pfizer and prepared as described in reference (279).

2.1.9 Chemicals

Standard laboratory reagents were purchased from MERCK or Sigma except where stated. Filter paper was from Whatman, ^{32}P radioisotopes (3000 Ci/mmol) and ^{33}P radioisotopes (3000 Ci/mmol) were from Amersham Life Sciences (UK) and ^{35}S radioisotopes (1000 Ci/mmol) were from ICN Ltd. Bacto-tryptone, yeast extract and Bacto-agar were purchased from Difco Labs. Hybond-C and PVDF nitrocellulose filters were from Amersham UK and Millipore respectively, Hybond-N nylon filter was from Amersham UK. Oligonucleotides were obtained from Oswell, and peptides were obtained from Genosys.

2.1.10 Antibiotics

Antibiotics used for bacterial cell culture were purchased from Sigma. Antibiotics were made as stock solutions 1000 times the working concentration, filter sterilised and were stored in aliquots at -20°C. The autoclaved media were cooled to 45°C or below prior to addition of antibiotic.

1000 X antibiotic stock solutions:

Ampicillin: 100 mg/ml in Milli Q water.

Kanomycin: 30 mg/ml in Milli Q water.

Chloramphenicol: 25 mg/ml in absolute ethanol.

2.1.11 Molecular Weight Standards

2.1.11a DNA Standards

HindIII or HindIII and EcoRI digested phage λ DNA purchased from Life Technologies were used to estimate the size of DNA fragments on agarose gels. HindIII digestion produces 8 bands of sizes (in bp); 23130, 9416, 6557, 4361, 2322, 2027, 564, 125. HindII and EcoRI digestion produces 13 bands of sizes (in bp); 21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564, 125.

2.1.11a Protein standards

Two types of protein standards were used. For gels to be stained with Coomassie Blue, high range markers from Life Technologies was used. For gels to be blotted onto nitrocellulose membranes, prestained markers from either BioRad or NEB were used. The sizes of prestained markers were subject to batch variation due to differences in dye uptake during manufacture. Each batch is precalibrated and comes supplied with apparent molecular masses, listed below are the sizes detailed in the suppliers catalogue. Please refer to individual figures for actual sizes.

Life Technologies high range (non-stained).

Protein	Size (Da)

Lysozyme	14300
β-lactoglobulin	18400
Carbonic anhydrase	29000
Ovalbumin	43000
Bovine serum albumin	68000
Phosphorylase B	97400
Myosin (H-chain)	200000

BioRad prestained markers

Protein	Size (Da)
Aprotinin	7200
Lysozyme	20400
Soyabean trypsin inhibitor	28300
Carbonic anhydrase	34800
Ovalbumin	49500
Bovine serum albumin	79500
β-galactosidase	115000
Myosin	208000

BioRad kaleidoscope prestained markers

Protein	Size (Da)
Aprotinin	6900
Lysozyme	17800
Soyabean trypsin inhibitor	30600
Carbonic anhydrase	41800
Bovine serum albumin	71000
β-galactosidase	133000
Myosin	202000

NEB prestained markers

Protein	Size (Da)
Aprotinin	65000
Lysozyme	165000
β-galactosidase	25000
Triosephosphate isomerase	32500
Aldolase	47500
Glutamic dehydrogenase	62000
MBP-paramyosin	83000
MBP-β-galactosidase	175000

2.1.12 Immunological Reagents

Monoclonal antibody against hexa-histidine tag (6xHis Monoclonal Antibody) was from Clontech Laboratories Inc. The polyclonal sera NS3-7 raised against the NS3 peptide FIPVENLETTMRS (amino acids 1195 - 1207), FIZ3 raised against the NS3 peptide PQSFQVAHLHAPTGSGKSTK (amino acids 1220 - 1239) and FIZ10 raised

against the NS5a peptide STSGITGDNMTTSSEPAPSG (amino acids 2356 - 2375) were raised in rabbits and were gifts from Pfizer Ltd. Rabbit antibodies against the NS3 helicase domain (α-NS3H) and the NS3 protease domain (α-NS3P) were also from Pfizer Ltd. The α-NS5 antibody was raised in a sheep against the region of NS5a/b (amino acids 2328-2516) expressed *in E. coli* (section 3.4).

2.1.13 Films and Photographic Reagents

Blue sensitive X-ray film was purchased from Genetic Research Instruments Ltd. (GRI). Cronex MD-Developer and Cronex MF-E fixer were both from DuPont.

2.1.14 Dialysis Tubing

Dialysis tube (30 mm width) was purchased in dried form from Medicell. It was prepared by first boiling for 10 minutes in 2% (w/v) sodium bicarbonate and 1 mM EDTA, then rinsed in Milli Q water and boiled again for 10 minutes in 1 mM EDTA. Storage was in this buffer at 4°C. The tubing was rinsed thoroughly in Milli Q water prior to use.

2.2 Techniques of Nucleic Acid Manipulation

The general molecular biology techniques employed during this work may be found in Molecular Cloning, A Laboratory Manual (439). However, these techniques were adapted to various degrees and each are described here.

Enzyme reactions were carried out in 1.5 ml or 0.4 ml microcentrifuge tubes and were mixed prior to incubation by gently flicking the base of the tube and the contents brought to the bottom of the tube with a brief spin in a microfuge.

2.2.1 Organic Extractions

2.2.1a Phenol Extraction

For volumes less than 100 µl, the volume was first made up to 100 µl with TE buffer or Milli Q water to facilitate the separation and removal of the aqueous phase. An equal volume of phenol was added to the sample, vigorously vortexed and then the aqueous and phenolic layers separated by centrifugation for 1 minute at 13,000g. The upper aqueous layer containing the DNA was then pipetted off, taking care to avoid disturbing the interface, and transferred to a fresh tube.

2.2.1b Phenol/Chloroform Extraction

An equal volume of a mixture of phenol:chloroform:iso-amyl alcohol (25:24:1) was added to the sample and mixed well by vortexing. The phases were separated by centrifugation for 1 minute at 13,000g and the upper aqueous layer transferred to a clean tube.

2.2.1c Chloroform Extraction

An equal volume of chloroform was added to the sample and mixed well by vortexing. The phases were separated by centrifugation for 1 minute at 13,000g and the upper aqueous layer transferred to a clean tube.

2.2.2 Ethanol Precipitation

To concentrate a DNA or RNA sample or purify it away from an undesirable solute, for example following a phenol extraction, the nucleic acid was precipitated in the presence of ethanol. A 1/10 volume of 3 M sodium acetate (pH 4.8) was added to the sample, followed by the addition of two and a half volumes of ice cold ethanol. The solution was mixed thoroughly before being placed in at -70°C for 30 minutes. The nucleic acid was then pelleted by centrifugation for 15 minutes at 13,000g at 4°C and the supernatant removed taking care not to disturb the pellet. The pellet was then washed by briefly vortexing with 1 ml ice cold 70% ethanol, spun as before and the supernatant removed. This wash was repeated and then the DNA pellets were dried in a vacuum dessicator. The DNA was finally resuspended in Milli Q water or TE.

A co-precipitant was sometimes added to improve precipitation of the nucleic acid and the visualisation of the pellet particularly when small quantities of nucleic acid were used. 0.2 μg/μl glycogen (Boehringer-Mannheim) was added to prior to addition of sodium acetate.

2.2.3 Restriction Enzyme Digestion of DNA

For the production of DNA fragments for subsequent cloning the following conditions were used. 5 μg of DNA was digested with 25 units of a restriction enzyme in 25 μl of 1X buffer (supplied as a 10X stock with the enzyme) in a 1.5 ml microcentrifuge tube. The reagents were mixed and then incubated at the optimal temperature for the enzyme until digestion was complete. The progress of enzymatic digestion was monitored by comparing an aliquot of the digestion mixture with a similar amount of uncut DNA by agarose gel electrophoresis (section 2.4.3).

For analytical studies, e.g. for the restriction analysis of potential recombinant constructs the reaction conditions were modified. In a 10 μl final volume, 2 - 5 μl of miniprep DNA (see 2.2.) was digested with 5 units of restriction enzyme in 1 X reaction buffer. The mixture was incubated for 1 hour at the optimal temperature for the enzyme.

2.2.4 Dephosphorylation of 5' Ends of DNA

DNA to be dephosphorylated was used directly following the restriction digest reaction described in 2.2.1. Calf intestinal alkaline phosphatase (CIAP) was added to the reaction mix at a ratio of 1 unit per picomole of DNA ends, gently mixed and then incubated at 37°C for 30 minutes. Following incubation the CIAP was inactivated by incubating at 75°C for 15 minutes in the presence of 10 mM EDTA.

2.2.5 End Filling of DNA Fragments

To ligate incompatible DNA ends the overhangs can be filled using Klenow polymerase to produce blunt ends. DNA to be end filled was resuspended in 1X NEBuffer (supplied with restriction enzymes from New England Biolabs) supplemented with 33 μM of each dNTP to 50 μg/ml. To this, 1 unit of Klenow polymerase per μg of DNA was added and incubated for 15 minutes at 25°C. The reaction was then stopped by the addition of EDTA to 10 mM and incubation at 75°C for 10 minutes.

2.2.6 Gel Purification of DNA Fragments from Agarose

DNA fragments from a restriction enzyme digest were resolved by agarose gel electrophoresis (section 2.4.3) and were visualised on a UV transilluminator. The desired bands were then excised using a clean scalpel blade and transferred to a microcentrifuge tube. The DNA was isolated from the gel slice using a QIAEX II gel extraction kit (Qiagen). The gel slice was solubilised at 55°C in buffer QXI and then 20 μl QXII buffer added which contains silica particles that bind the DNA. The particles were then washed once in 500 μl buffer QXI and twice in 500 μl buffer PE by vortexing until the particles were resuspended and then centrifuging for 30 seconds at 13,000 g. The DNA/silica pellet was then resuspended in 20 μl Milli Q water by vortexing and the DNA eluted from the silica particles by incubation for 5 minutes at 50°C. The silica particles were pelleted by centrifugation as before and the DNA solution transferred to a clean microcentrifuge tube. The quality and quantity of the purified DNA was estimated by comparison with known standards following agarose gel electrophoresis.

2.2.7 Ligation of DNA

DNA molecules possessing compatible terminals and a 5' phosphate group may be joined together using T4 DNA ligase. Prior to ligation, insert and vector fragments were gel purified (section 2.2.6) and the vector fragment dephosphorylated (section 2.2.4). 50 ng linearised, dephosphorylated vector was added to insert DNA at molar ratios of between 5:1 and 10:1, (insert : vector). To this 1 µl of 10x T4 DNA ligase buffer and 1 µl T4 DNA ligase (400 U) was added, and the volume made up to 10 µl with Milli Q water. This mixture was gently mixed and incubated either overnight in a 16°C water bath in the cold room, or on the bench-top for 1 - 2 hours. Half of each ligation mix (5 µl) was then used to transform competent *E. coli* cells (section 2.5.3).

2.2.8 Radiolabelling of DNA

Oligonucleotides were end labelled with ^{32}P for subsequent use in the bandshift assay using method 1 and with ^{33}P for subsequent use in the helicase unwinding assay using method 2.

2.2.8a Method 1

20 pmol of oligonucleotide was transferred to a microfuge tube, and 2 µl T4 PNK buffer, 20 µCi $\gamma[^{32}P]$-ATP and 1 µl of T4 PNK added. The volume was made up to 20 µl with Milli Q water and the tube was incubated at 37°C for 30 minutes and the reaction stopped by the addition of 5µl 250 mM EDTA. Unincorporated label was removed by gel purification of the labelled oligonucleotide on a TBE polyacrylamide gel (section 2.2.9).

2.2.8b Method 2

30 pmol of oligonucleotide A9938 was incubated with 10 units of T4 PNK (Pharmacia) for 30 minutes at 37°C in a buffer containing 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA and 0.2 μM ATP containing 10 μCi γ[^{33}P]-ATP. The reaction was stopped with 25 mM EDTA.

The labelled oligonucleotide was then annealed to a longer oligonucleotide for use in the helicase assay. 25 pmol of oligonucleotide A9937 was added to the labelling mix and the DNA ethanol precipitated at -20 °C for 2 hours. The DNA was washed twice with 70 % ethanol and dried and resuspended in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgSO_4$ by vortexing for 1 minute, heating to 37°C and vortexing again. The mixture was incubated for 10 minutes at 95°C and then cooled to room temperature of three hours. The annealed substrate was purified using G-50® Sephedex columns (Boehringer Mannheim).

2.2.9 Purification of Radiolabelled Oligonucleotides

2.2.9a gel purification

The ^{32}P-labelled oligonucleotide was adjusted to 1 X agarose gel sample buffer and was resolved by TBE PAGE (section 2.4.2). The apparatus was dismantled and the gel covered with plastic film then exposed to X-ray film. The autoradiograph was developed and the gel then replaced upon it. The region of the gel corresponding to a desired oligonucleotide as identified by aligning the autoradiograph with the gel and the

oligonucleotide was then excised with a clean scalpel blade. This gel fragment was then cut into small pieces and transferred to a microfuge tube and the oligonucleotide eluted from the acrylamide by diffusion into 500 µl 0.3 M sodium acetate, pH 4.8 on a rotary shaker. The oligonucleotide was then ethanol precipitated and resuspended in TE buffer.

2.2.9b using G-50® Sephedex columns

G-50® Sephedex columns were supplied by Boehringer Mannheim. The top then bottom caps were removed from the column in that order. The column was then placed in a 15 ml falcon tube and centrifuged at 1100 g for 2 minutes. The eluted buffer was discarded. Taking care to keep the column upright the oligonucleotide sample (up to 100 µl) was the applied to the centre of the column ensuring no sample was applied to the sides. The column was centrifuged as before but for 4 minutes and the eluate containing the oligonucleotide retained. The column was discarded as radioactive waste since it contained unincorporated radionucleotides.

2.2.10 Site Directed Mutagenesis

In the work presented here site directed mutagenesis was carried out using the Chameleon kit (Stratagene) which was based on the unique site-elimination technique of Deng and Nickloff (440). Mutagenesis buffer, enzyme mix, enzyme dilution buffer and competent *E. coli* were provided with the kit. Two primers were required for this technique, a mutagenic primer to introduce the required nucleotide change and a selection primer to allow the selection of mutant DNA (section 2.1.5). The selection primer introduces a base change which eliminates a unique restriction site and as such

can be designed for any plasmid. Both primers must be designed to anneal to the same DNA strand.

In a 1.5 ml microcentrifuge tube 0.25 pmol of the plasmid to be mutated was mixed with 25 pmol of selection primer and 25 pmol of mutagenic primer in 20 µl of mutagenesis buffer. The primers were annealed to the plasmid by placing the tube in a boiling water bath for 5 minutes, then on ice for five minutes and then incubating the mixture for 30 minutes at 25°C. 7 µl of the nucleotide and 1 µl of enzyme mix was added to the reaction mixture and this was mixed thoroughly then incubated for 1 hour at 37 °C. After incubation the mixture was placed at 80°C for 10 minutes. 20 units of NruI restriction endonuclease and 2 µl of 10 X NruI reaction buffer was added and the volume made up to 50 µl with MilliQ water. The mixture was then incubated for 2 hours at 37°C after which, 5 µl of this mixture was used to transform 90 µl of *E. coli* XL*mutS* competent cells (section 2.5.3). 100 µl of the transformation was plated onto LB agar plates containing ampicillin (100 µg/ml) which were incubated overnight at 37°C. Individual colonies were picked into 3 ml of 2YT broth containing ampicillin (100 µg/ml), grown overnight at 37°C and then plasmid DNA extracted from the bacteria as described in section 2.2.13. 0.5 µg of the plasmid DNA was then digested by incubating the DNA with 20 units of NruI in 20 µl of 1 X NruI reaction buffer for 2 hours at 37°C. 2 µl of this reaction mixture was then used to transform 40 µl of *E. coli* XL1-Blue competent cells (section 2.5.3) and the transformation mixture grown overnight as before. Individual colonies were grown overnight as before and then plasmid DNA extracted from the bacteria as described in section 2.2.12. The presence of the desired mutation was confirmed by automated DNA sequencing (section 2.2.11).

2.2.11 DNA Sequencing

2.2.11a Method 1 - Manual Sequencing

The dideoxy chain termination method of DNA sequencing, first described by Sanger *et al.* (441), was used in the form of the Sequenase® Version 1.0 kit (Amersham). Using this kit, up to 400 bases of sequence may be obtained from a single reaction. Sequenase® reaction buffer, termination mixes, labelling mix, Sequenase® enzyme, enzyme dilution buffer, DTT and stop solution were supplied as part of this kit.

The template (1 - 2 μg of maxiprep DNA (in 9 μl) or 9 μl of miniprep DNA) was transferred to a microfuge tube and 1 μl denaturant (0.2 M NaOH 2 mM EDTA) added. The tube was incubated at 37°C for 30 minutes to denature the DNA, which was then ethanol precipitated (section 2.2.2) and resuspended in 7 μl of Milli Q water. To this solution, 2 μl of Sequenase® reaction buffer and 1 μl primer were added and the mixture heated to 65°C for two minutes, then cooled slowly usually over 30 minutes to < 30°C to anneal primer and template.

2.5 μl aliquots of each of the four termination mixes, ddATP, ddTTP, ddCTP and ddGTP were placed into four labelled tubes and prewarmed to 37°C. The labelling mix was diluted 1:4 in Milli Q water and the Sequenase® enzyme was diluted 1:8 in enzyme dilution buffer, and stored on ice. To the annealed DNA/template 1 μl of DTT was added followed by 2μl of diluted labelling mix, 10 μCi of $\alpha[^{35}S]$-ATP and 2 μl of diluted Sequenase® enzyme in that order. The tubes were then incubated at room temperature for 2 - 5 minutes depending on the length of sequence to be read. Next 3.5 μl of reaction mix was transferred to each of the termination mixes and incubated at 37°C for a further 5 minutes and the reaction stopped by the addition of 3.5 μl of stop

solution. The tubes were then stored at -20°C until required, or used immediately for TBE-PAGE analysis (section 2.4.1).

2.2.11b Method 2 - Automated Sequencing

Automated sequencing reactions were performed using the ABI Prism™ dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems). The kit contains AmpliTaq DNA polymerase FS, terminator ready reaction mix and plasmid and primer control. The terminator ready reaction mix contains the dRhodamine terminators. This system is based on the dideoxy method of DNA sequencing but each dideoxynucleotide is tagged with a different dye [ddG-EO-dR110 (blue), ddU-EO-dROX (red), ddA-dR6G (green) and ddC-EO-dTAMRA (yellow)]. During electrophoresis the products of the cycle sequencing reaction are separated according to size and the ABI 377 apparatus and software 'read' the order of the dyes and hence the DNA sequence.

In a 0.2 ml MicroAmp® PCR tube (PE Applied Biosystems) 500 ng of miniprep (section 2.2.12) or CsCl pure (section 2.2.14) plasmid DNA and 3.2 pmol of sequencing primer were added to 8 µl of terminator ready reaction mix and the volume made up to 20 µl with MilliQ water. The tubes were placed in a GeneAmp 9600 thermal cycler and the DNA was then polymerised by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The tubes were then held at 4°C until ready for the next step. The reaction volumes were transferred to a 1.5 ml microcentrifuge tube and the DNA was precipitated with 74 µl of 70 % ethanol containing 0.5 mM $MgCl_2$ at room temperature for 15 minutes. After drying the pellet was resuspended in 4 µl

autosequencing load buffer and heated at 95°C for 2 minutes before electrophoresis on the ABI Prism 377 sequencer (section 2.4.1b).

2.2.12 Alkaline Lysis Method of DNA Preparation (Miniprep)

This method is based on that of Birnboim and Doly (442). Single colonies of *E. coli* were individually picked from an agar plate into 5 ml of LB broth in a 30 ml universal tube and grown overnight at 37 °C with shaking at 200 rpm. The bacteria from 3 ml of overnight culture was pelleted in a 1.5 ml microcentrifuge tube (13,000g, 30 seconds) by two sequential additions of 1.5 ml of culture and the supernatant aspirated. The bacterial pellet was then resuspended in 200 µl miniprep solution I containing 1 mg/ml lysozyme. This was left at room temperature for 5 minutes and then 400 µl miniprep solution II was added, mixed by inversion and incubated for a further 5 minutes. Finally 300 µl of miniprep solution III was added, the tube vortexed and left on ice for 10 minutes. The white precipitate was then pelleted (13,000g, 10 minutes) and the supernatant was transferred to a fresh tube. 1 µl RNaseA (10 mg/ml) was then added and this was incubated for 15 minutes at 37°C. The DNA was then ethanol precipitated and then washed twice with 70 % ice cold ethanol. The resulting DNA pellet was resuspended in 30 µl TE buffer.

2.2.13 Boiling Miniprep method of plasmid DNA preparation

This method was first described by Holmes and Quigley (443). A bacterial pellet was prepared from an overnight culture as described in section 2.2.12. The supernatant was aspirated and the pellet was then resuspended by vigorous vortexing in 180 µl STET buffer and 20 µl lysozyme solution. The sample was boiled for 1 minute, then centrifuged (13,000g, 15 minutes). The glutinous pellet of cell debris was then removed with a sterile toothpick and discarded. Plasmid DNA was then precipitated by the addition of 200 µl of ice cold isopropanol and pelleted by centrifugation (13,000g, 15 minutes). The supernatant was removed and the DNA pellet was washed twice with 70% ethanol and the resulting DNA pellet was dried in a vacuum desiccator and resuspended in 50 µl of TE buffer.

2.2.14 Large Scale Preparation of Plasmid DNA

A 5 ml overnight culture of the *E. coli* transformed with the plasmid of interest (section 2.5.3) was used to inoculate a 2 L conical flask containing 1 L of 2YT media and this was incubated overnight at 37°C with shaking at 200 rpm. The cells were then harvested by centrifugation (2,000g, 30 minutes, MSE Mistral 3000i, 4°C), the supernatant was discarded, and the pellet was then resuspended in 6 ml Tris sucrose supplemented with 1 ml of lysozyme solution. The suspension was incubated on ice for 5 minutes, then 1 ml of 0.5 M EDTA (pH 8.0) was added, and then incubated on ice for a further 10 minutes. The cells were lysed by the dropwise addition and continuous mixing of 10 ml of lytic mix.

The lysate was then transferred to a 50.38 Ti Beckman ultracentrifuge tube, and clarified by ultracentrifugation (100,000g, 30 minutes, Sorval 50.38 rotor, 4 °C). The

supernatant was then transferred to a 50 ml thick walled polycarbonate Oakridge tube and 15 ml of phenol was added. The phases were thoroughly mixed by inversion, then separated by centrifugation (3,000g, 15 minutes Sorval S-34 rotor, +4°C). The aqueous layer was transferred to a clean Oakridge tube and an equal volume of ice-cold ethanol added. A heavy precipitate was typically observed. If this did not occur, the tube was placed at -70°C for 30 minutes. The DNA was then pelleted by centrifugation (10,000g, 10 minutes, Sorval SS-34 rotor, +4 °C) and the pellet was dried in a vacuum desiccator.

The DNA was resuspended in 10 ml of TE and 10.7 g of CsCl was added. The mixture was vortexed to dissolve the CsCl and then 150 µl ethidium bromide solution (10 mg/ml) was added. The solution was transferred to a 12 ml Sorval quick seal centrifuge tube and the plasmid DNA was then separated from contaminants by centrifugation (300,000g, 4 hrs, Sorval NVT 65 rotor, 15°C). The tube typically contained three visible species of nucleic acid. The linear and open circle DNA appeared as a brown band near the top of the tube, whilst the RNA was pelleted to the bottom. The plasmid band was represented by a broad, pink band beneath the chromosomal DNA.

The top of the centrifuge tube was pierced with a 23 gauge syringe and the plasmid band drawn off with a similar needle and syringe by piercing the tube just below the band. The DNA was washed several times with an equal volume of isopropanol, saturated with CsCl, to remove the ethidium bromide, then dialysed against several changes of TE at 4°C, to remove the CsCl. The concentration of the DNA sample was then calculated and aliquots were stored at 4°C for current use or minus 20°C for long term storage.

2.2.15 Extraction of DNA from Insect Cells

Cells were detached from the surface of a T25 cell culture flask (section 2.6.5) and washed twice with sterile PBS to remove any FCS, then resuspended in 1 ml TE buffer and 1 ml of DNA lysis buffer added. The contents of the tube were gently mixed until the viscosity of the mixture was seen to increase as the cells lysed and released DNA. To this, 12.5 µl of proteinase K (10 mg/ml in TE) and 2.5 µl ribonuclease A (10 mg/ml) were added and the tube was incubated at 37°C for 2 hours. Contaminating proteins were then removed from the DNA by two rounds of phenol/chloroform extraction followed by two rounds of chloroform extraction (section 2.2.1). Care was taken during the phenol extraction not to shear the high molecular weight DNA during the mixing of the phases. Also, the genomic DNA produced was extremely viscous, so removal of the aqueous layer was performed with caution using a wide-bore pipette to avoid protein carry-over into the fresh tube. The DNA was then ethanol precipitated in the presence of 0.3 M sodium acetate which produces a visible clot of DNA. The DNA was pelleted by centrifugation (4,000 g, 10 minutes) and the supernatant was discarded. The pellet was washed twice with 70% ethanol, then dried. The pellet was soaked in 200 µl TE overnight, then incubated at 37°C for 15 minutes or until the DNA was fully solubilised. The DNA concentration was then calculated (section 2.2.16).

2.2.16 Determination of DNA Concentration

DNA samples were diluted so that an A_{260} of between 0.1 and 1.0 was obtained. The absorbence of the sample was determined at 260 nm and 280 nm in a quartz cuvette using an Ultraspec 2000 spectrophotometer (Pharmacia) and the DNA concentration calculated from the standard estimation that 1.0 A_{260} absorbence unit is equal to 50 μg/ml double stranded DNA or 33 μg/ml single stranded DNA. The purity of the sample was determined from the A_{260}/A_{280} ration. A ratio of 1.8 indicated that the DNA sample contained little contaminating protein.

2.2.17 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is an *in vitro* method for the amplification of a specific DNA sequence. Two primers are annealed to the complementary strands of a DNA molecule at positions which flank the region of interest and this region of DNA can then be amplified through multiple cycles of denaturing, annealing and elongation reactions.

Standard PCR

The standard PCR reactions were carried out in 1 X polymerase buffer (supplied with the enzyme) supplemented with 2 mM $MgCl_2$ in a 0.5 ml microfuge tube. To this, 200 ng template, 500 μM each dNTP (A, G, C, T), 100 pmole each primer and 10 units of Taq polymerase (Boehringer Mannheim) were added and the volume adjusted to 100 μl with Milli Q water. The reaction mixture was overlaid with sterile mineral oil to prevent evaporation during the PCR. The tube was then placed in a Hybaid Omnigene PCR machine. The DNA template was melted at 94°C for four minutes and then

amplified by 25 rounds of 1 minute at the annealing temperature, 2.5 minutes at 72°C (elongation step), and 1 minute at 94°C (denaturation step). Following the final annealing step, the polymerisation was allowed to proceed for 5 minutes. This was done to minimise the formation of single strands at the ends of the amplified DNA molecules. The PCR products were stored at 4°C and 10 µl analysed by agarose gel electrophoresis.

Annealing temperature

To calculate the optimal annealing temperature for the primer, 4°C was added for each G or C residues, and 2°C for each A or T, then 5°C subtracted from the total (439). The annealing temperatures were designed to be within 2°C for each pair of primers to ensure annealing of each primer and to prevent non-specific binding.

Primer Design

PCR primers consisted of 16 - 19 bases that were complimentary to the template strand with nucleotides added to the 5' end to create a unique restriction site and initiation or termination codons if required. Engineering of restriction sites to the 5' end of PCR products simplified sub-cloning.

2.2.18 Capture Cloning of PCR Products

Two commercially produced kits were employed during this study to facilitate the cloning of DNA molecules produced by PCR, the TA cloning kit (Invitrogen) and the pCRScript kit (Stratagene). The TA cloning kit takes advantage of the template

independent activity of some thermostable polymerases which add an adenosine residue to the 3' ends of PCR products (e.g. *Taq*). The PCR product is ligated into linearised pCRII vector DNA which contains single 5' 'T' overhangs. PCR products of reactions using polymerases which produce blunt ended DNA molecules (e.g. Vent) were cloned using the pCRScript kit. The PCR product was ligated into the pCRScript vector which was supplied linearised by digestion with SrfI restriction endonuclease to produce blunt ends. During ligation of the PCR product to the pCRScript vector, religation of the vector was prevented by the inclusion of 10 units of SrfI enzyme in the ligation reaction.

Selection of recombinant plasmids was the same for both capture cloning methods. Both plasmids carry the gene encoding the β-galactosidase α-peptide, which can complement a deletion in the host *lac* to produce a functional enzyme. Insertion of a fragment into the multiple cloning site of these plasmids destroys the *lac* function. β-galactosidase catalyses the cleavage of X-gal to produce a blue colour, thus clones containing an insert appear white when plated on to agar containing IPTG and X-gal compared to non-recombinants which are blue. Blue/white selection was achieved by the addition of 30 µl X-gal (20mg/ml in dimethylformamide) and 20 µl 0.2 M IPTG to the agar plates 30 minutes prior to use. IPTG and X-gal were added separately to prevent precipitation of X-gal.

Plasmid minipreps were made from overnight cultures seeded with a single white colony (section 2.2.13) and the presence of the desired insert confirmed by restriction analysis (section 2.2.3) and DNA sequencing (section 2.2.11).

In some instances the PCR products were digested with enzymes corresponding to the engineered restriction sites then ligated directly into the desired vector (section 2.2.7).

2.3 Techniques of Protein Manipulations

2.3.1 Production of Protein Extracts

2.3.1a Extractions from bacterial cells

The bacteria which had been induced to express recombinant proteins were pelleted (2,000g, 15 minutes Mistral 3000i 4°C) and then washed in 1ml PBS per 10 ml original culture. The resulting pellet was then resuspended in 1ml protein lysis buffer per 10 ml original culture and the cells lysed by sonication on ice. The suspension was sonicated at 20 microns using a MSE Soniprep for four pulses of 15 seconds each, pausing between each pulse to prevent heat denaturation of the sample. For analysis of total cell protein, 20 µl protein sample in 1 X SDS-PAGE load buffer was analysed by SDS-PAGE (section 2.4.4). For analysis of soluble protein the sample was first clarified with a 30 minute, 70,000 g centrifugation step and the supernatant retained for SDS-PAGE analysis. The pellet was resuspended in 8 M urea for analysis of the insoluble fraction. To ensure the fidelity of the protein expressed, SDS-PAGE analysis was normally carried out in duplicate; one gel was stained with Coomassie Blue (section 2.3.4) and the other used for a Western blot (section 2.3.5/6).

2.3.1b Extractions from insect cells

To harvest the protein, the cells were removed from the surface of T25 cell culture flasks (section 2.6.5) with a Pasteur pipette, then the suspension transferred to a 15 ml Falcon tube and the cells pelleted (150g, 10 minutes, MSE Mistral 3000i). If the cells had been grown in medium containing FCS, the cells were washed twice by

resuspending the pellet in PBS then pelleting as before. Finally, the cells were resuspended in 100 µl protein lysis buffer per ml of original culture. Lysis was achieved by 4 rounds of sonication of the sample. Each round of sonication was carried out on ice with a 15 second pulse followed by a 15 second interval to prevent overheating of the samples. Analysis of protein expressed was as for section 2.3.1a.

2.3.2 Isolation of Protein Sequestered in Inclusion Bodies

E. coli which express recombinant protein were harvested by centrifugation (2,000g, 15 minutes Mistral 3000i 4°C) and resuspended in 10 ml ice cold PBS per 100 ml culture. Resuspended cells were then transferred to 30 ml Oakridge tubes in 10 ml aliquots and cooled on ice and the remainder of the isolation procedure carried out at 4°C. The cells were then sonicated on ice for 4 pulses of 15 seconds at an amplitude of 20 microns using a MSE Soniprep sonicator, with pauses of 15 seconds between pulses. The lysate was the clarified by centrifugation (10 minutes, 8,000g, 4°C, SS34 rotor) and then pellet resuspended in 5ml ice cold PBS. This sonication/resuspension wash was repeated 4 times. The final pellet was resuspended in 5 ml PBS containing 8 M urea, 1 mM DTT, sonicated as before, then the insoluble cell debris removed by centrifugation (15 minutes, 8,000g, 4°C, SS34 rotor). The supernatant, containing the solubilised inclusion body material was retained for refolding.

2.3.3 Bradford Estimation of Protein Concentration

This method is essentially the same as that described previously (444). In a 1 ml plastic disposable cuvette 1 ml of Bradford reagent (Sigma) was added to 33 µl of the protein sample to be tested. After 5 minutes the OD at 595 nm was measured. The protein concentration was estimated by using a standard curve of BSA concentrations prepared in the same buffer and measured in the same way.

2.3.4 Coomassie Blue Staining of SDS-PAGE Gels

SDS-PAGE gels were placed in a plastic box and covered with Coomassie Blue R stain, a wide spectrum protein stain which enables the detection of ≥ 200 ng of protein. This was left shaking for 2 - 4 hours then the stain was poured off and the gel rinsed in Milli Q water. The gel was then covered with destain 1, left shaking for one hour and then replaced with destain 2. A destain bag (MoBiTech) or sponge bung cut into pieces was placed in the box which was then shaken until the gel had destained thoroughly. If required the gel was photographed and then dried onto blotting paper using a DrygelSr. SE1160 gel drier (Hoeffer Scientific Instruments) at 80°C for 120 minutes to be stored for reference.

2.3.5 Western Transfer of Protein Extracts

Protein samples were resolved by SDS-PAGE and then the gel was soaked in transfer buffer for 15 minutes. Three sheets of Whatman 3MM paper cut to the size of the gel were also soaked and placed smoothly onto the cathode of the Western blotting apparatus. A piece of nitrocellulose membrane cut to the size of the gel soaked in buffer

was placed onto the paper, and the gel placed on top of that. This was then covered by three more sheets of Whatman paper, cut to size and similarly soaked. During the assembly of the stack, care was taken to ensure that no air bubbles were introduced between the layers which would impair the transfer of the protein on to the nitrocellulose membrane. The anode was then placed on top of the stack and the apparatus was run at 35 mA overnight or 150 mA for 2 hours, then the membrane was transferred to blocking buffer. After the transfer was completed, the gel was stained with Coomassie Blue (section 2.3.4) to check the efficiency of transfer. The immobilised proteins were then detected using specific antibodies (section 2.3.6).

2.3.6 Immunological Detection of Immobilised Proteins

Following Western transfer of the proteins (section 2.3.5), the nitrocellulose membrane was washed with blocking buffer for at least 2 hours (preferably overnight) to prevent non-specific binding of the antibodies. The membrane was then covered with primary antibody appropriately diluted in blocking buffer and incubated with shaking for 2 hours. This was then washed in Western blot wash buffer, twice for 1 minute and then for 10 minutes with shaking. The membrane was then incubated for 2 hours with a secondary antibody conjugated to either HRP or AP appropriately diluted 1/2000 in blocking buffer, then washed as before and rinsed for 10 minutes in PBS. The method used to visualise immunoreactive proteins depended on the secondary antibody used. When using HRP-conjugated secondary antibody the membrane was dried between sheets of 3MM paper and then covered with equal volumes of HRPL substrate solutions A and B and agitated for 2 minutes. After this time the membrane was placed on a sheet

of 3MM paper, covered with cling film and exposed to X ray film for 10, 30 and 90 seconds and the film developed. When an AP-conjugated secondary antibody was used the membrane was rinsed in deionised water, and then covered with BCIP/NBT liquid substrate (Sigma). When colour had started to develop, the substrate was poured off and the membrane rinsed in Milli Q water before drying between 3MM paper and storing in the dark.

2.3.7 N-terminal Protein Sequencing

N terminal sequencing was done by Alta Bioscience (University of Birmingham). The protein sample to be sequenced was first resolved by SDS-PAGE (section 2.4.4) and transferred to PVDF membrane (section 2.3.5). The protein was then stained by washing the membrane in PVDF protein stain for two minutes followed by destaining in Milli Q water. The membrane was then dried between blotting paper and the band representing the protein to be sequenced was cut out and sent to Alta Bioscience. Sequencing was carried out using the method of Edman (445) on a Perkim Elmer 473A protein sequencer.

2.3.8 Protein Purification: Column Chromatography

Heparin sepharose and metal chelate HiTrap chromatography columns were purchased from Pharmacia Biotech and prepared following the manufacturers protocol. Throughout the purification procedure care was taken not to introduce air bubbles into the column. Using a peristaltic pump the columns were washed with 5 column volumes

of Milli Q water at a rate of 1 ml per minute. For the metal chelate column only the matrix was then charged with half a column volume of 0.1 M $NiSO_4$ and then washed again with 5 column volumes of Milli Q water. The column was then equilibrated with 5 column volumes of the protein sample buffer and then the protein sample was applied to the column. After the protein had been applied, the column was washed with ten column volumes of protein sample buffer and the bound protein eluted with an appropriate gradient (section 3.2). At the point of applying the protein sample to the column 1.5 ml fractions of the column eluate were collected using a Frac-100 fraction collector (Pharmacia Biotech) and analysed by SDS-PAGE and Western blot.

2.4 Electrophoretic Techniques

2.4.1 Electrophoresis of DNA Sequencing Products

2.4.1a Method 1 - Manual Sequencing

Two glass plates (420 mm x 330 mm and 395 mm x 330 mm) were cleaned with detergent and water, then with ethanol and the smaller plate coated with dimethyl dichlorosilane in a fume hood. This was left for 10 minutes and then washed a further time with Milli Q water. The plates were then placed with their cleaned/silanised faces together, either side of two 0.4 mm spacers. These spacers also had a piece of foam attached to the top in such a way so as to protrude from the top of the smaller plate. The foam pieces ensured a firm seal was formed between the plates and the upper reservoir tank of the electrophoresis apparatus. The plates were then sealed around the sides and bottom with yellow Scotch electrical tape taking care to ensure that the potential for leaks was minimised.

The acrylamide was then prepared by mixing 18 ml of SequaGel concentrate with 49.5 ml of SequaGel diluent and 7.5 ml SequaGel buffer in a small lipped beaker. 30 µl of TEMED and 600 µl 10% ammonium persulphate was then added and the gel solution mixed, avoiding aeration. The gel mix was then poured into the spaces between the assembled plates with the assembly tilted in such a way as to guide the gel down one side of the plates and into the bottom corner, taking care not to introduce air bubbles. When the solution had reached the top of the smaller plate the apparatus was laid back almost flat (at an angle of 10°) and the excess acrylamide was absorbed using paper towels. Two sharkstooth combs were then inverted and pushed into the space between the two plates and bulldog clips were placed on the top of the apparatus to hold the combs firmly in place.

After the acrylamide had polymerised, the tape sealing the bottom of the plates and bulldog clips were removed and the top of the gel was washed with 1X TBE to remove unpolymerized acrylamide and crystallised urea. The plates were then attached to an S2 sequencing gel apparatus (Gibco BRL) and the top and bottom reservoirs each filled with 1X TBE. The combs were removed, and the wells were washed with 1X TBE. The combs were then reinserted such that the teeth made contact with the polyacrylamide without piercing it, forming wells between each pair of teeth. A blank run was the performed as described below to preheat the gel prior to loading the samples. This helps prevent reannealing of the samples at the start of the run when the gel would otherwise be cold. The samples to be electrophoresed were boiled for two minutes and then 4 µl of each were loaded into the wells in the order G, A, T, C.

The gel was run at 60 Watts until the bromophenol blue dye front had reached the bottom of the plates, which usually took from 1.5-2.0 hours. To read more sequence

from the same reaction a second loading of the same samples was made in empty lanes at this point. The run was then continued again until the bromophenol blue dye front from the second loading reached the bottom of the gel. This double loading enables an extra 100 - 200 bases of sequence to be read, depending on the quality of the sequencing reactions. The apparatus was then dismantled and the buffer discarded. The remaining tape was removed from around the plates and these were carefully prised apart using a thin spatula then the larger plate with the gel attached was then placed in a tray containing fixer for 15 minutes. The plate and gel were then removed from the tray and excess fixer was carefully drained off. A sheet of Whatman 3MM paper cut to the size of the large plate was then laid onto the gel, smoothed down to ensure full contact and rolled off the plate with the gel attached. The gel was covered with Saran wrap and dried using a DrygelSr. SE1160 gel drier (Hoeffer Scientific Instruments) at 80°C for 60 minutes. The Saran wrap was removed and the gel exposed to X-ray film.

2.4.1b Method 2 - Automated Sequencing

Electrophoresis and data analysis was carried out using an ABI Prism 377 DNA sequencer version 3.0 sequencer and software. Two glass plates were cleaned with deionised water and assembled into the casting apparatus supplied. A 4.25 % acrylamide sequencing gel was prepared by mixing 18g of urea (Amresco ultra pure), 0.5g of amberlite MB150 resin and 5.3 ml acrylamide:bis mix 19:1 (Amresco ultra pure) in 27.5 ml of Milli Q water. 5 ml of 10 X TBE was passed through a Nalgene 0.2 μm filter unit and then the acrylamide mix filtered into it through the same filter. This was then mixed and degassed under vacuum and 250 μl of 10% ammonium persulphate and 35 μl of

TEMED were added. The mix was taken into a 50 ml syringe and then the gel poured using the assembled casting apparatus and an inverted comb was then inserted.

After polymerisation the gel was clamped into the electrophoresis apparatus and the top and bottom reservoirs filled with 1 X TBE. The comb was removed and the gel cleaned with 1 X TBE and then the comb was replaced so that the teeth just touch the surface of the gel. The sequencing samples were boiled for two minutes and then loaded into the wells. The gel was run for 7 hours using the standard run module, mobility file and matrix file for dRhodamine dye terminators according to the manufacturers protocol and the DNA sequence read from the created sequence file.

2.4.2 TBE Polyacrylamide Gel Electrophoresis

TBE polyacrylamide gel electrophoresis was performed using the Bio-Rad mini-Protean electrophoresis apparatus for gel purification of radiolabelled oligonucleotides and Protean II kit for band-shift and helicase assays. Two clean, dry glass plates which were separated by suitable spacers were clamped together and locked into the casting tray. The gel was prepared (**Table 2.1**), mixed well taking care to avoid aeration and poured to 5mm below the top of the smaller plate. A suitable comb was inserted and the gel left to polymerise and after polymerisation the comb was removed and the wells rinsed with 0.5x TBE. The gel apparatus was then assembled as described by the manufacturer and immersed in 0.5 X TBE. The samples were adjusted to 1 X agarose gel sample buffer, loaded into the wells and the gel run at 15 mA per gel for 2-4 hours (Protean II) or at 10 mA for up to 1 hour (Mini-protean) depending on the size of the molecules and complexes to be separated. Progression of electrophoresis was monitored

by the migration of xylene cyanol and bromophenol blue bands. Gels for band shift and helicase assays were run in the cold room to prevent disruption of the protein:DNA or DNA:DNA complexes.

	Volume (ml for each gel)	
	mini-gel	full size gel
40% Acrylamide bis	2.5	12.5
5 X TBE	1	5
distilled water	6.4	32
10 % Ammonium persulphate	0.1	0.5
TEMED	0.01	0.05

Table 2.1 Solutions and volumes used for the preparation of a 10% polyacrylamide gel.

2.4.3 Agarose Gel Electrophoresis of DNA

Agarose gels were formed using a Horizon gel electrophoresis apparatus (Gibco BRL). 0.7 - 2.0% agarose (w/v) in 1X TAE or 1 X TBE buffer containing 5 μg/ml ethidium bromide was melted in a microwave oven, allowed to cool to 65°C and then poured into the assembled apparatus taking care not to introduce air bubbles. Once the gel had set it was covered with 5 mm of appropriate 1X buffer and the combs removed to reveal the wells. Generally, agarose of 1% (w/v) was used but for separation of larger

fragments (>5 kbp) 0.7 % (w/v) was used and for smaller fragments (<1 kbp) up to 2% (w/v) was used.

Prior to loading, the samples were adjusted to 1X agarose gel sample buffer and then added to the wells by careful pipetting. The gel was run at a constant current of 150 mA until the sample (as seen by the appearance of the bromophenol blue tracking dye) had migrated the required distance along the gel. The DNA bands were visualised on a UV transilluminator and photographed if required. Due to the migration of ethidium bromide during electrophoresis staining was not always sufficient. To remedy this the gel was soaked in 1.5 μg/ml ethidium bromide for 15 minutes, then washed in distilled water for a further 15 minutes before viewing. For preparative gels, UV exposure time was kept to minimum to prevent DNA damage.

2.4.4 SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Two basic designs of gel apparatus were employed; the Bio-Rad Protean II apparatus, and the Novex precast gel electrophoresis kit. The BioRad system required the preparation of SDS-PAGE gels. The two glass plates separated by 1.5 mm spacers were clamped together ensuring that the bottoms of the plates were flush and this assembly was then locked into the casting tray and the gel poured.

SDS-PAGE gels consisted of a stacking gel, which concentrated the protein mix into a tight band before it reached the resolving gel, and a resolving gel which separated the proteins. The resolving gel was made first (**Table 2.2**), poured into the gap between the glass plates to approximately 4 cm from the top of the small plate and overlaid with a thin layer of water saturated butanol. The excess acrylamide was kept and used to

monitor the progress of the polymerisation. Once the resolving gel had polymerised, it was washed with Milli Q water to remove the butanol and non-polymerised acrylamide. The stacking gel was then prepared (**Table 2.2**), poured to 5 mm from the top of the smaller plate and a comb with the required number of wells was selected and inserted into the liquid stacking gel. Care was taken not to introduce bubbles at this stage. Once the stacking gel had set, the comb was removed and the wells washed twice with SDS-PAGE running buffer.

	Volume per gel (ml)	
	Running gel	Stacking gel
Milli Q water	13.2	6.1
Protogel	11.1	1.3
Protogel buffer	8.7	-
Protogel stacking buffer	-	2.5
TEMED	33 µl	10 µl
10% Ammonium persulphate	330 µl	50 µl

Table 2.2 Volumes (per gel) used to make 10% SDS-PAGE stacking and running gels.

The gels required for the Novex gel apparatus are purchased ready made from R & D Systems. The combs were removed from the gels and the wells washed thoroughly with Milli Q water and then 1X SDS-PAGE running buffer prior to use. The gel

apparatus was assembled and the upper and lower reservoirs filled with 1 X SDS-PAGE running buffer. In the case of the BioRad kit cold water was run through the centre unit throughout electrophoresis to prevent overheating.

The protein samples in 1 X SDS-PAGE sample buffer were boiled for two minutes prior to being loaded into the wells. Electrophoresis using the BioRad system was for 1.5 - 2 hours at 60 mA per gel and the Novex system was run for 1 - 1.5 hours at a constant 125 volts. After the bromophenol blue band had reached the base of the gel, the apparatus was dismantled and the stacking gel cut from the running gel. The running gel was then either stained with Coomassie stain as described (section 2.3.4) or soaked in transfer buffer prior to Western blotting (section 2.3.5).

2.5 Bacterial Techniques

2.5.1 Maintenance of *E. coli* Strains

E. coli strains were maintained on LB agar plates containing the appropriate antibiotic at 4°C for up to two weeks. For long term storage, 0.75 ml of 30% glycerol was added to 0.75 ml of an overnight culture of the *E. coli* strain in a 2 ml screw cap microcentrifuge tube. The tubes were then placed at -80°C for storage.

2.5.2 Production of Competent *E. coli*

For the transformation of ligation reactions commercially available XL-1 Blue (Stratagene) or TOP10F' (Invitrogen) supercompetent cells were used. For transformation of plasmid DNA, competent cells were made using two methods.

2.5.2a Preparation of competent cells by the calcium chloride method

An overnight culture of *E. coli* was used to inoculate 50 ml of 2YT medium at a dilution of 1:100 and grown at 37°C with vigorous shaking (200 rpm) to an OD_{600} of 0.6 - 0.8. At this point the cells were chilled on ice for 15 - 30 minutes then pelleted by centrifugation (2,000g, 15 minutes Mistral 3000i 4°C). The supernatant was removed, and the cells gently resuspended in 10 ml of ice-cold sterile 50 mM $CaCl_2$ and again incubated on ice for 15 - 30 minutes then harvested as above. Finally the cells were carefully resuspended in 2.5 ml ice-cold sterile 50 mM $CaCl_2$, 10 % glycerol. Cells produced in this way were suitable for immediate use (100 µl per transformation), but could also be snap-frozen in a methanol/dry ice bath in 100 µl aliquots and stored at -80°C for future use.

2.5.2b Preparation of electrocompetent cells

An overnight culture of *E. coli* was used to inoculate 100 ml of 2YT medium at a dilution of 1:100 and growth continued at 37°C with vigorous shaking (200 rpm) until an OD_{600} of 0.6 - 0.8 was reached. The cells were then placed on ice for 15 - 30 minutes and harvested by centrifugation (2,000g, 15 minutes Mistral 3000I, 4°C) in two sterile 50 ml falcon tubes. The supernatant was discarded and each pellet washed by resuspending in 50 ml ice cold sterile Milli Q water and then harvested as before. The pellets were then washed again in 25 ml of ice cold water and finally resuspended in 2

ml ice cold 10% glycerol. This suspension was divided into 50 µl aliquots and used immediately or snap frozen (section 2.5.2a) and stored at -70°C.

2.5.3 Transformation of $CaCl_2$ Competent *E. coli* Cells with Plasmid DNA

Aliquots of competent cells prepared as described above (section 2.5.2) were removed from -80°C storage and thawed on ice. Commercially bought supercompetent cells were treated with 0.85 µl β-mercaptoethanol per 50 µl cells and left on ice for a further ten minutes. The recombinant plasmid was then introduced into the microcentrifuge tube containing the cells, mixed gently by hand, and left on ice for 30 minutes. 10-100 ng (5 µl of a ligation mixture) of DNA for a cloning transformation was used or 0.1 - 1 ng of plasmid DNA. The cells were then heat-shocked for 45 seconds in a 42°C water bath before replacing on ice for 2 minutes. 0.9 ml of SOC medium was then added per 100 µl of cells and this was incubated at 37°C with gentle shaking for 45 - 60 minutes. Aliquots were then plated on to LB agar plates containing the appropriate antibiotic for selection and grown with the plates inverted overnight at 37°C.

2.5.4 Transformation of Electro-competent *E. coli* Cells with Recombinant DNA

Plasmid DNA (0.1 - 1 ng) was added to an aliquot of electrocompetent cells (section 2.5.2) that had previously been thawed on ice and this was left on ice for 10 minutes. The mixture was then transferred to a pre-cooled electroporation cuvette (0.2 cm electrode gap) and pulsed once at 25 µF, 2.1 kV and 200Ω using a BioRad Gene

Pulsar. Immediately afterwards 0.9 ml of SOC medium was added to the cells and mixed gently by pipetting. The cells were then transferred to a microcentrifuge tube and incubated at 37°C for 45 - 60 minutes with gentle shaking. Aliquots were then plated on LB agar plates containing the appropriate antibiotic for selection and grown with the plates inverted overnight at 37°C.

2.6 Handling of Insect Cells and Baculoviruses

2.6.1 General Handling Considerations

Cells of the insect cell line *Spodoptera frugiperda* (Sf9) were maintained either in monolayers in plastic tissue culture vessels (35 mm plates/clusters, 60 mm plates, or T25 and T75 canted neck flasks) or in suspension culture in plastic Erlenmyer flasks or sterile glass spinner vessels (Techne). Cultures were grown at 27-28°C and did not require CO_2. When healthy, cells were seen to double every 18-22 hours and to be >95% viable, were spherical in appearance and attached readily to plastic tissue culture vessels. Cells infected with recombinant baculoviruses became enlarged and detached from the monolayer. Suspension cultures could be grown to densities of up to 10^8 but usually 10^7 before 'clumping' of cells was observed and viability affected. Care was taken when centrifuging (150g, 10 minutes, MSE Mistral 3000i), resuspending and pipetting the cells to prevent shearing. To reduce the shearing of cells grown in suspension culture, Pluronic F-68, a surfactant, was added to TC100 media to a concentration of 0.01% (w/v).

2.6.2 Freezing and Defrosting of Sf9 Cell Stocks

Frozen stocks of Sf9 cells were stored in liquid nitrogen. Cells from a viable, log phase culture were pelleted and the supernatant discarded. The pellet was then resuspended in 90% FCS and 10% dimethyl sulphoxide (DMSO) to a final concentration of 1 x 10^7 cells/ml and then dispensed as 1 ml aliquots into cryovials (Costar). The cells were frozen initially at -20°C for 1 hours, then transferred to a -80°C freezer overnight after which the vials were stored in liquid nitrogen.

Frozen Sf9 stocks were thawed at room temperature and transferred to a T25 flask containing 4-5 ml of pre-warmed media. The flask was rocked gently to ensure even distribution of the cells then left at room temperature for 1 hour. The flask was then transferred to a 27°C incubator for 4 hours, after which time the media was aspirated to remove any non-viable cells and replaced with 5 ml of pre-warmed media which also diluted the DMSO around the cells. The flask was then incubated at 27°C for 2 days after which the cells had recovered enough to be routinely subcultured. Alternatively the 1 ml stock of Sf9 cells was added to 25 ml pre-warmed media in suspension culture and incubated with gentle shaking (90 rpm) at 27°C for 5 days or until the cell density had reached 1 x 10^7 cells/ml. The cells were then ready for further subculture.

2.6.3 Routine Maintenance of SF9 Cells

To maintain a constant cell line which could be used to seed larger scale suspension cultures, two flasks of Sf9 cells were seeded and one was subcultured twice weekly to produce two new cultures each time. The remaining flask kept for one week

as a back-up stock in case of future contamination. In this way, the possibility of all seed stocks being simultaneously contaminated was reduced.

Typically, cells from one flask of the previous split (subculture) were counted and then cells from this culture were added to fresh pre-warmed media to produce a culture of not more then 5 x 10^5 cells/ml. Generally a dilution of 1 in 3 was required twice weekly to maintain the suspension culture. At each split the cell viability was also monitored to ensure cells were healthy. Occasionally, if the cells were allowed to grow too dense 'clumping' of cells was observed whereby large numbers of cells stick together to form a clearly visible clump in the culture. This can be overcome by allowing the cells to settle for a few minutes The clumps fall to the bottom more quickly and single cells can then be carefully pipetted from the top of the culture and used to seed fresh media.

Subculturing of cells maintained in monolayer culture was done similarly. Cells were resuspended by gentle pipetting of the media across the monolayer. Care was taken not to touch the monolayer with the pipette tip nor to introduce bubbles into the culture. Cell viability and number was assessed, then T25 or T75 flasks were seeded at 50% confluence, approximately 8 x 10^4 cells per cm^2. To maintain cell viability it was necessary to subculture the cells every 3 - 4 days.

2.6.4 Cell Counting and Viability Estimation

The concentration and viability of a population of Sf9 cells was assayed using Trypan blue, which is taken up only by non-viable cells. The 10 X stock (0.4% w/v) was added to a diluted sample of cells and incubated for 5 minutes to allow uptake of the

dye. An aliquot of the mixture was then analysed using a haemocytometer (Sigma) and inverse phase microscope (Kodak). The volume beneath the coverslip of a haemocytometer is calibrated so that the volume contained in each etched square is 1 x 10^{-4} ml. Therefore, a count of the number cells within a predetermined area of the haemocytometer was equal to that number of cells multiplied by 10^4 per ml. This figure was then multiplied by the inverse of the dilution used prior to the cell count which provides a figure for the density of the cells in the source vessel. To calculate the viability, the blue, non-viable cells were counted and compared to the total number of cells and expressed as percentage viability.

2.6.5 Infection of Sf9 Cells

The vessels and cell seeding densities chosen for infection depended on the purpose of the experiment. For the production of viral stocks a multiplicity of infection (MOI) of 0.1 was used and cells harvested after 6 - 7 days. For the production of recombinant protein the cells were infected with an MOI optimised for production of the protein of interest and for a period of time similarly optimised.

2.6.5a Infection of Sf9 Cells in Monolayer Culture

The desired vessel was seeded with the appropriate number and volume of cells (**Table 2.3**) and left for 1-2 hours in order for the cells to attach to the surface and form a monolayer.

Size of Vessel	Cell Number	Inoculum volume (min-max)	Medium volume
35mm dish	1.5 x 10^6	0.1-0.2 ml	1.5-2.0 ml
60mm dish	2.5 x 10^6	0.4-1.0 ml	3.0-5.0 ml
25 cm^2 flask	2.0 x 10^6	0.4-1.0 ml	3.0-5.0 ml
75 cm^2 flask	6.0 x 10^6	1.0-2.0 ml	10-15 ml

Table 2.3 Seeding densities for commonly used tissue culture vessels.

The vessel was then tilted and the medium aspirated taking care not to disturb the monolayer. The inoculum was then added. The method of addition of inoculum to the cells varied with the vessel used. For T75 and T25 flasks, the inoculum was pipetted to the bottom of the flask then the flask gently laid flat until the inoculum had covered the monolayer. For 35 mm and 60 mm dishes the inoculum was added dropwise to the centre of the dish. The flask or dish was then left at room temperature for 1 - 2 hours ensuring that the inoculum was evenly distributed and that the cells did not dry out. This was achieved by either gently rocking the vessel several times every 15 minutes, or placing it on a rocking platform. The inoculum was then aspirated and the appropriate volume of media added. The infection was then left for a period of time depending on the experiment.

2.6.5b Infection of Sf9 Cells in Suspension Culture

To ensure simultaneous infection of the suspension cultures, cells should be pelleted by centrifugation and resuspended in medium plus inoculum. However, in practice the deleterious effect this had on cell viability in addition to the practical implications of spinning down and resuspending large volumes of cells in a sterile manner meant that this protocol was avoided. Accordingly, the following method was used. The vessel to be infected was seeded at 1-2 x 10^5 cells/ml and incubated at 28°C until cell density had reached 5 x 10^5 cells/ml for the generation of virus stocks, or to 1-2 x 10^6 cells/ml for recombinant protein production. The amount of inoculum used was then calculated from the cell density ([CELLS]) in cells/ml, the total volume of cell culture (V_C) in ml, the concentration of the virus stock used ([VIRUS]) in plaque forming units/ml (pfu/ml), and the multiplicity of infection wanted (MOI) in pfu / cell. Thus:

$$\text{Volume of inoculum required} = \frac{\text{MOI} \times V_C \times [\text{CELLS}]}{[\text{VIRUS}]}$$

With virus stocks of titres less than 1 x 10^7 pfu/ml the inoculum would make up a large proportion of the total volume. In this situation the cells were pelleted by centrifugation and resuspended in the inoculum.

2.6.6 Plaque Assay

The plaque assay was used for the determination of viral titre of a virus sample and the purification of recombinant virus from wild type virus. The required number of 60 mm tissue culture dishes were seeded with 2.5 x 10^6 exponentially growing cells with a viability of > 90% (e.g. 4 ml of culture at 6.25 x 10^5 cells/ml) and the cells allowed to attach for 1 - 2 hours at room temperature. During this time serial dilutions of the virus stock to be assayed or purified were made in microcentrifuge tubes to give a final volume of 1 ml. Typically, if the virus was obtained by picking an individual plaque, dilutions from 10^{-1} to 10^{-3} were used. With co-transfection supernatants, 10^{-3} to 10^{-6} dilutions were used. With P1 and P2 virus stocks, derived from monolayer culture, dilutions of 10^{-4} to 10^{-7} were plated out. Dilutions of 10^{-6} to 10^{-9} were used for large scale virus stocks. The dishes were then checked under a microscope to ensure 50% confluence and the media removed. 800 µl of the appropriate virus dilution was then added drop-wise to the centre of the dish taking care not to disrupt the monolayer. The cells were then incubated at 27°C for one to two hours with occasional gentle shaking to ensure that the entire monolayer remained covered with the virus suspension. The overlay was prepared during the infection step. 4% (w/v) low melting point agarose (LMP; type VII, Sigma), in Milli Q water was melted and kept at 37°C with 1.3X Sf900 SFM media (Life Technologies), for cells maintained in Sf900 SFM media or TC100 for cells maintained in TC100 media. The agarose was then added to the media (1:3 agarose:media) and kept at 37°C until ready for addition to the dishes. Following infection, the virus was removed and 4 ml of molten overlay added dropwise to the centre of the dish, taking care not to disrupt the monolayer and the overlay allowed to set. The dishes were then incubated in a sealed plastic box lined with damp paper towels at 27°C for 6 - 7 days. After this period the dishes were examined for plaques. The

plaques were visualised by overlaying each dish with 2 ml of MTT stock solution (5 mg/ml in PBS) diluted in PBS to 1 mg/ml. This was left for 45 minutes or until full colour had developed and then removed. Plaques, visible as yellow spots on a dark purple/black background were counted and the virus titre calculated as plaque forming units per ml (pfu/ml).

2.6.7 Production of Recombinant Baculoviruses

For the production of a recombinant baculovirus, linearised AcNMPV genome was introduced into insect cells with a transfer vector containing the gene of interest. The gene of interest was then transferred into the viral genome by homologous recombination between the transfer vector and virus genome using the host cell machinery. Introduction of nucleic acid into cells was done using liposomes, the method detailed here uses the Lipofectin reagent supplied by Life Technologies. For this protocol, a 35 mm dish was seeded. 1 μg transfer vector and 100 ng linear AcMNPV DNA (BaculoGold, Pharmingen) were diluted in 100 μl FCS free media and mixed in a microfuge tube. To this, 5 μl of Lipofectin diluted in 100 μl FCS free media was then added, mixed gently and incubated at room temperature for 15 minutes. The medium was removed from the dish, and the cells washed twice with FCS free media. This was done to remove any protein from the culture which would inhibit the formation of liposomes. 0.8 ml FCS free media was then added to the DNA/Lipofectin, mixed gently and carefully pipetted onto the cells. The dish was swirled to mix the contents and incubated for 4 hours at 28°C. Finally, a further 1 ml of fully supplemented media was

added and then the dish left for 48 hours, after which time the supernatant was harvested and screened for recombinant baculovirus.

2.6.8 Screening for Recombinant Baculoviruses

A plaque assay was performed as described (section 2.6.6) using the supernatant from the co-transfection (section 2.6.7). Following staining with neutral red, occlusion negative plaques were picked and transferred into 300 µl of medium using a sterile glass Pasteur pipette, vortexed and left overnight to allow the virus particles to diffuse out of the agarose plug and into the medium. The desired serial dilutions were then made and the plaque assay repeated. Occlusion negative plaques were picked as before and again the plaque assay repeated. Finally a plaque was picked as before and this was used to produce a high titre vital stock (section 2.6.9)

2.6.9 Production of Viral Stocks

The process of producing viral stocks was one of amplification. In all the infections, a multiplicity of infection of less than 1 (typically 0.05-0.1) was used in order to allow serial infection of the cells and thus greater final virus titre.

Plaques from virus purification (section 2.6.8) were picked from the final plate and transferred to 300 µl of culture medium, vortexed and left overnight to allow the virus particles to diffuse out of the agarose plug and into the medium. Half of this stock was then used to infect 1.5 x 10^6 Sf9 cells in a T25 flask (section 2.6.5) for 4-6 days, after which the supernatant was harvested by centrifugation. This supernatant was

termed the passage 1 (P1) or seed stock and was not used for experimentation. To ensure a back up of recombinant virus, 1 ml of the seed stock was taken and stored at -80°C.

The seed stock was then used to make the passage 2 (P2) or intermediate stock by the infection of 5 x 10^6 cells in a T75 flask with 250 µl of P1 virus. Again, the infection was left for 6 days, then the supernatant collected as above.

The P2 stock was then used for a final round of amplification. A 1 litre spinner vessel (Techne) was seeded with 500 ml at 1 x 10^5 cells/ml and grown until the cell density reached 5 x 10^5 cells/ml. The cells were then infected with P2 inoculum, allowed to infect for 6 days, and the supernatant harvested as above and stored in 50 ml aliquots at 4°C. This was termed the passage 3 (P3) virus stock which was used for subsequent experiments.

2.7 Assays

2.7.1 Band Shift Assay

15 pmol NS3 was incubated with 1.5 pmol of ^{32}P radiolabelled oligonucleotide (section 2.2.8) in 20 µl of 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 0.25 mg/ml BSA buffer for 30 minutes at 37°C. To this, 4 µl of 6 X agarose gel load buffer was added and the samples subjected to TBE-PAGE using a 6 % gel prepared with 19:1 acrylamide:bis. Following electrophoresis the gels were dried and autoradiographed.

2.7.2 Helicase Assay

0.3 pmol of double stranded helicase substrate (section 2.2.8) was incubated with 4.5 pmol NS3 at 37°C for 60 minutes in 15 µl of 50 mM MOPS (pH 6.5) , 5 mM ATP, 3 mM $MgCl_2$, 2 mM DTT, 0.25 mg/ml BSA buffer. 10 µl of the reaction was added to 5 µl of helicase load buffer and resolved by TBE-PAGE using a 15 % gel prepared with 37.5:1 acrylamide:bis. 0.3 pmol of helicase substrate was heat denatured by heating to 100°C for 2 minutes and run alongside the reaction samples on the TBE-PAGE gel to act as a marker for the migration of the released strand. Following electrophoresis the gel was dried and autoradiographed and unwinding was quantified by PhosphorImage analysis using IamgeQuant software.

2.7.3 Malachite Green NTPase Assay

This assay was based on the methodology of Hess and Derr (446) and was performed in a 96 well plate. In a 20 µl volume, 6 pmol enzyme was added to 25 mM Tris pH 7.5, 5 mM $MgCl_2$, 0.5 mM ATP, 5 µM polyuridylic acid and incubated for 30 minutes at 25 °C. The reaction was stopped by the addition of 5 µl 250 mM EDTA. 200 µl AM/MG reagent was then added and after 5 minutes, 30 µl 34% (w/v) trisodium citrate added. The OD at 630 nm was read after 30 minutes using a Dynatech Laboratories plate reader. The amount of NTP hydrolysed was calculated from an inorganic phosphate standard curve.

2.7.4 Radioactive ATPase Assay

This assay was performed in 1.5 ml microcentrifuge tubes in a 5 µl volume. 3 pmol of enzyme was added to 25 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.1 mM ATP, 5 µM polyuridylic acid, 0.125 µCi γ-[^{32}P] or α-[^{32}P] ATP and incubated for 30 minutes at 25°C. The reaction was stopped with the addition of 1 µl 250 mM EDTA. 2 µl of the reaction was dotted 2 cm from the bottom of a sheet of PEI cellulose and 2 cm apart. This was done 1 µl at a time, allowing the first dots to dry before adding the second. The chromatogram was developed with 0.4 M KH_2PO_4 pH 3.4, air dried and exposed to X-ray film. The autoradiograph was laid over the chromatogram and the regions corresponding to inorganic phosphate and ATP were cut out and the Cerenkov radiation counted using a Packard 1900CA liquid scintillation counter. To demonstrate antibody inhibition of NS3 associated NTPase activity, NS3 was preincubated with an equal volume of HCV +ve or HCV -ve human antisera diluted 1/3 in Tris-HCl pH 7.5 prior to the assay.

2.7.5 PKA assay

In a microcentrifuge tube, 40 µU (0.17 pmol) cAMP-dependent protein kinase A was added to 20 µl of buffer consisting of 50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.1 mg/ml (w/v) BSA, 10 µM ATP (containing 1 µCi γ-[^{32}P] ATP) and 5 µM Kemptide substrate. The reaction mix may also contain an inhibitor protein or peptide. The mixture was incubated for 20 minutes at 30°C and then 15 µl of the mixture placed on a phosphocellulose disc. The disc was then washed twice in 0.5% (v/v) phosphoric acid and twice in MilliQ water for 5 minutes each time. The disc was

then transferred into plastic vials and the radioactivity counted using a Packard 1900CA liquid scintillation counter.

2.7.6 Immunofluorescence of HepG2 Cells for NS3

A glass coverslip was placed into a 35 mm cell culture dish (or 6 well plate was used). HepG2 cells were seeded onto these and grown to 80% confluence, then infected with vT7-3 at a multiplicity of infection of ten pfu/cell. The cells were incubated for 1 hour at 37°C and during this time 4 μg pcDNS3 and 10 μl of lipofectin were mixed with 1 ml of OPTI-MEM culture media. The inoculum was removed from the cells and replaced with the OPTI-MEM and the cells returned to 37°C for a further two hours. The OPTI-MEM was then replaced with 3 ml of fully supplemented RPMI and the cells incubated for 4 hours at 37°C. The cells were then washed with PBS, fixed in 50:50 (v/v) cold methanol:acetone and stored overnight at -20°C. The methanol:acetone was removed and the cells rehydrated in PBS at room temperature for 20 minutes. After this the coverslip was removed from the dish using tweezers and placed face down onto 30 μl of NS3-7 antibody diluted 1/20 in PBS which had been placed of a piece of parafilm for 20 minutes. The coverslip was washed 3 times in fresh PBS and then placed face down onto 30 μl of FITC conjugated swine anti-rabbit antibody (DAKO Ltd) diluted 1/20 in PBS as before. The slide was then washed again 3 times in fresh PBS and then in MilliQ water then placed face down onto 15% glycerol on a clean slide. The slide was then viewed under 400 X magnification with an inverted microscope and tungsten lamp UV light source. Photographs were taken using standard 400 ASA photographic film.

2.7.7 Immunostaining of Hepatocytes for NS3

Liver biopsy specimens were fixed overnight in 10% neutral buffered formalin. The sample was impregnated with paraffin wax using a Tissue Tek Vacuum Impregnating Processor and the biopsy encased in paraffin wax using a Tissue Tek II dispensing console embedding centre. Paraffin sections of 3 - 4 μm thickness were cut on a microtome and transferred to clean glass slides and dried at room temperature for 5 minutes. The slides were then placed in a 56°C oven for 12 - 18 hours, the paraffin wax was removed by washing twice in xylene, twice in ethanol and then under running tap water. The liver sections were circled with wax using a PAP pen and the slides then transferred to a humidity chamber. Endogenous peroxidase activity was blocked by covering the sections with fresh 0.5% H_2O_2 in methanol for 15 minutes and then the sections were washed in water for 5 minutes. The slides were then covered with TBS for 5 minutes then the TBS replaced with fresh TBS for a further 5 minutes. The slides were then incubated with normal swine serum diluted 1/10 in TBS for 10 minutes then replaced with NS3-7 antibody (1/250 dilution in TBS) for 30 minutes. The slides were washed in TBS for 5 minutes and then incubated with biotinylated swine anti-rabbit serum (1/500 dilution in TBS containing normal 1/25 diluted normal human serum) for 30 minutes and then washed again in TBS. The TBS was removed and replaced with streptavidin-biotin complex diluted 1/125 in TBS for 30 minutes and again the slides washed in TBS for 5 minutes. The slides were then incubated for 10 minutes in fresh 0.1% (v/v) diaminobenzidine/TBS solution washed in TBS and then water for 5 minutes each and finally counterstained with Mayer's haematoxylin for up to 5 minutes.

After this, the slides were rinsed in water, then air dried and covered with Tissue-Tek mountant and a glass coverslip placed on top.

As a control, the NS3-7 antibody was depleted of anti-NS3 antibodies by first incubating NS3-7 with NS3 protein (0.1 mg/ml) at a 1:1 ratio for 30 minutes prior to staining.

Slides were viewed and photographed using an Olympus BH-2 microscope. Sites of peroxidase activity were stained brown and the nuclei were stained blue.

Chapter 3: Expression and Purification of Recombinant Proteins

3.1 Expression of NS3 in *E. coli*

Where referred to, all HCV amino acids positions refer to the sequence published by Inchauspe *et al* of HCV H strain (369).

3.1.1 Construction of NS3 Expression Plasmids

The full length NS3 coding sequence was amplified by PCR from plasmid pBRTM/HCV1-3011 using the sense primer NS3-16b1 and antisense primer NS3-16b2 which were designed to introduce a 5' *Nde*I restriction site and 3' *Bam*HI restriction site and initiation and termination codons. The PCR product was ligated into pCRII vector to create the construct pCRNS3 and the sequence of this plasmid was confirmed by DNA sequence analysis to ensure errors had not been introduced during PCR.

To create the construct pAW3(1027-1657) the NS3 gene was excised as an *Nde*I - *Bam*HI fragment and ligated into *Nde*I and *Bam*HI digested pET16b. This plasmid contains the full NS3 coding sequence fused to an N-terminal 10 histidine tag which can be used for identification and purification of the recombinant protein. The plasmid pRS3(1027-1657) was constructed by ligation of the same NS3 coding sequence into *Nde*I and *Bam*HI digested pRSETB vector and contains the full length NS3 coding sequence but no N-terminal tag. The 5' and 3' insert/vector junctions of both plasmids were confirmed by DNA sequence analysis. Plasmid pET3(1038-1657) was constructed by Helen Lavender (Pfizer Ltd) and contains the coding sequence for amino acids 1038

- 1657 of NS3, cloned into pET16b at the *Nde*I and *Bam*HI restriction sites in frame with the 10 histidine tag.

An additional construct that contained the NS3 coding sequence was prepared from the infectious HCV clone of Yanagi *et al* (428). The full length coding sequence for NS3 was generated by PCR as described above. The resulting fragment was digested with *Nde*I and *Bam*HI restriction enzymes and ligated into similarly cut pET16b vector to produce pETRP3(1027-1657). The full nucleotide sequence of the insert was confirmed by DNA sequence analysis.

3.1.2 Construction of NS3 Mutants

3.1.2a Construction of NS3 Helicase/NTPase Mutants

Mutations were made to six amino acids within conserved domains of DEAD box helicases using the Chameleon site directed mutagenesis kit (Stratagene) based on the procedure of Deng and Nickloff, (440) (Section 2.2.10). Amino acid substitutions were made at six positions (K_{1236}, D_{1316}, E_{1317}, C_{1318}, Q_{1486} and R_{1493}). These were selected on the basis of previous mutational studies of HCV NS3 and related helicases (287,289,290) and crystallographic analysis of HCV helicase (306) and were expected to be required for full helicase/NTPase activity (**Figure 3.1**). These residues are proposed to be involved in either the binding and hydrolysis of ATP (K_{1236} [motif I], D_{1316}, E_{1317} and C_{1318} [motif II]) or the coupling of unwinding to NTPase activity (Q_{1486} and R_{1493} [motif VI]). Mutants were named "$X_{1234}Y$" where X is the wild type amino acid, $_{1234}$ is the amino acid position and Y is the mutant amino acid. The mutations constructed were $K_{1236}A$, $D_{1316}A$, $E_{1317}A$, $C_{1318}S$, $Q_{1486}H$ and $R_{1493}K$ and presence of

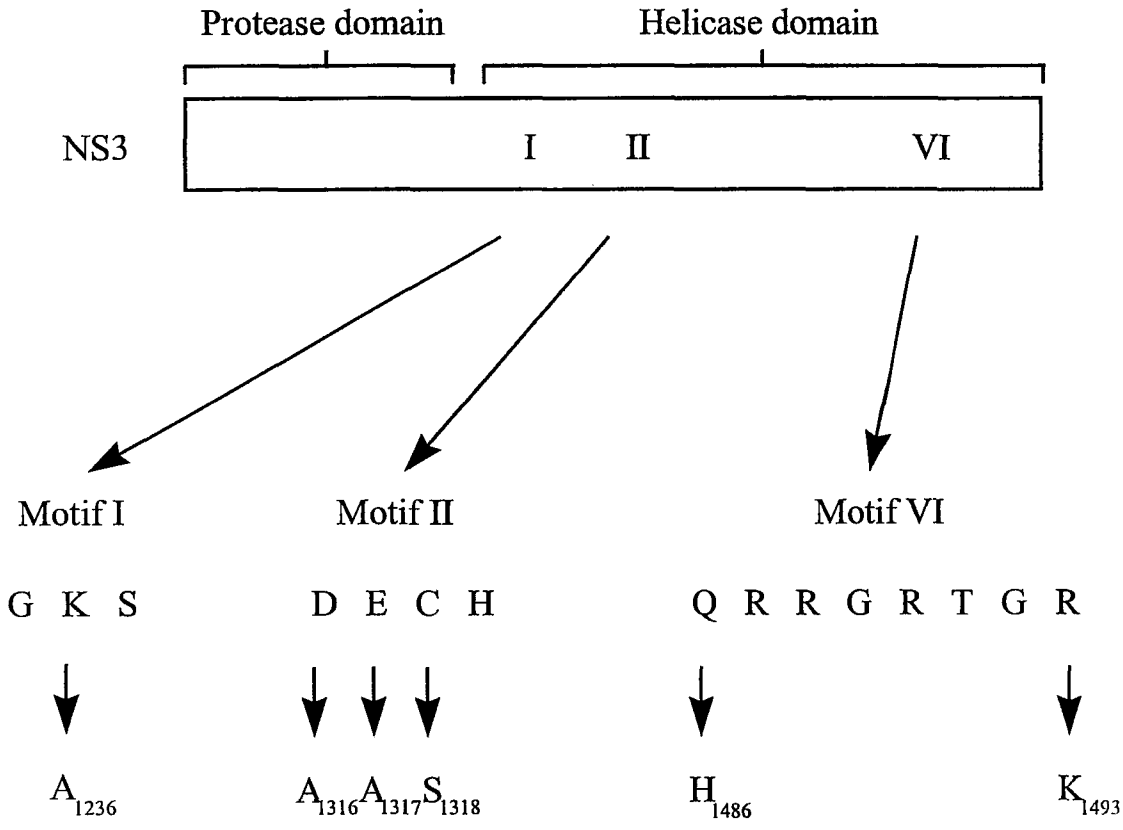


Figure 3.1 Amino acid substitutions made to the conserved DEAD-box helicase motifs I, II and VI of NS3. Single letter code and amino acid position in NS3 are shown.

the mutations was confirmed by DNA sequence analysis. In addition construction of mutant $C_{1318}S$ introduced a novel *Hinf*I restriction site and so the presence of this mutation was confirmed by restriction digest analysis (**Figure 3.2**). The creation of this novel site produced a DNA band of 1.0 kb in place of a 1.5 kb DNA fragment which was observed with wild type sequence (Lane C) following *Hinf*I digestion. Lanes 1, 5, 7, 12 - 14 and 16 each show a 1.5 kb fragment and are wild type NS3, lanes 3, 4, 6, 8 - 11, 15, 17 - 20 and 22 - 24 show a 1.0 kb in place of the 1.5 kb fragment and are $C_{1318}S$ mutants.

3.1.2b Construction of an NS3 Mutant for the Study of PKA Inhibition

The NS3 mutant NS3-RR was created by site directed mutagenesis of pAW3(1027-1657) using the Chameleon site directed mutagenesis kit. This construct was designed to create the HCV type 1b NS3 sequence at the proposed PKA inhibition site. This was done by substitution of amino acids K_{1495} and P_{1496} for R_{1495} and R_{1496} respectively. The presence of the desired mutation was confirmed by DNA sequence analysis.

The parental plasmid, mutagenesis and selection primers, and the created mutant plasmid and NS3 proteins are shown in **Table 3.1**.

3.1.3 Expression of NS3 Protein

Plasmid pAW3(1027-1657) was induced to express NS3 protein in *E. coli* strain JM109 (DE3). To determine the optimum time of expression a time course was

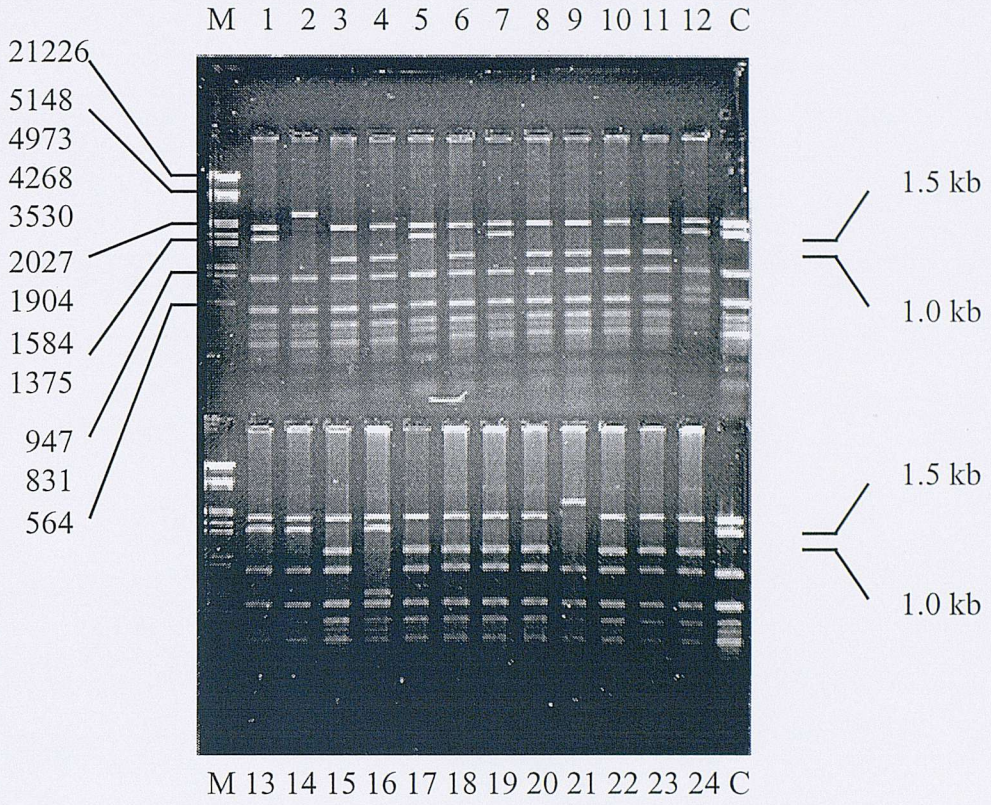


Figure 3.2 Restriction digest of putative $C_{1318}S$ NS3 mutants. Putative mutant clones were incubated with the restriction enzyme *Hinf*I. Samples were adjusted to 1 X agarose gel load buffer and DNA fragments resolved using a 1% agarose gel in 1 X TBE then stained with ethidium bromide. Lanes M; molecular weight marker with sizes shown to the left, lanes C; pAW3(1027-1657) H*inf*I digest, remaining lanes show putative mutant clones numbered 1 - 24. 1.5 kb and 1.0 kb bands are identified.

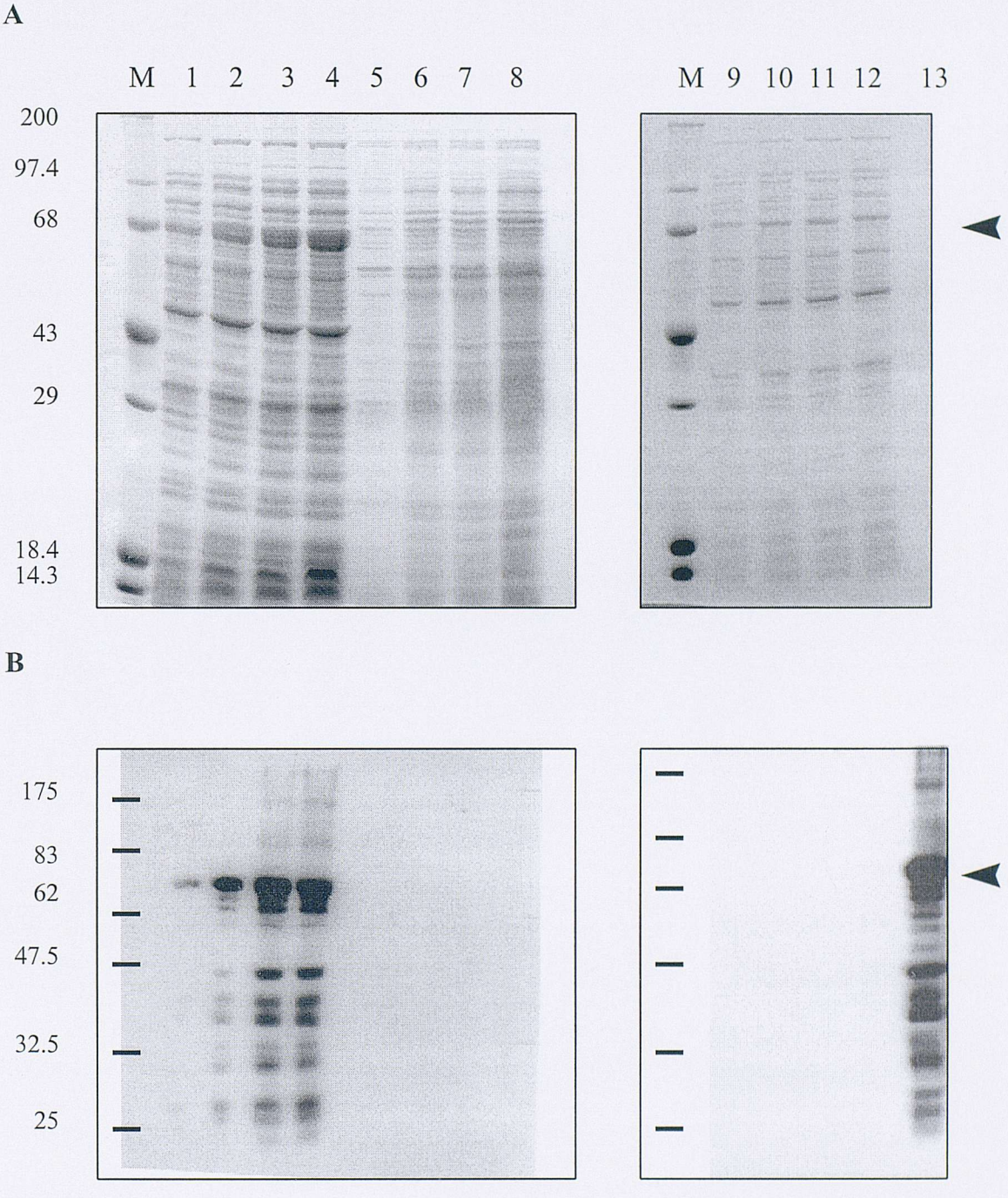
Parental Plasmid	Selection Primer	Mutant Primer	Mutant Plasmid	Mutant NS3 Protein
pAW3(1027-1657)	A6302	B0534	pAW3-KA	$K_{1236}A$
pAW3(1027-1657)	A6302	B0535	pAW3-DA	$D_{1316}A$
pAW3(1027-1657)	A6302	B0536	pAW3-EA	$E_{1317}A$
pAW3(1027-1657)	A6302	A0849	pAW3-CS	$C_{1318}S$
pAW3(1027-1657)	A6302	B0537	pAW3-QH	$Q_{1486}H$
pAW3(1027-1657)	A6302	B0538	pAW3-RK	$R_{1493}K$
pAW3(1027-1657)	A6302	B4913	pAW3-RR	NS3-RR

Table 3.1 Site directed mutagenesis of NS3. Amino acid substitutions were made to plasmid pAW3(1027-1657) by site directed mutagenesis. Shown are the parental plasmid, mutagenesis and selection primers, and the created mutant plasmids and NS3 proteins.

carried out in which samples of culture expressing pAW3(1027-1657) were taken at hourly intervals between zero and 3 hours post induction. *E. coli* strain JM109 (DE3) harbouring pAW3(1027-1657) designated JM109(DE3)[pAW3(1027-1657)] was grown at 37°C in 50 ml LB media containing 100 μg/ml ampicillin in a 250 ml conical flask. Growth was monitored by measuring the optical density at 600 nm (OD_{600}). At an OD_{600} of 0.6 - 0.8 expression of NS3 was induced by the addition of 0.5 mM IPTG and growth continued for 3 hours and 10 ml samples were taken at 0, 1, 2 and 3 hours post induction. Total cell and soluble protein extracts were made as described (Section 2.3.1a) and these were analysed by SDS-PAGE and Western blot and compared to those of *E. coli* strain JM109(DE3) expressing the parental plasmid pET16b (**Figure 3.3A**). By Coomassie Blue stain protein consistent with the expected size of 69 kDa can be seen to accumulate with time in protein samples from JM109(DE3)[pAW3(1027-1657)] (lanes 1 - 4) but is absent in the control expression (lanes 5 - 8). Western blotting of these extracts with the anti-NS3 antibody NS3-7 identified this protein as NS3 (**Figure 3.3B**). Analysis of the soluble fraction of JM109(DE3)[pAW3(1027-1657)] failed to detect any NS3 protein by Coomassie stain or Western blot (Lanes 9 - 12) which demonstrated that all recombinant protein was insoluble. Western blot analysis also detected smaller immunoreactive species not present in control expressions which probably correspond to degradation products of NS3 (**Figure 3.3A** lanes 1 - 4). Immunoblotting with an anti-histidine tag antibody identified only a single band of the expected size for full length NS3 (**Figure 3.4**). This suggests that most degradation originates at the N-terminus, which is consistent with the observations of Steinkuhler *et al* (447) who showed by mass spectrometry and N-terminal sequencing that three major species of NS3 existed, full length and two species truncated at amino acids 1028



Figure 3.3 Time course of expression of NS3 in JM109 (DE3). Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blot was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lanes M, molecular weight markers with sizes given in kDa. Lanes 1 - 4, total cell extract of pAW3(1027-1657) expression, 0 - 3 hours post induction; lanes 5 - 8, total cell extract of pET16b expression, 0 - 3 hours post induction; lanes 9 - 12, soluble protein extract from pAW3(1027-1657) expression, 0 - 3 hours post induction. Lane 13, total cell extract of pAW3(1027-1657) expression included for Western blot positive control. Accumulation of protein representing NS3 is arrowed.


A
M 1 2 3 4 5 6 7 8
M 9 10 11 12 13
200
97.4
68
43
29
18.4
14.3
B
175
83
62
47.5
32.5
25

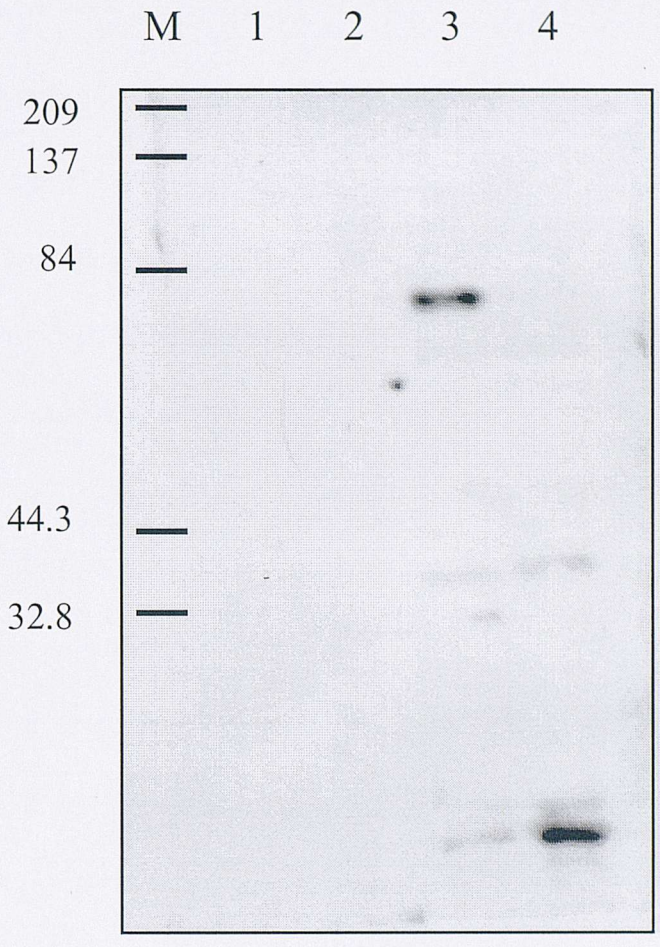


Figure 3.4 Detection of NS3 with an anti-histidine tag antibody. Samples of *E. coli* JM109 (DE3) expressing pAW3(1027-1657) were resolved by SDS-PAGE using a 10% gel before Western transfer. Western blotting performed with anti-histidine tag antibody (1/5000) and HRP conjugated secondary detected with HRPL substrate. Lane M, molecular weight markers; lane 1, uninduced pAW3(1027-1657); lane 2, uninduced pET16b; lane 3, induced pAW3(1027-1657); lane 4, induced pET16b.

(proline) or 1033 (serine). In this report additional degradation products were observed, similar to NS3 degradation observed by others when expressed in *E. coli* (277,278) and insect cells (279,281).

Expression of NS3 using this construct in other *E. coli* strains was unsuccessful. Expression was attempted using BL21 (DE3), BL21 (DE3) pLysS and HMS174 (DE3) using the same conditions described for *E. coli* JM109 (DE3) but NS3 could not be detected by Coomassie stain or Western blot. Western blots of protein samples of pAW3(1027-1657) expression in BL21 (DE3) (**Figure 3.5**), HMS174 (DE3) (**Figure 3.6A**) and BL21 (DE3) pLysS (**Figure 3.6B**) are shown. A smeared band was visible across the western blot in figures 3.6A and B at approximately 70 - 80 kDa. This band appeared to be a non specific reaction with the HRPL substrate since this was also observed with unrelated protein samples (data not shown). However, in cells expressing pAW3(1027-1657) an immunoreactive protein of approximately 45 kDa was detected (**Figure 3.5**). N-terminal sequencing of this protein identified the sequence NH_2-M N L T E L K N T P V S E L I-COOH which was identified as *E. coli* transcription termination factor Rho (Genbank accession number P03002) by a BLASTA homologous sequence search.

3.1.4 Production of Soluble NS3 Protein

Expression of foreign genes in *E. coli* often results in the production of insoluble recombinant protein and so the absence of soluble NS3 protein was not entirely unexpected (448-450). Several strategies were undertaken to improve the yield of soluble protein. The effect of reducing the temperature and IPTG concentration, co-

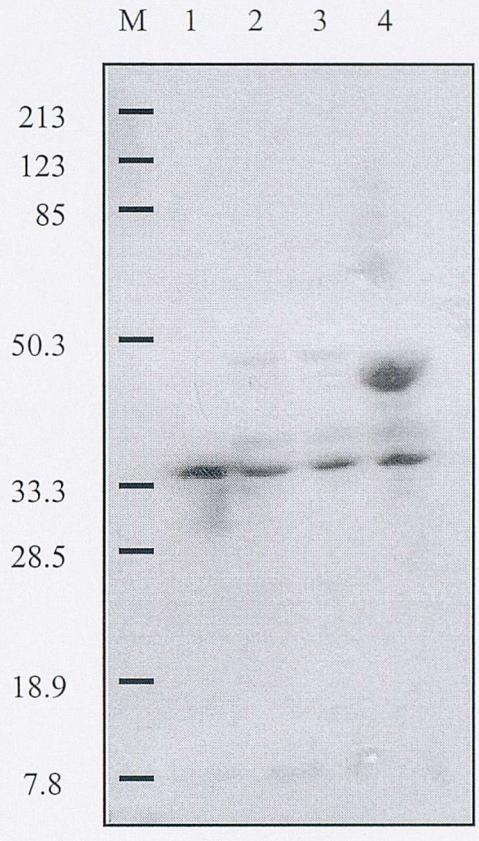
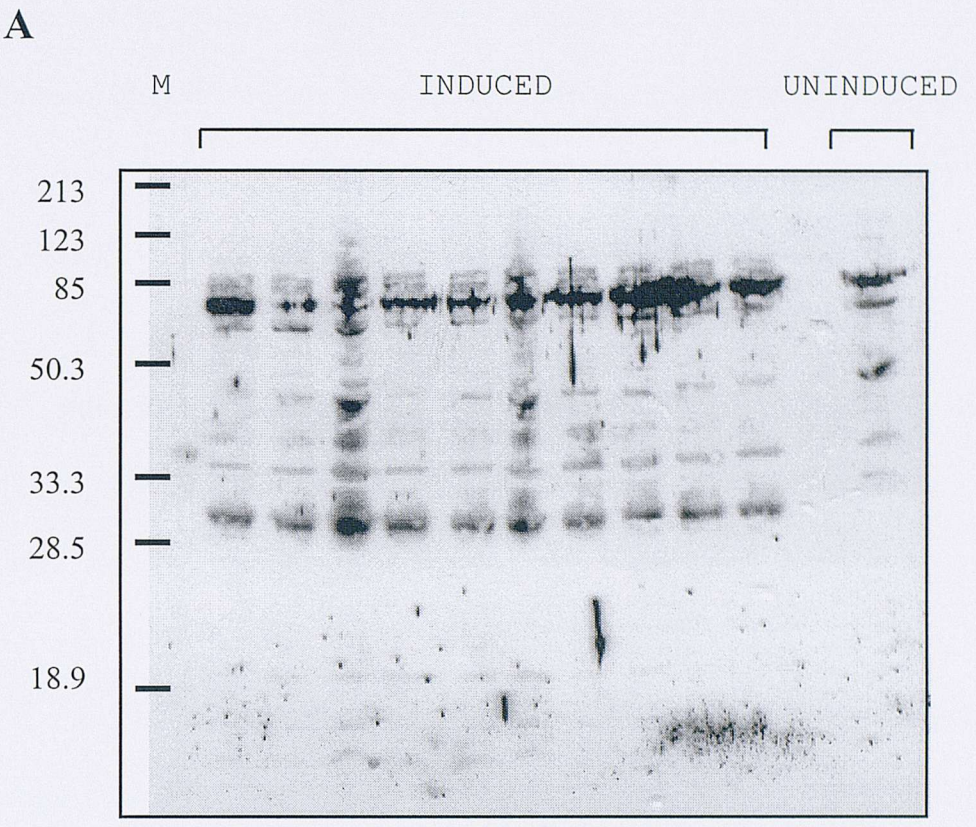
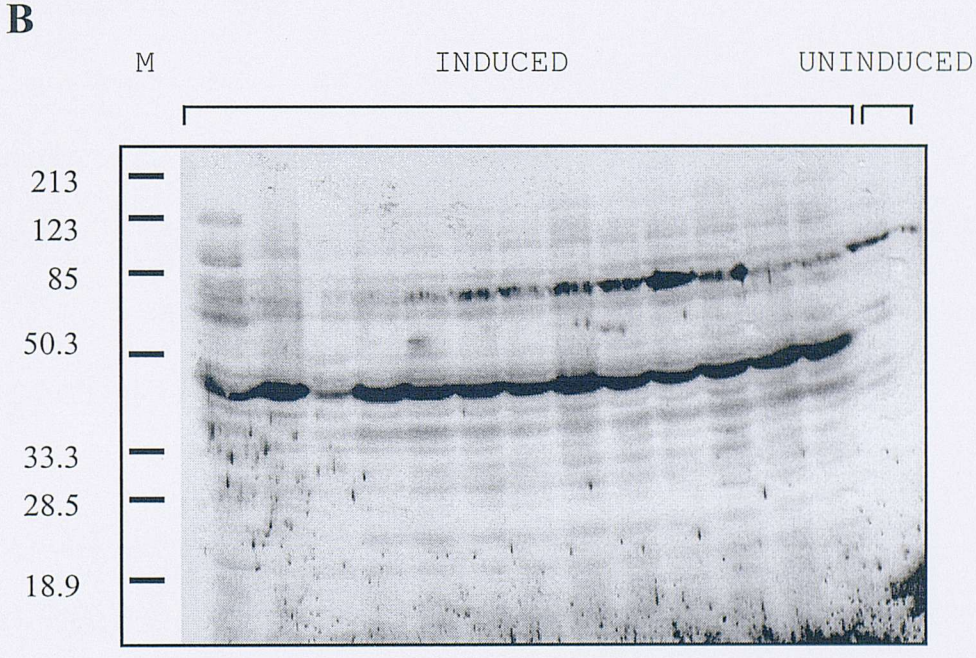


Figure 3.5 Expression of pAW3(1027-1657) in *E. coli* BL21 (DE3). Samples resolved by SDS-PAGE on a 10% gel before Western transfer. Western blotting was performed with FIZ-3 antibody (1/1000) and HRP conjugated secondary was detected with HRPL substrate. Lane M, molecular weight markers with sizes given in kDa. Lane 1, pET16b uninduced; lane 2, pET16b 3 hour induction; lane 3, pAW3(1027-1657) uninduced; lane 4, pAW3(1027-1657) 3 hour induction.



Figure 3.6 Expression of pAW3(1027-1657) in *E. coli* BL21 (DE3) pLysS and HMS174 (DE3). (A); HMS174 (DE3) or (B); BL21 (DE3) pLysS colonies, freshly transformed with pAW3(1027-1657) were picked into 2 ml of LB media, grown to and OD_{600} of 0.6 at 37°C and expression induced for three hours with 0.5 mM IPTG. Each lane represents the extract of a culture grown from an individual colony. Samples were resolved by SDS-PAGE on a 10% gel before Western transfer. Western blotting was performed with FIZ-3 antibody (1/1000) and HRP conjugated secondary was detected with HRPL substrate. Lane M; molecular weight markers with sizes given in kDa.


A
M
INDUCED
UNINDUCED
213
123
85
50.3
33.3
28.5
18.9


B
M
INDUCED
UNINDUCED
213
123
85
50.3
33.3
28.5
18.9

expression of molecular chaperones and use of an enriched growth medium were investigated. Also effects of an N-terminal truncation to the NS3 protein, presence of the N-terminal histidine tag and use of detergents in the cell lysis buffer were examined.

3.1.4a Effect of Reducing the Temperature of Expression

To study the effects of reducing the temperature of expression on NS3 solubility JM109(DE3)[pAW3(1027-1657)] was grown to an OD_{600} of 0.6 at 37°C and the temperature reduced to either 30°C or 25°C. Expression was induced with 0.5 mM IPTG when the desired culture temperature had been attained. 10 ml samples were taken at 0, 1, 2 and 3 hours post induction and total and soluble extracts made. These were analysed by SDS-PAGE and Western blot (**Figure 3.7**). Reducing the temperature had no effect on the solubility of NS3, as protein was not detectable even by Western blot in the soluble fraction.

Reduction of culture temperature during expression studies has been associated with reduced proteolytic degradation of the target protein (451). However in this study NS3 degradation was still observed at lower culture temperatures (**Figure 3.7**).

3.1.4b Induction of Protein Expression With 0.1 mM IPTG

Solubility and correct folding of recombinant proteins may be enhanced by a reduction in IPTG inducer concentration (451). JM109(DE3)[pAW3(1027-1657)] was grown to an OD_{600} at 37°C at which point a zero hour post induction sample was taken and protein expression induced with 0.1 mM IPTG. As before samples were taken at one,

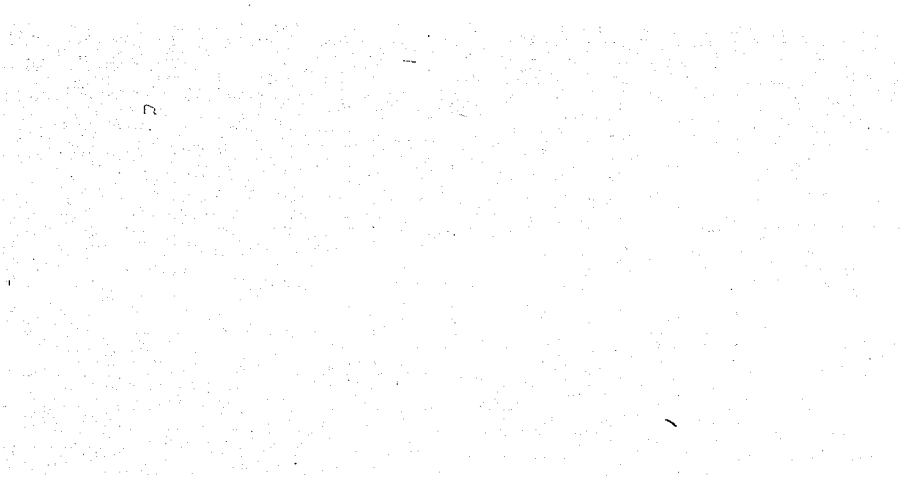
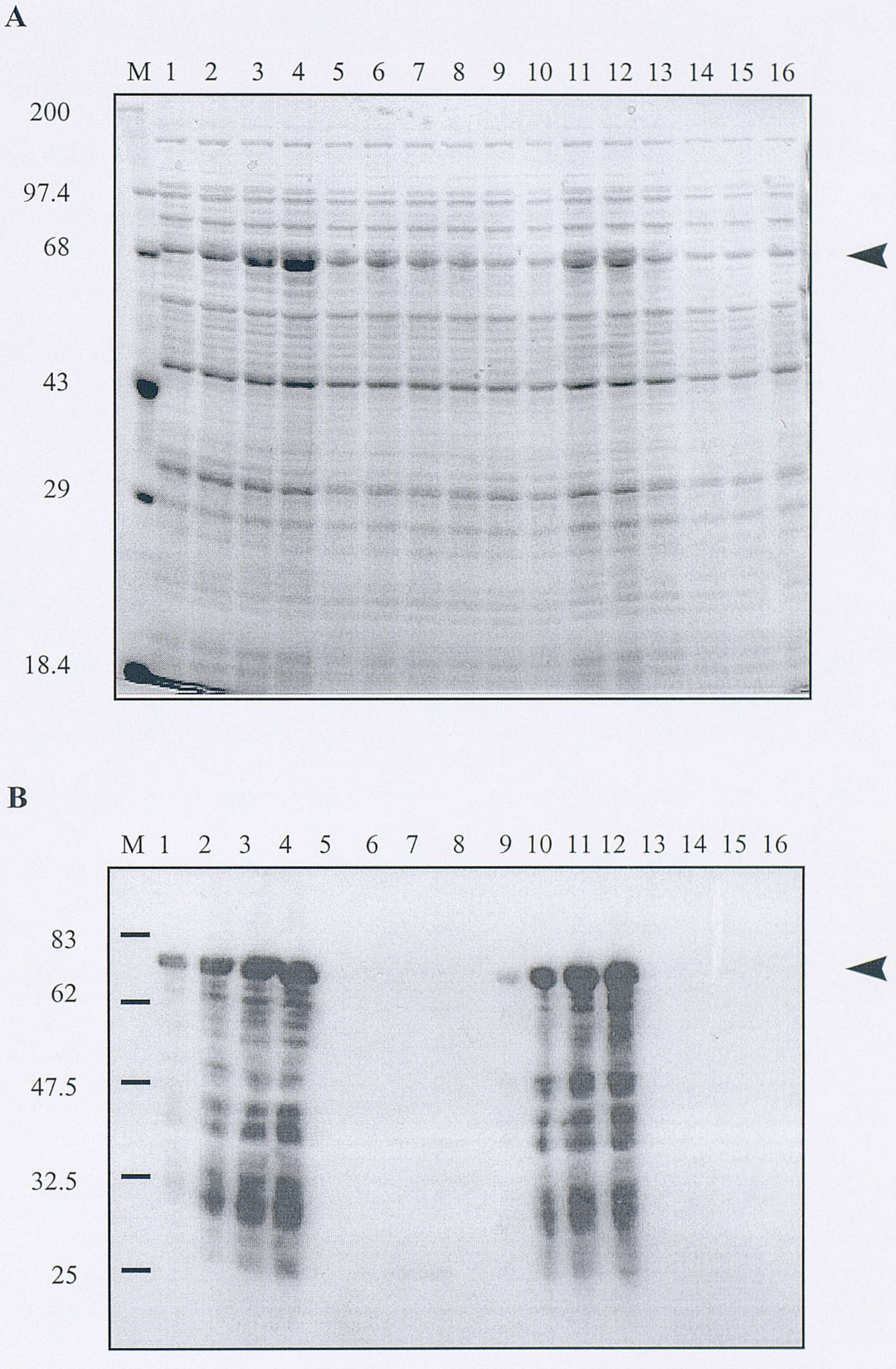


Figure 3.7 Time course of expression of NS3 in JM109(DE3) at 25°C and 30°C. Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lanes M, molecular weight markers with sizes given in kDa. (A) Lanes 1 - 8 show expression at 30°C and 9 - 16 show expression at 25°C. (B) Lanes 1 - 8 show expression at 25°C and 9 - 16 show expression at 30°C. Lanes 1 - 4 and 9 - 12, total cell extract 0 - 3 hours post induction; lanes 5 - 8 and 13 - 16, soluble protein extract 0 - 3 hours post induction. Migration of NS3 is arrowed.


A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
200
97.4
68
43
29
18.4
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
83
62
47.5
32.5
25

two and three hours post induction and total and soluble extracts made. SDS-PAGE and Western blot analysis (**Figure 3.8**) showed similar quantities of NS3 were detected following induction with 0.1 mM IPTG as for 0.5 mM IPTG induction in the total cell extract, but no soluble protein was detected by either Coomassie stain or Western blot (**Figure 3.8** lanes 5 - 8).

3.1.4c Use of Detergents in Lysis Buffer

Detergents can be used to release membrane bound proteins and to prevent protein aggregation. In this study the effects of four detergents were evaluated, Triton-X-100, Nonidet-P40 (N-P40), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulphonate (CHAPS) and n-octyl β-D-glucopyranoside (BOG). A time course of NS3 expression from pAW3(1027-1657) at 37°C was carried out as described above but in 0.5 litre LB. 10 ml samples were taken at each time point. Total cell extracts were prepared in the absence of detergent and soluble extracts made in the presence of standard lysis buffer containing the desired detergent at 1%. **Figure 3.9** shows SDS-PAGE and Western blot of these samples. Soluble NS3 was not detectable in the soluble fraction when extracted in the presence of any of the detergents.

3.1.4d Co-expression of NS3 with the *E. coli* Chaperone GroELS

The *E. coli* chaperone complex GroELS is involved in the folding and stability bacterial and bacteriophage proteins (452,453). GroELS is also effective with heterologous proteins, for example in mediating the assembly of ribulose bisphosphate

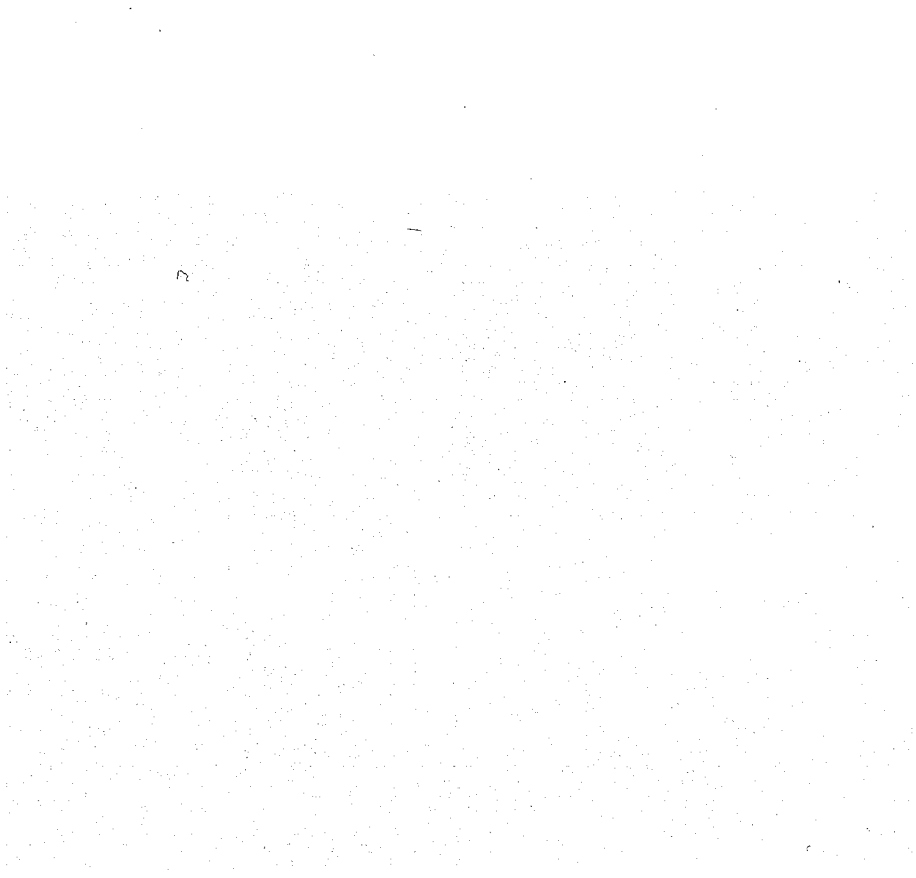
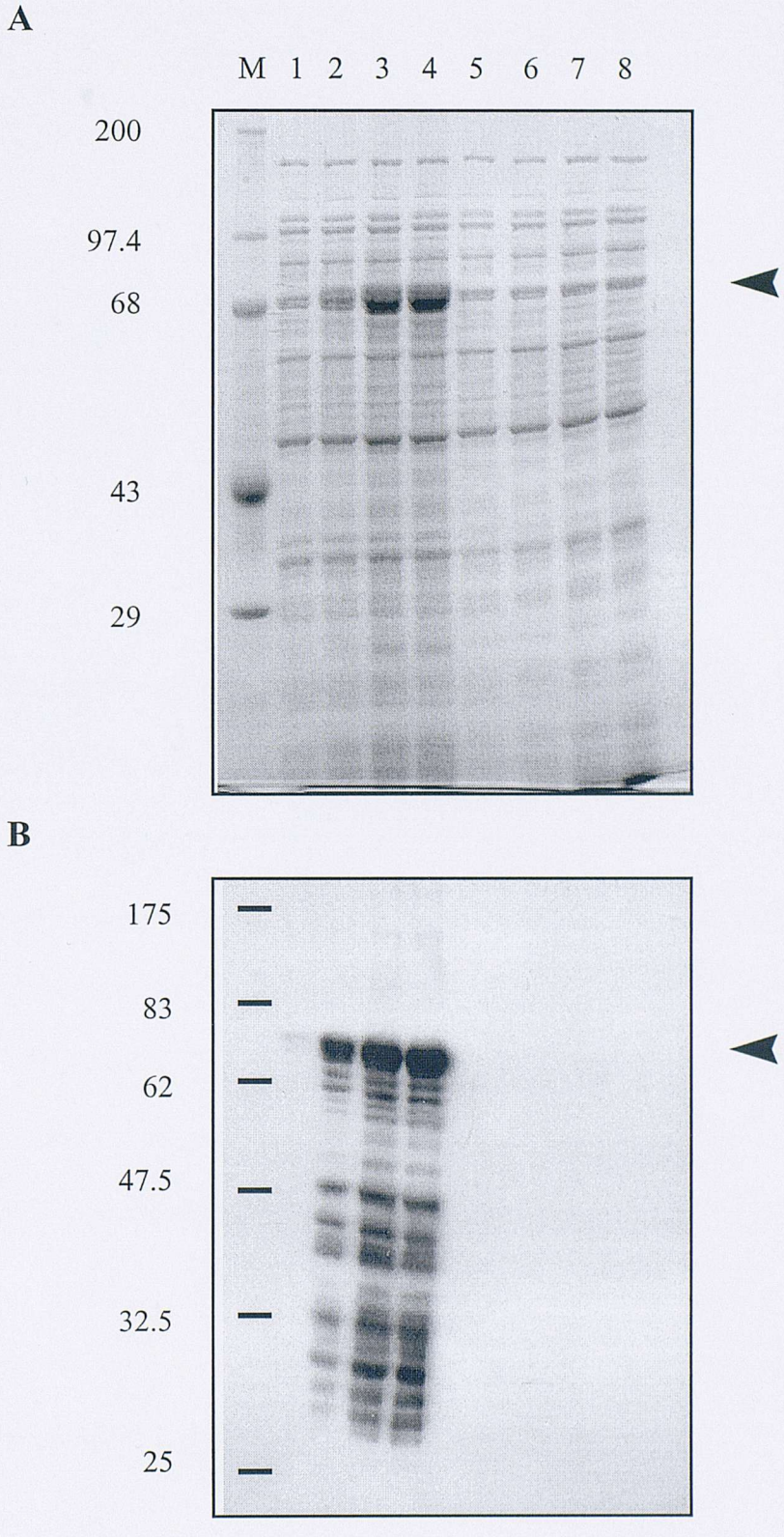


Figure 3.8 Time course of NS3 expression induced with 0.1 mM IPTG. Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blotting was performed with NS3-7 (1/3000 dilution) and HRP conjugated secondary detected with HRPL substrate. Lanes M, molecular weight markers with sizes given in kDa. Lanes 1 - 4, total cell extract 0 - 3 hours post induction; lanes 5 - 8, soluble protein extract 0 - 3 hours post induction. Migration of NS3 is arrowed.


A
M 1 2 3 4 5 6 7 8
200
97.4
68
43
29
B
175
83
62
47.5
32.5
25

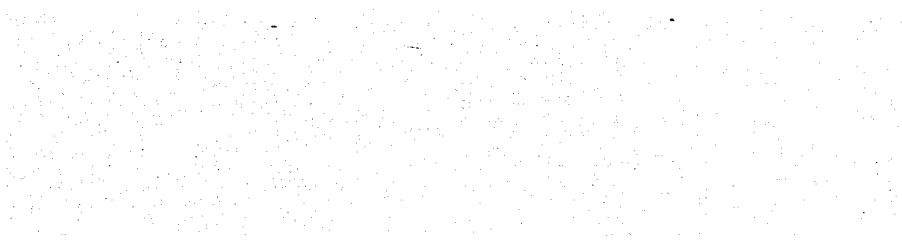
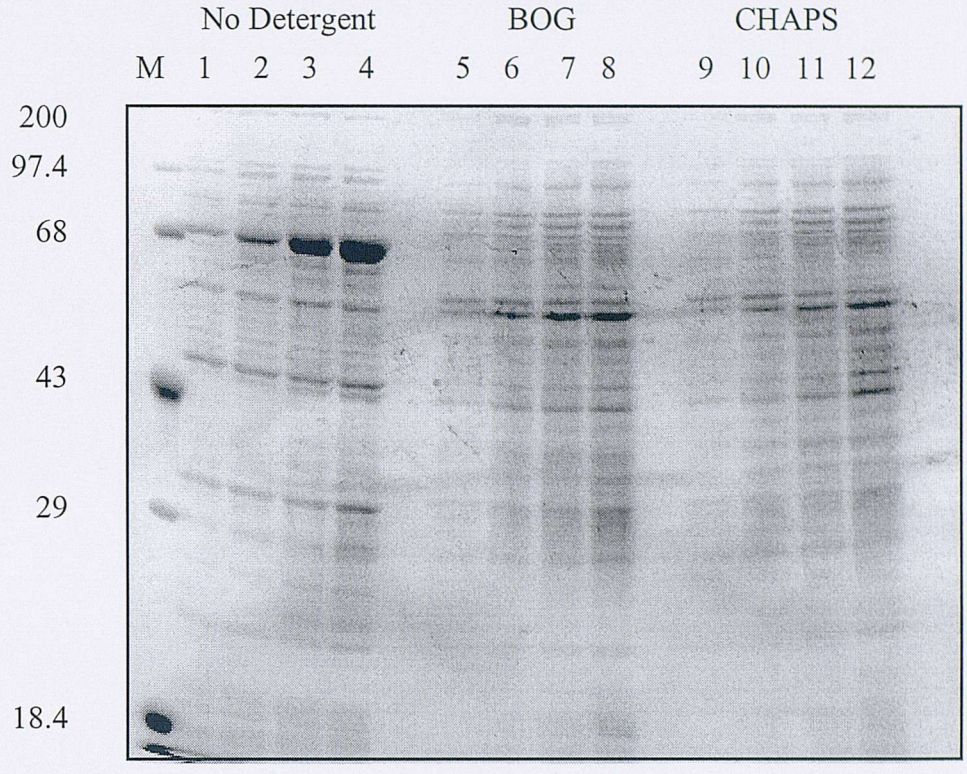
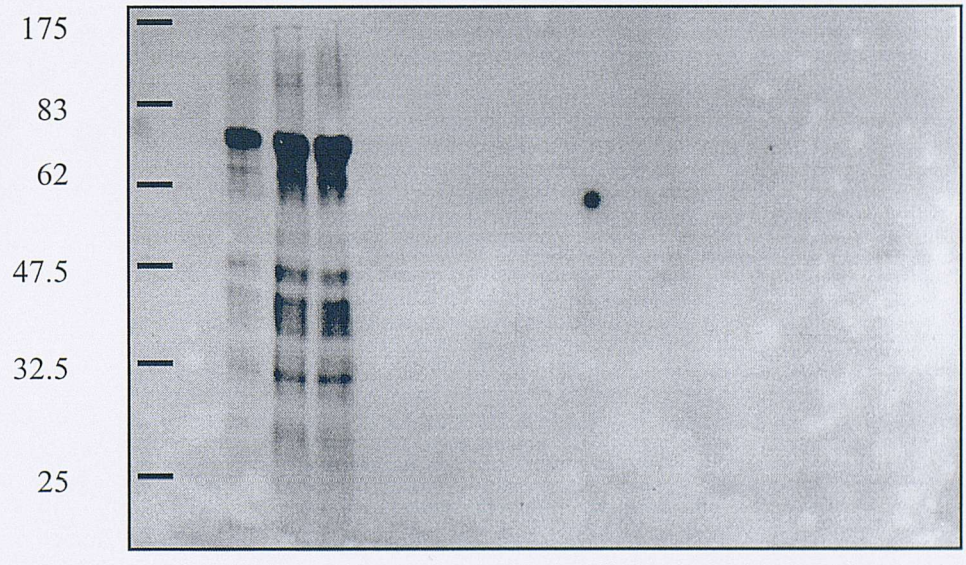


Figure 3.9 Extraction of NS3 protein in the presence of detergents. Samples resolved by SDS-PAGE on a 10 % gel were stained with Coomassie Blue or analysed by Western blotting. Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. (A) Coomassie stained gel and (B) Western blot of soluble extracts prepared in the presence of 1% (w/v) BOG (lanes 5 - 8) or 1% (w/v) CHAPS (lanes 9 - 12) at times 0, 1, 2, 3 hours post induction respectively. (C) Coomassie Blue stained gel and (D) Western blot of soluble extracts prepared in the presence of 1% (w/v) Nonidet-P40 (lanes 5 - 8) and Triton-X-100 1% (w/v) (lanes 9 - 12) at times 0, 1, 2, 3 hours post induction respectively. In all figures, lane M, molecular weight markers with sizes given in kDa; lanes 1 - 4, pAW3(1027-1657) total cell extract 0 - 3 hours post induction control samples.

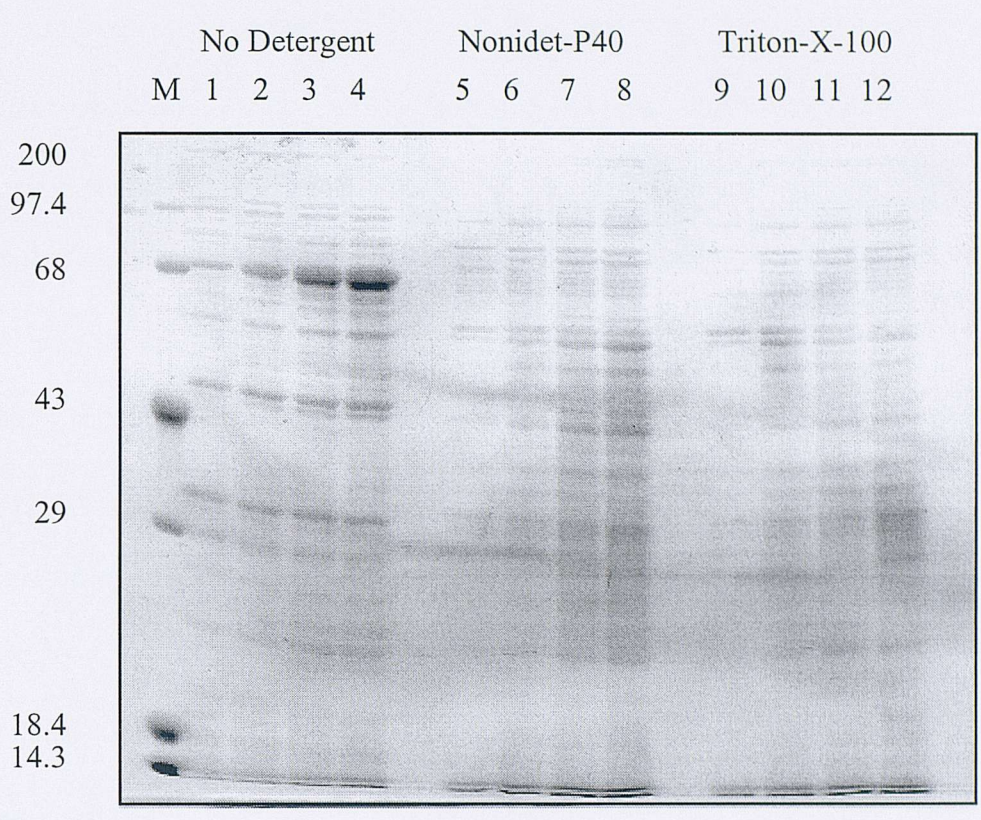
A


No Detergent
BOG
CHAPS
M 1 2 3 4 5 6 7 8 9 10 11 12
200
97.4
68
43
29
18.4

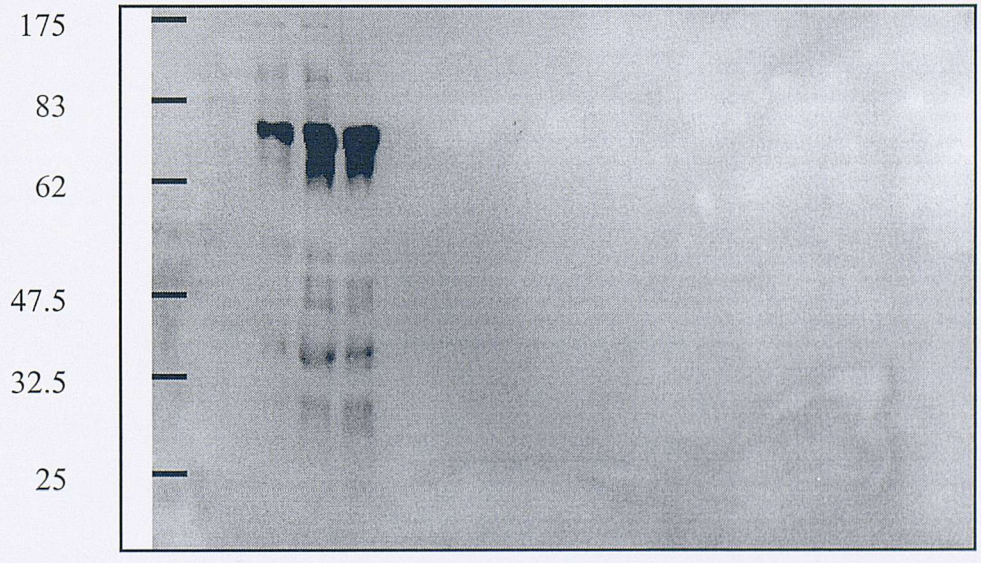
B


175
83
62
47.5
32.5
25

C


No Detergent
Nonidet-P40
Triton-X-100
M 1 2 3 4
5 6 7 8
9 10 11 12
200
97.4
68
43
29
18.4
14.3

D


175
83
62
47.5
32.5
25

carboxylase/oxgenase photosynthetic complexes and procollagenase (438,454). The GroELS complex is composed of the GroEL (~60 kDa) and GroES (~10 kDa) polypeptides encoded by the *groE* operon. This has been cloned in plasmid pMON15903 which is selectable for kanomycin resistance (438). *E. coli* strain JM109(DE3) was co-transformed using pAW3(1027-1657) and pMON15903 and a time course of protein expression carried out as described previously (section 3.1.3) with kanomycin (30 μg/ml) added to the media. Soluble extracts were made and compared to the corresponding insoluble extracts using SDS-PAGE and Western blot (**Figure 3.10**). In the soluble extract protein of approximately 60 kDa corresponding to GroEL was detectable by Coomassie stain (lanes 1 - 4) but absent from the control expression (JM109(DE3)[pET16b]) (lanes 9 - 12). The 10 kDa protein corresponding to GroES has presumably run off the end of the gel. It is therefore clear that this protein was expressed to high levels. However, recombinant NS3 was only detected in the insoluble fraction even in the presence of high levels of GroELS (lanes 5 - 8). Soluble NS3 was not detectable either by Coomassie stain or Western blot.

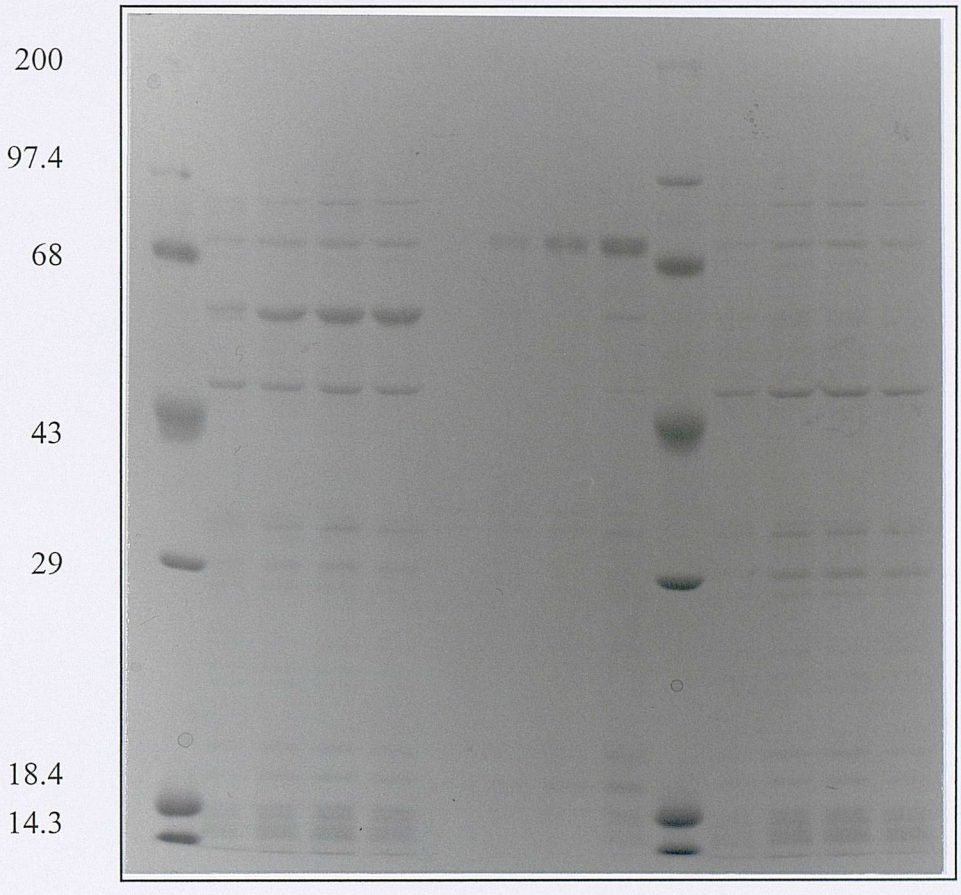
3.1.4e Expression of NS3 in Rich Media

The use of rich media during protein expression has been shown to help to overcome the production of inclusion bodies (448). In this study NS3 was expressed at 37°C and 25°C in Super Broth (**Figure 3.11**). As with previous attempts to improve the yield of soluble protein, no soluble protein was detected at either 37°C or 25°C. Total protein expressed was also greatly reduced compared to previous expression experiments with NS3 just detectable by Coomassie stain in extracts from the 37°C



Figure 3.10 Co-expression of NS3 with the *E. coli* chaperone complex groELS. Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (A) or analysed Western blotting (B). Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lane M, molecular weight markers with sizes given in kDA; lanes 1 - 4, soluble protein extract 0 - 3 hours post induction; lanes 5 - 8, insoluble protein extract 0 - 3 hours post induction; lanes, 9 - 12 (A only) control soluble protein extract from JM109(DE3)[pET16b] 0 - 3 hours post induction.

A


M 1 2 3 4 5 6 7 8 9 10 11 12 13
200
97.4
68
43
29
18.4
14.3

B

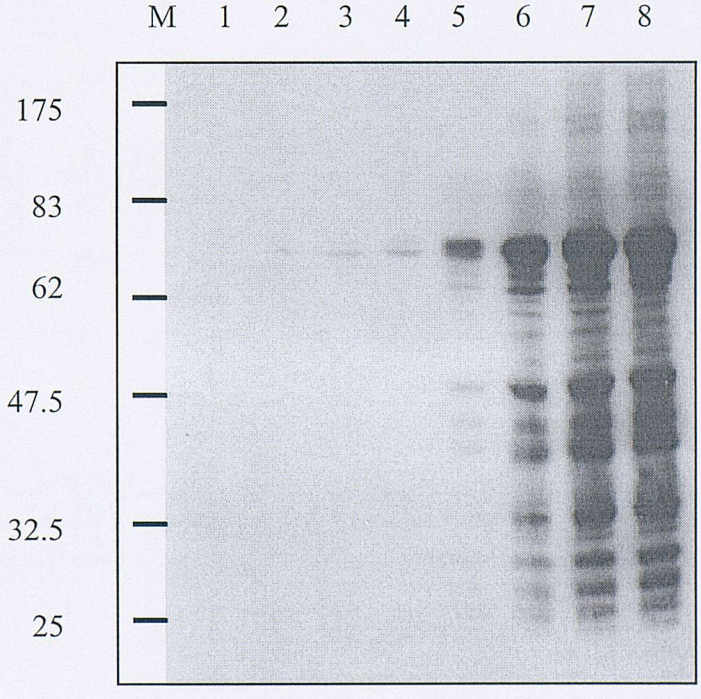
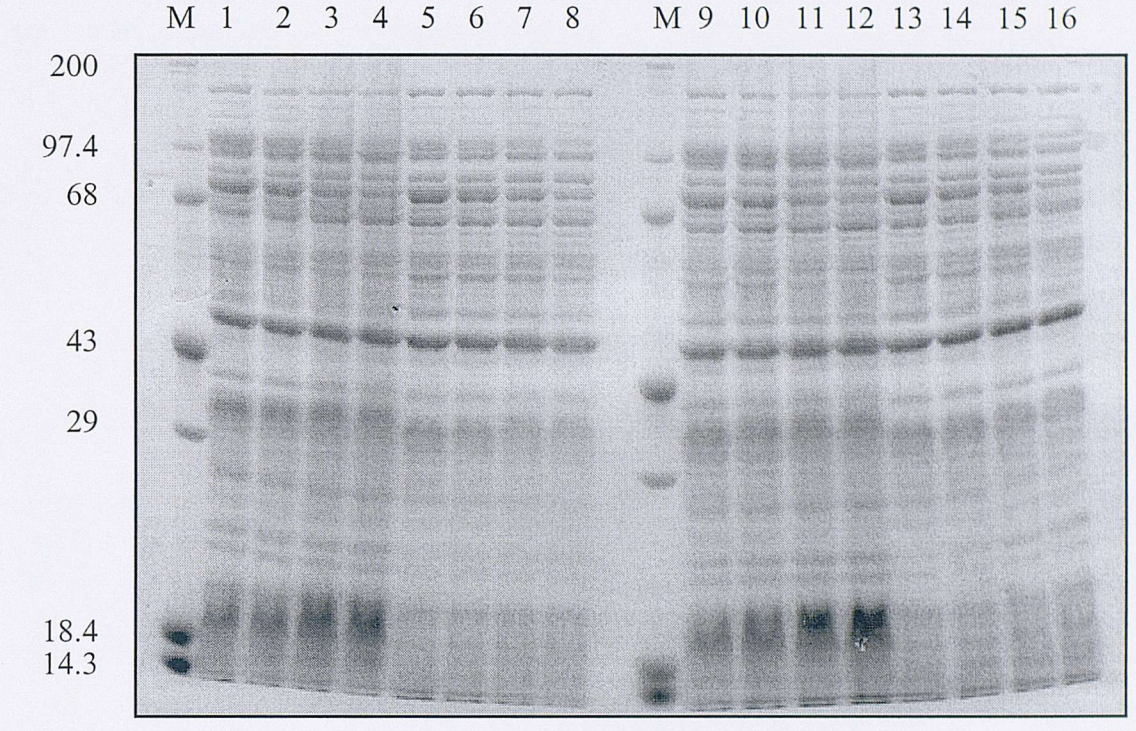

M 1 2 3 4 5 6 7 8
175
83
62
47.5
32.5
25

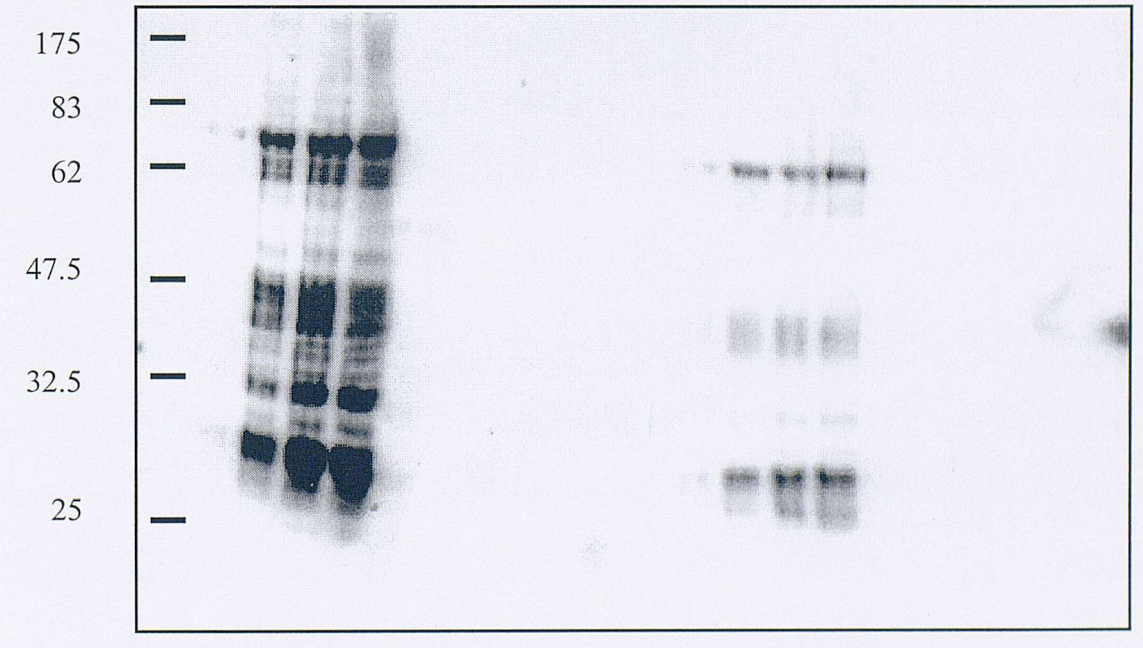


Figure 3.11 Expression of NS3 in Super Broth at 37°C and 25°C. Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (A) or analysed Western blotting (B). Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lanes M, molecular weight markers with sizes given in kDa. Lanes 1 - 8 show expression at 37°C and 9 - 16 show expression at 25°C. Lanes 1 - 4 and 9 - 12, total cell extract 0 - 3 hours post induction; lanes 5 - 8 and 13 - 16, soluble protein extract 0 - 3 hours post induction.

A


M 1 2 3 4 5 6 7 8 M 9 10 11 12 13 14 15 16
200
97.4
68
43
29
18.4
14.3

B


175
83
62
47.5
32.5
25

expression. This may have been caused by the addition of glucose to the media which would have a repressive effect on the *lac* promoter. Also a much greater level of degradation was observed when using Super Broth (**Figure 3.11B**) which may also have contributed to the low level of detectable protein.

3.1.4f Expression of N-terminally Truncated NS3

Plasmid pET3(1038-1657) has the 11 N-terminal amino acids of NS3 deleted which contain hydrophobic amino acids that may affect protein folding and therefore production of soluble protein. A time course of expression was carried out at 37°C and total and soluble extracts analysed by SDS-PAGE and Western blot (**Figure 3.12A**). Expression of this construct produced NS3 detected only in the insoluble fraction.

3.1.4g Expression of NS3 without an N-terminal Tag

Plasmid pRS3(1027-1657) encodes full length NS3 without a histidine tag which may interfere with protein folding and therefore affect solubility. A time course of expression was carried out at 37°C and total and soluble extracts analysed by SDS-PAGE and Western blot (**Figure 3.12B**). NS3 expressed from this construct was predominantly detected in the insoluble fraction. At 2 and 3 hours post induction a band corresponding to NS3 is faintly visible by Western blot (**Figure 3.12B** lanes 7 and 8) however this band represents an insignificant amount of NS3 and is likely to represent carry-over of insoluble protein during preparation of the soluble extract.

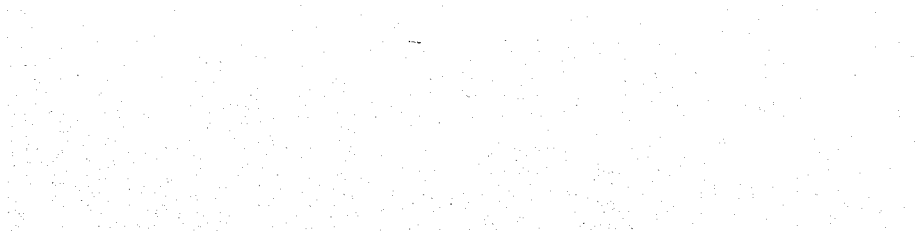
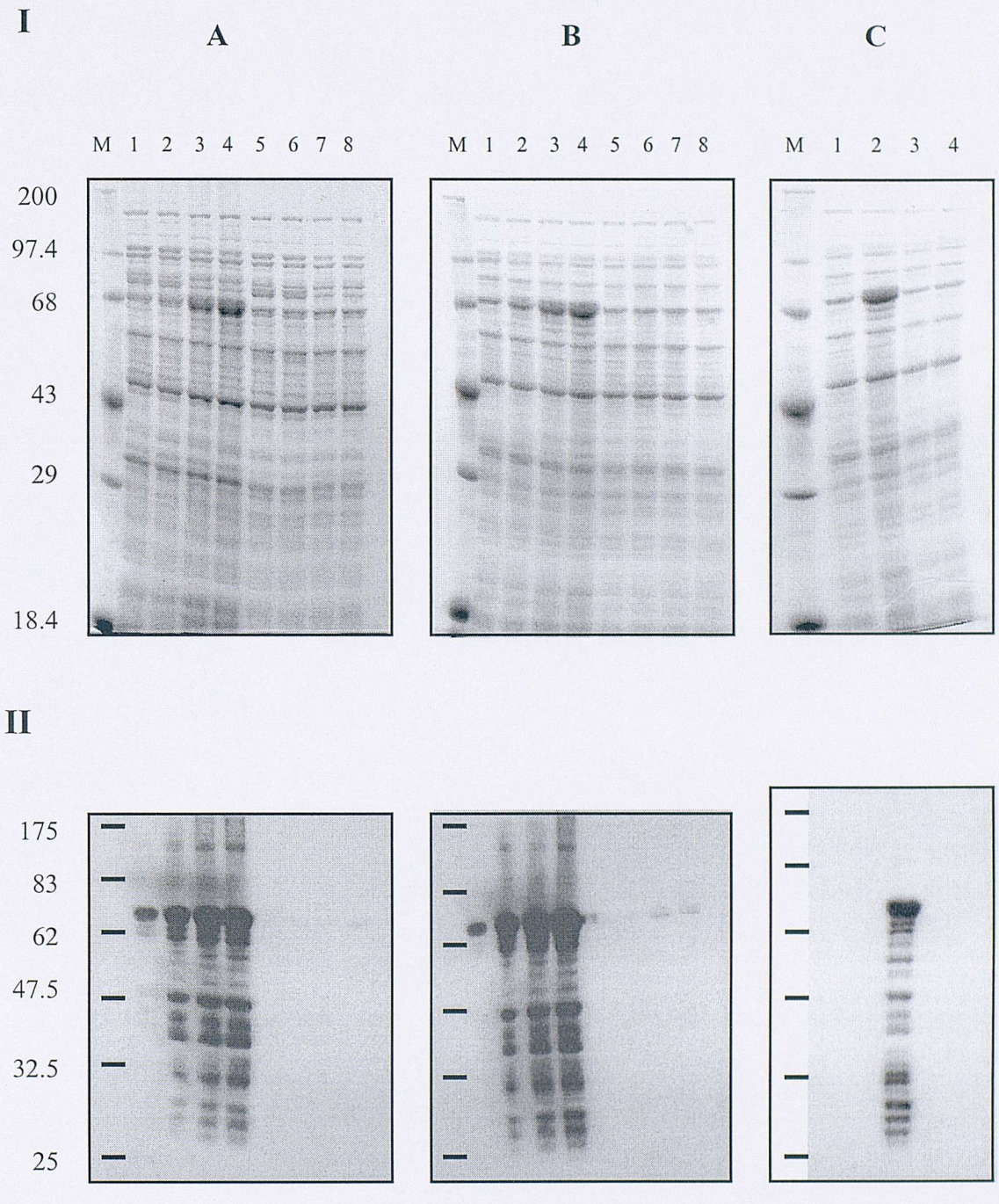


Figure 3.12 Expression of NS3 from pET3(1038-1657), pRS3(1027-1657) and pETRP3(1027-1657). Samples resolved by SDS-PAGE on a 10% gel were stained with Coomassie Blue (I) or analysed Western blotting (II). Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lanes M; molecular weight markers with sizes given in kDa. (A) NS3 expression from plasmid pET3(1038-1657) in *E. coli* JM109 (DE3). Lanes 1 - 4, total cell extract 0 - 3 hours post induction; lanes 5 - 9 soluble protein extract 0 - 3 hours post induction. (B) NS3 expression from plasmid pRS3(1027-1657) in *E. coli* JM109 (DE3). Lanes 1 - 4, total cell extract 0 - 3 hours post induction; lanes 5 - 9, soluble protein extract 0 - 3 hours post induction. (C) NS3 expression from plasmid pETRP3(1027-1657) in *E. coli* JM109 (DE3). Lane 1, total protein 0 hours post induction; lane 2, total protein 3 hours post induction; lane 3, soluble protein 0 hours post induction; lane 4, soluble protein 3 hours post induction.


I
A
B
C
M 1 2 3 4 5 6 7 8
M 1 2 3 4 5 6 7 8
M 1 2 3 4
200
97.4
68
43
29
18.4
II
175
83
62
47.5
32.5
25

3.1.4h Expression of NS3 from an 'Infectious HCV Clone'

Plasmid pETRP3(1027-1657) (section 3.1.1) encodes the sequence for full length NS3 isolated from a HCV cDNA clone (pCV-H77C) (428). A full-length genomic RNA transcribed from this clone was shown to be infectious when introduced into the livers of chimpanzees (428). This sequence was used since it is infectious in chimps and all components of the viral replication machinery must be active. An amino acid sequence comparison of this NS3 sequence and the NS3 sequence coded for by pAW3(1027-1657) was performed and four amino changes were noted which may affect NS3 solubility. These changes, in the order pAW3(1027-1657) - pETRP3(1027-1657), are T_{1190} - A, E_{1202} - G, N_{1429} - S and M_{1531} - T. The crystal structure of NS3 protease domain has been deduced for HCV type 1a and 1b (266,267) and these sequences contained alanine at position 1190. Position 1190, 1429 and 1531 are highly conserved among HCV genotypes (**Figure 3.13**). Polar residues are located at both positions however, a hydrophobic residue (Met) is located at position 1531 in pAW3(1027-1657). The changes at these positions may affect correct folding of NS3 and therefore NS3 protein derived from pCV-H77C may be more likely to form soluble protein. pETRP3(1027-1657) was expressed in *E. coli* JM109(DE3) maintained at 37°C by the addition of 0.5 mM IPTG and samples were taken at zero and three hours post induction (**Figure 3.12C**). NS3 expressed from pETRP3(1027-1657) was detectable by Coomassie stain and Western blot and was only present in the insoluble fraction.

3.1.5 Purification of Insoluble NS3 Protein

```
HVC-H(pAW3...)      RGVTKAVDFI---------PTNGDVVVVS---------WYELMPAETT
pCV-H77C            RGVAKAVDFI---------PTSGCVVVVS---------WYELTPAETT
HCV-J1              RGVAKAVDFI---------PTSGDVVVVA---------WYELTPAETT
HCV-1               RGVAKAVDFI---------PTSGDVVVVA---------WYELTPAETT
HCV-H77             RGVAKAVDFI---------PTSGDVVVVS---------WYELTPAETT

HCV-C2              RGVAKAVDFV---------PTSGCVVVVA---------WYELTPAETS
HCV-KI              RGVAKAVDVF---------PASGCVVVVA---------WYELTPAETS
HCV-J               RGVAKAVDFI---------PTSGDVVVVA---------WYELTPAETS
HCV-BK              RGVAKAVDFV---------PTIGCVVVVA---------WYELTPAETS

HCV-G9              RGVAKAVDVF---------PTTGDVVVVA---------WYELRPAETT

HCV-J6              RGVAKSIDFI---------PTQGCVVVVA---------WYELTPAETT

HCV-J8              RGVAKSIDFI---------PTQGDVVVVA---------WYELTPAETT

HCV-BEBE1           RGVAKSIDFI---------PTQGDVVVVA---------WYELTPAETT

HCV-NZL1            RGVAKSLQFI---------PTTGCVVVCA---------WYDLQPAETT
HCV-K3a             RGVAKALQFI---------PTTGCVVVCA---------WYDLQPAETT

HCV-Tr              RGVAKALQFI---------PVSGDVVVCA---------WYDLQPAETT

HCV-ED43            RGVAKAVDFV---------PTSGDVVVCA---------WYELTPAETT

HCV-EUH1480         RGVAKALEFV---------PATGDVVVCS---------WYDLTPAETT

HCV-EUHK2           RGVAKSLDFI---------PTSGDVVVCA---------WYELTPSETT

HCV-JK046           RGVAKALDFV---------PTSGDVVVCA---------WYELTPAETT

CONSENSUS             A                   X                   X

POSITION            1190                1429                1531
```

Figure 3.13 Comparison of the NS3 sequence of different HCV isolates. Amino acid residues at positions 1190, 1429 and 1531 compared between different isolates of HCV. Sequence pCV-H77C represents the 'infectious' HCV sequence constructed by Yanagi *et al* (428) used for the construction of pETRP3(1027-1657) The consensus amino acid for each position is indicated where X = a polar amino acid. Sequence source; Strain-H (143), pCV-H77C (428), -J1 (373), -1 (132), -H77 (427), -C2 (455), -K1 (169), -J (2), -BK (133), -G9 (456), -J6 (390), -J8 (335), -BEBE1 (457), -NZL1 (458), -K3a (459), -Tr (460), -ED43 (461), -EUH1480 (87), -EUHK2 (462), -JK046 (463).

Due to the inability to detect soluble NS3 and the high level of full-length protein present in the insoluble fraction, it was decided to purify this protein and then refold it for use in activity studies. As mentioned above (section 3.1.4) it is common for recombinant proteins expressed in a bacterial system to form insoluble complexes known as inclusion bodies. Whilst this introduces the problem of obtaining soluble, enzymatically active protein it is possible to isolate these inclusion bodies intact enabling a simple purification strategy to be used since the inclusion bodies consist almost entirely of the recombinant protein.

An overnight culture of JM109(DE3)[pAW3(1027-1657)] was used to inoculate 500 ml LB containing ampicillin and this was grown to an OD_{600} of 0.5. The culture was then allowed to cool to 30°C which typically took 20 minutes. Protein expression was induced by the addition of IPTG to a final concentration of 0.5 mM and the culture transferred to a 25°C incubator. After three hours the cells were harvested by centrifugation. Protein expression was carried out at 25°C in an attempt to reduce the degradation observed previously, however, this temperature produced lower levels of protein (**Figure 3.11**) and so protein expression was induced at 30°C and then the temperature reduced to 25°C.

Inclusion bodies containing NS3 were isolated by a series of sonication washes as described in section 2.3.2. Samples were retained at each stage which corresponded to the total protein extract, soluble protein, washes 1 - 4 and insoluble protein, for SDS-PAGE analysis (**Figure 3.14**). There is no protein present in the supernatant of the final wash prior to solubilisation of the inclusion bodies with 8 M urea (lane 6), demonstrating the removal of contaminating soluble protein. The final lane shows the purified inclusion bodies containing NS3 diluted 1 in 3 with 8 M urea prior to refolding.

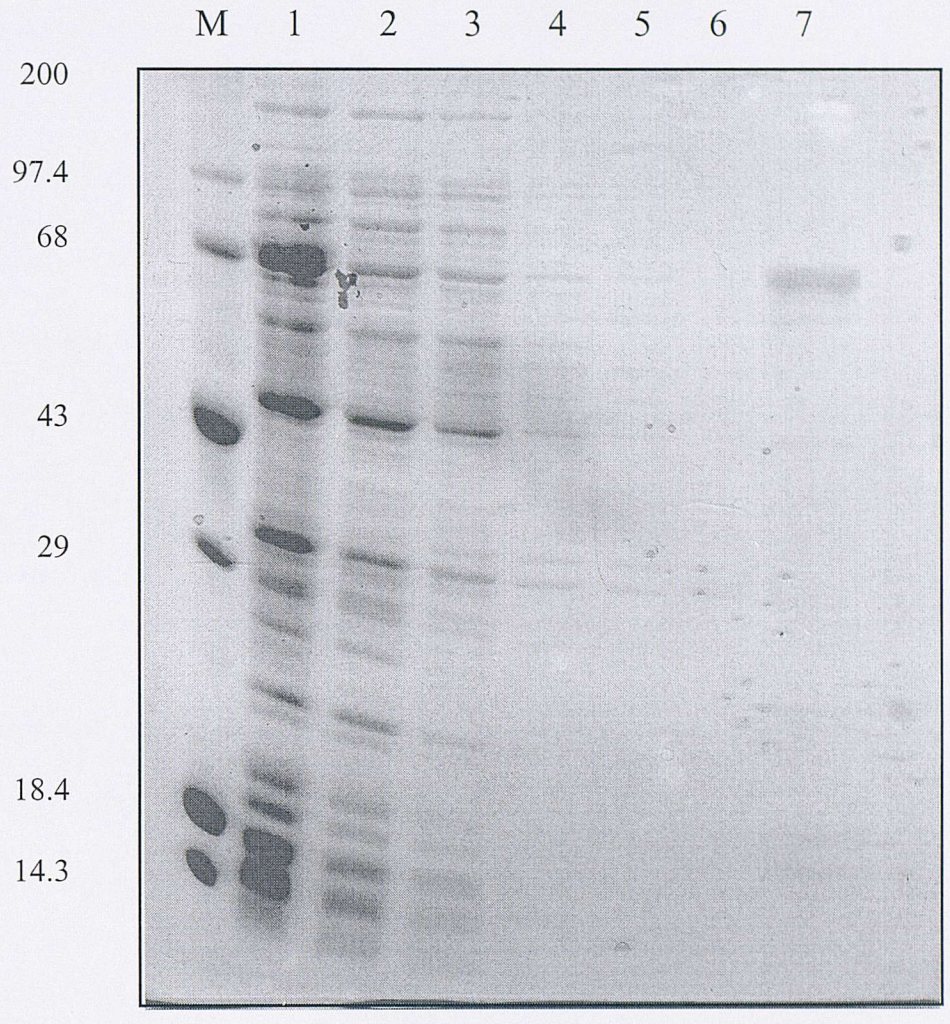


Figure 3.14 Purification of inclusion bodies containing insoluble NS3. Samples were resolved by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Lane M, molecular weight markers with sizes given in kDa; lane 1, total cell extract; lane 2, soluble protein (wash 1); lanes 3 - 6, washes 2 - 5; lane 7, NS3 inclusion bodies solublised with 8 M urea and diluted to 0.2 mg/ml prior to refolding protocol.

The concentration of urea solubilised NS3 was typically 0.8 mg/ml and varied less than 15%, giving an overall yield of 20 mg of recombinant protein from 500 ml of start culture.

Protein degradation was still evident when protein expression was conducted at 25°C. A number of serine protease inhibitors have been shown to have no or little effect on NS3 protease activity (249). It was therefore possible to use inhibitors which would not affect downstream studies of NS3 serine protease activity during protein isolation to reduce degradation of NS3. NS3 inclusion body preparations were therefore prepared in the presence of 1 mM PMSF or Complete™ protease inhibitor cocktail (1 X concentration) and the samples analysed by Western blot and compared to a control sample prepared in the absence of inhibitors (**Figure 3.15**). No reduction in degradation of NS3 was observed in the presence of the protease inhibitors used. It was concluded that the smaller species of NS3 observed were the result of degradation prior to cell lysis.

NS3 mutants were expressed and isolated as inclusion bodies by the same procedure used for wild type NS3 (section 3.1.5). Each mutant was analysed by SDS-PAGE and visualised with Coomassie Blue stain (**Figure 3.16**).

3.1.6 Refolding of Solubilised NS3

Soluble, active NS3 was recovered by dialysis of the urea solubilised protein sample to remove the urea and allow the refolding of the protein. To this end urea was removed from the sample buffer by stepwise dialysis against Buffer R (25 mM Tris-HCl

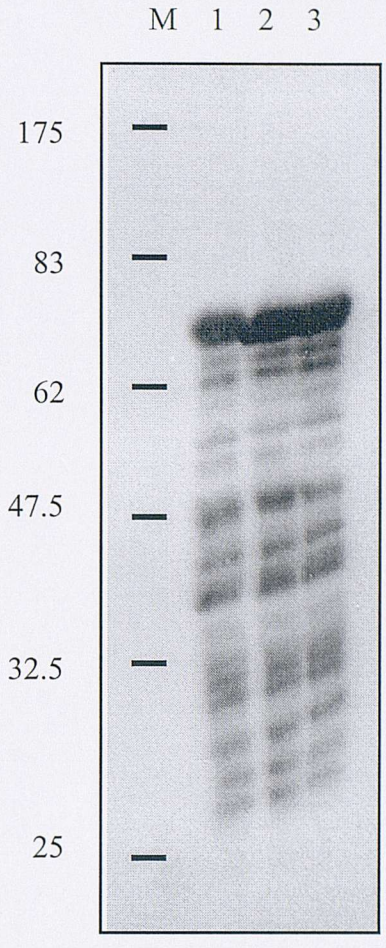


Figure 3.15 NS3 extraction in the presence of protease inhibitors. Samples were resolved by SDS-PAGE on a 10% gel before Western transfer. Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lane M, molecular weight markers with sizes given in kDa; lane 1, NS3 extraction in the absence of protease inhibitors; lane 2, NS3 extraction in the presence of 0.1 mM PMSF; lane 3, NS3 extraction in the presence of protease inhibitor cocktail.

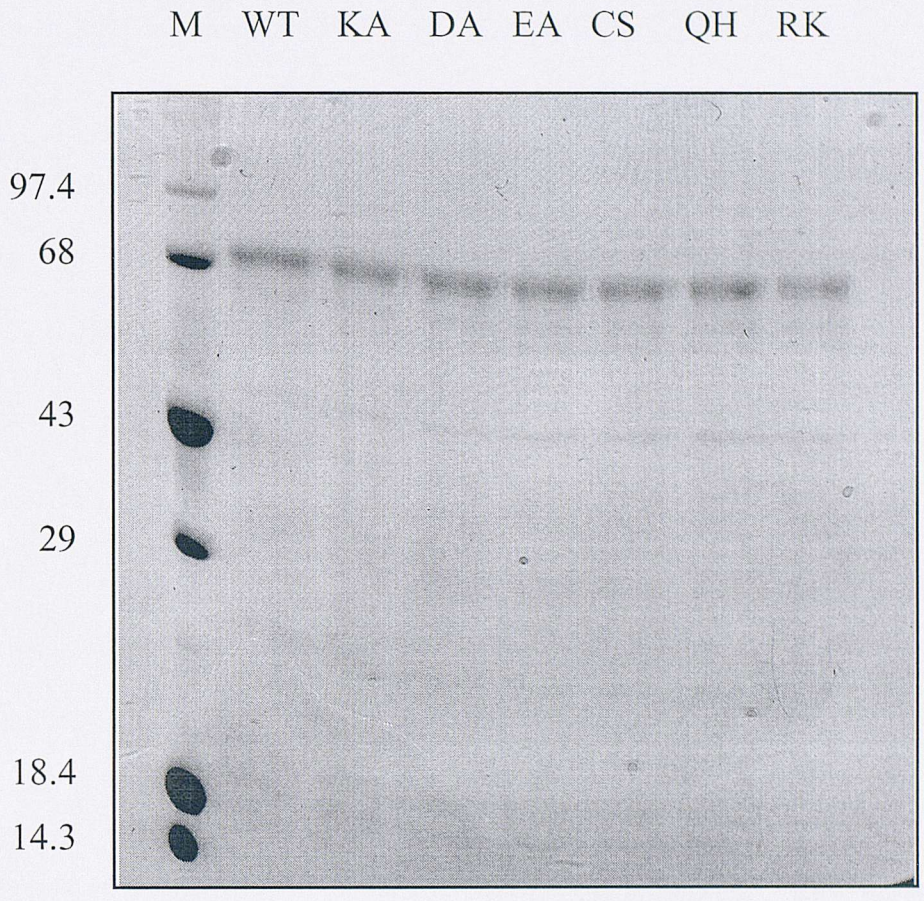


Figure 3.16 NS3 mutant proteins. Samples were resolved by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Lane WT, wild type NS3; KA, mutant $K_{1236}A$; DA, mutant $D_{1316}A$; EA, $E_{1317}A$; CS, $C_{1318}S$; QH, $Q_{1486}H$; RK, $R_{1493}K$.

(pH 7.6), 10% (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.1% CHAPS), which was designed to optimise refolding of NS3.

The protein sample was first dialysed against 20 volumes of Buffer R containing 4 M urea, then 2 M urea, 1M urea and finally 3 changes of Buffer R with no urea to give a final urea concentration of 125 μM. All dialysis was carried out at 4°C and each buffer change was for a minimum of 2 hours. At this stage a large amount of precipitated protein was visible. This was removed by centrifugation at 70,000 g for 30 minutes at 4°C and the supernatant assayed for soluble protein using the Bradford method (section 2.3.3). The typical yield of soluble protein using this method was 25 - 30 μg/ml.

3.1.7 Improving Refolding Efficiency

From a starting concentration of approximately 0.8 mg/ml, 25 μg/ml soluble NS3 was recovered after dialysis. Given that there was no volume change and therefore no dilution effect this represented a recovery of only 3% of the total protein. To improve the recovery of soluble protein, alternate refolding conditions were investigated. Refolding was monitored in the presence of either 0.1% (v/v) Triton-X-100, 0.1% (v/v) Nonidet-P 40, 500 mM NaCl or 10 mM DTT in Buffer R (section 3.1.6) and in the absence of DTT in Buffer R. The method of dialysis was also investigated. In addition to step-wise dialysis, dialysis was carried out directly against Buffer R or the protein sample was diluted first to 1 M urea and then dialysed against Buffer R. The effect of $ZnCl_2$ on refolding was also investigated. X-ray crystallography of the protease domain of NS3 has shown that it has an associated zinc ion (266), and that this zinc ion is essential for protease activity (223). In order to improve the solubility of $ZnCl_2$ it was

necessary to reduce the pH of Buffer R to 7.2 and a control at pH 7.2 in the absence of zinc was also included. Finally the effect of protein concentration prior to refolding was investigated. An equal quantity of NS3 was used for each refolding variable investigated and the success of protein refolding was judged by the concentration of the soluble NS3 recovered.

Following refolding the samples were clarified as described above and the protein present in the soluble fraction was quantified using the Bradford method. The results are shown in **Table 3.2**. There was little difference in refolding observed with any of the buffer variables, as determined by the concentration of the soluble protein recovered and final NS3 concentration was not affected by the protein concentration at the start of the refolding procedure. However, a reduction of the starting protein concentration facilitated the recovery of a greater percentage of protein in a soluble form giving an increase in the yield of protein recovered. Reduction of the buffer pH from 7.6 to 7.2 had no effect on refolding. Based on these results the refolding protocol for future protein preparations was as follows. Protein was diluted from approximately 0.8 mg/ml to approximately 200 μg/ml in 8 M urea, PBS and the urea was removed by stepwise dialysis into 20 volumes of buffer R (pH 7.2) either in the presence or absence of 0.1 mM $ZnCl_2$ containing 4, 2, 1 M urea sequentially. Each buffer change was for two hours minimum and all steps were carried out at 4°C. The protein sample was then dialysed against a buffer suitable for further purification or 20 volumes of Buffer R containing 50% (w/v) glycerol for storage at -20°C which also reduced the urea concentration of the sample to less than 7 μM. The yield of soluble protein was 25 - 30 μg/ml and was concentrated to approximately 120 μg/ml by dialysis against 50% glycerol storage buffer.

Dialysis Buffer	Mode of Refolding	Concentration of Soluble Protein (μg/ml)
Buffer R (standard protocol)	**Step-wise Dialysis**	**30**
R + 0.1% (v/v) Triton X 100	**Step-wise Dialysis**	**25**
R + 0.1% (v/v) Nonidet P40	**Step-wise Dialysis**	**30**
R + 500 mM NaCl	**Step-wise Dialysis**	**25**
R + 10 mM DTT	**Step-wise Dialysis**	**25**
R - DTT	**Step-wise Dialysis**	**25**
R, pH 7.2	**Step-wise Dialysis**	**30**
R, pH 7.2 + 100 μM $ZnCl_2$	**Step-wise Dialysis**	**30**
Buffer R	**One-step Dialysis**	**10**
Buffer R	**Dilution**	**10**
Buffer R (500 μg/ml NS3)	**Step-wise Dialysis**	**30**
Buffer R (250 μg/ml NS3)	**Step-wise Dialysis**	**30**

Table 3.2 Summary of attempts to refold denatured NS3 purified as inclusion bodies. Buffer R (R) as described in the text. Mode of refolding refers to the way in which urea was removed from the buffer or the concentration altered. Following dialysis the precipitated material was removed by centrifugation and the supernatant assayed for protein content using Bradford reagent.

3.1.8 Refolding of NS3 Protein from Different Clones

Protein expressed from plasmids pRS3(1027-1657), pET3(1038-1657) and pETRP3(1027-1657) were analysed for refolding under the same conditions as for full length NS3 expressed from pAW3(1027-1657). Inclusion bodies containing the protein of interest were isolated as described above and refolded as described in section 3.1.6. As before, soluble protein recovered following refolding was quantified using Bradford reagent and compared to that of pAW3(1027-1657) (**Table 3.3**). No improvement in yield of soluble protein was observed with any of these alternative clones.

3.2 Further Purification of Refolded NS3

3.2.1 Metal Chelate Chromatography

A 1 ml HiTrap chelating column (Pharmacia) consisting of reactive IDA (iminodiacetic acid) groups linked to sepharose was prepared as described (section 2.) and equilibrated with buffer R (pH 7.2) minus DTT. NS3, refolded in the absence of $ZnCl_2$ was dialysed to remove the DTT and 1 mg of NS3 applied to the column. During application of the NS3 protein sample, the nickel on the column began to precipitate. This was apparent by the appearance of a brown precipitate and was likely to be caused by residual DTT in the protein preparation. However, column flow was not affected and the purification run was continued. The flow-through containing unbound protein was collected and the column washed with a further 5 volumes of buffer R to ensure residual unbound protein was removed. The bound protein was then eluted using a stepwise

Plasmid	Concentration of Soluble Protein (μg/ml)
pAW3(1027-1657)	30
pRS3(1027-1657)	25
pET(1038-1657)	30
pETRP(1027-1657)	30

Table 3.3 Amount of soluble NS3 recovered following refolding of NS3 expressed from different plasmids.

gradient of 0 - 250 mM imidazole. The collected fractions were analysed by SDS-PAGE and western blot and assayed for NTPase activity using the malachite green assay (**Figure 3.17**). Full length NS3 was found only in the flow through in a purified form and was shown to be active for NTPase activity. The presence of NS3 in the flow-through was not unexpected since the affinity ligand, nickel had precipitated on the column and so was not available to bind the histidine tag to the column. The degradation products of NS3 were found almost exclusively in the first of the elution volumes and only low level NTPase activity was associated with these fractions

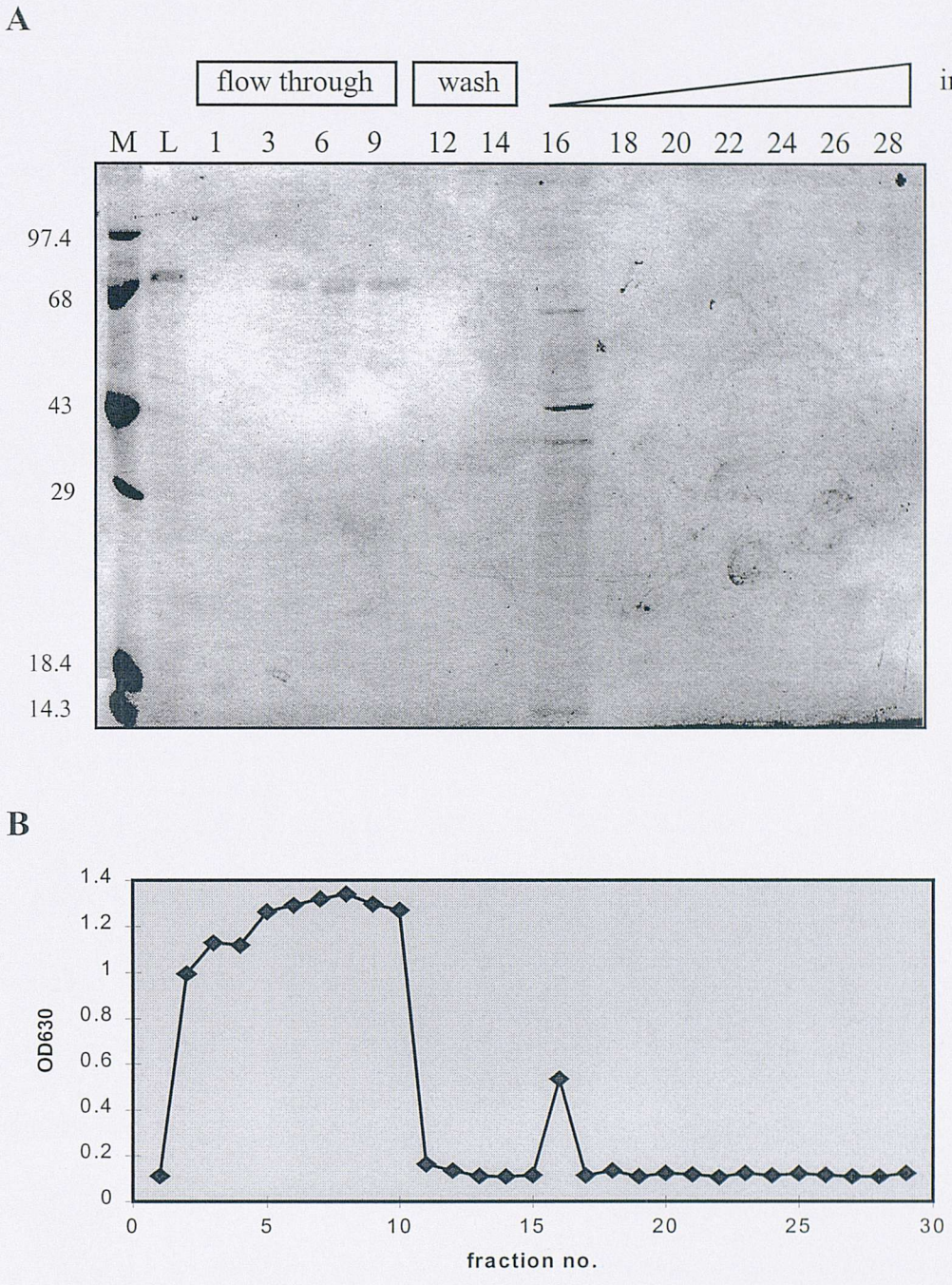


Figure 3.17 NS3 purification by metal chelate chromatography. (A) Eluted fractions were resolved by SDS-PAGE using a 10% gel and stained with Coomassie Blue. Lane M, molecular weight markers with sizes shown to the left; lane L, load sample; numbered lanes refer to the number of the collected fraction. Flow through, wash and eluted fractions are shown. (B) NTPase activity of eluted fractions. 15 µl of each fraction was assayed for NTPase activity using the malachite green method.

(fraction number 16). This confirmed that the observed NTPase activity was a property of full length NS3 and not the degraded proteins. However the elution profile indicated that NS3 was not adsorbed to the affinity ligand and that the NS3 degradation products were adsorbed by non-specific interactions. It was therefore necessary to refine this purification process.

The purification protocol was repeated with two amendments, a fresh protein sample was prepared taking care to avoid DTT contamination and the sample was finally dialysed against the same buffer containing 0.5 M NaCl. This was done to prevent non-specific binding of contaminating proteins (of bacterial origin or NS3 degradation proteins) to the column since it was anticipated that full length NS3 would specifically bind to the column via a polyhisitidine-nickel interaction. Protein was applied to the column and eluted on an imidazole gradient as before, with all buffers containing 0.5 M NaCl. Flow-through and eluted fractions were analysed by SDS-PAGE and Western blot and assayed for NTPase activity. A similar profile was again observed, with the majority of full length NS3 present in the flow-through fractions only and the contaminating proteins in the first elution volumes. A small amount of full length NS3 was observed to elute at 250 mM imidazole, it is assumed that this protein was bound specifically to the column. NTPase activity was not detected in any of the eluted fractions. A likely cause of this was the presence of sodium chloride which has been shown to be inhibitory to NS3 NTPase activity (278) (section 4.2.2). To avoid the possibility that sodium chloride present in the eluates was inhibiting NTPase activity of NS3 the purification procedure was repeated in the absence of NaCl. The eluted samples were treated as before and an identical profile observed. Again the samples were assayed for NTPase activity and again no activity was detected. This result suggested an

alternate/additional cause of NS3 NTPase inhibition. It was hypothesised that nickel ions leaching from the column during chromatography may be responsible for inhibition of activity. Inhibition by nickel and other divalent transition metals of other classes of NTPase has been observed (464) and inhibition of NS3 protease activity by nickel has also been described (251).

To investigate this hypothesis the elution fractions representing flow-through, contaminating protein and eluted full length NS3 were dialysed against buffer R, to look for presence of nickel ions. After two hours dialysis a brown precipitate was observed in all samples as a result of nickel(II) reduction by DTT confirming the presence of nickel in all samples eluted from the metal chelate column. To test for inhibition of NTPase activity by nickel, NTPase activity was assayed in the presence of increasing amounts of nickel and the results shown in **Figure 3.18**. NTPase activity is severely reduced in the presence of 1 mM $NiSO_4$ and completely absent in 10 mM $NiSO_4$. The results indicate that NS3 NTPase activity is inhibited by the presence of nickel at concentrations comparable to those expected from the metal chelate column. In anticipation of the presence of nickel in the eluted samples removal of nickel by dialysis was attempted to restore NTPase activity. Following dialysis the protein samples were assayed for NTPase activity (**Figure 3.18**). No activity was observed for these samples and it was concluded that nickel was likely to be bound to NS3 preventing its removal by dialysis. Dialysis was also performed in the presence of excess magnesium in an attempt to compete out nickel binding but it was still not possible to restore NTPase activity (**Figure 3.18**).

In response to the inability to further purify active full length NS3 using metal chelate chromatography an alternate protocol was investigated.

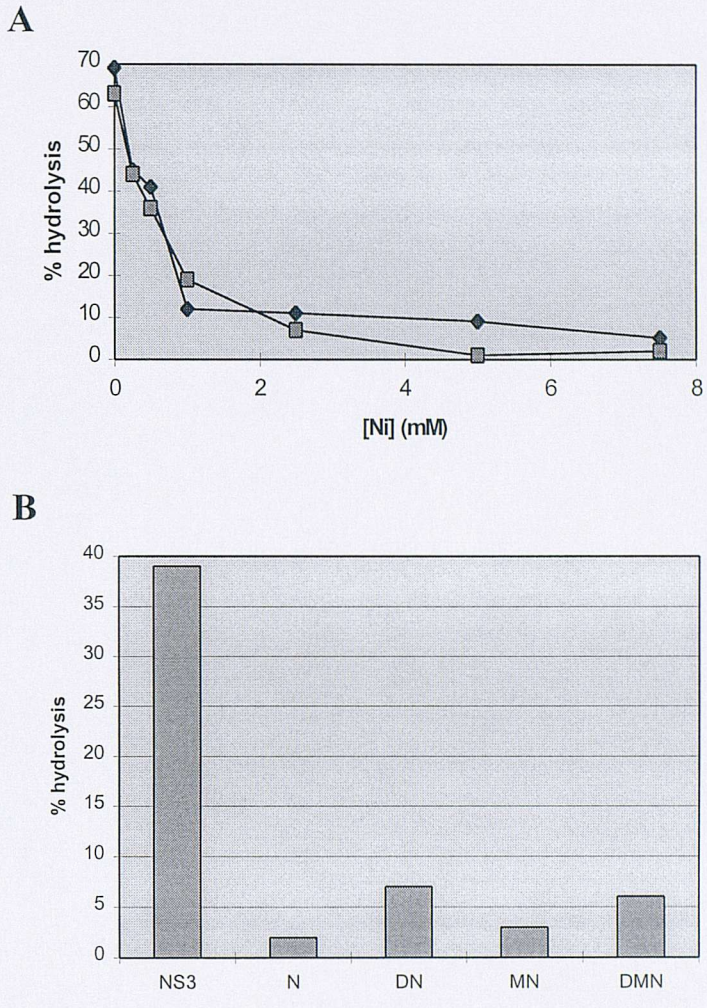


Figure 3.18 Nickel inhibition of NS3 NTPase activity. (A) NTPase activity was monitored in the presence of increasing concentrations of $NiSO_4$ using the radioactive method of NTPase assay at 2.5 mM (■) and 5 mM $MgCl_2$ (◆). (B) Recovery of NS3 NTPase activity following dialysis to remove nickel contaminants. Refolded NS3 (NS3) was adjusted to 0.1 M $NiSO_4$ (N) and dialysed against buffer R (DN) or adjusted to 0.1 M $NiSO_4$, 0.1 M $MgCl_2$ (MN) and dialysed against buffer R (DMN). NTPase activity of each sample (NS3, N, DN, MN, DMN) was assayed. NTPase activity assayed by hydrolysis of $\gamma[^{32}P]ATP$ to $\gamma[^{32}P]Pi$.

3.2.2 Heparin Sepharose Chromatography

A 1 ml HiTrap Heparin sepharose column (Pharmacia) was prepared as described (section 2.) and equilibrated with buffer R. Refolded NS3 protein was prepared as described above in section 3.1.6 and 2 mg applied to the column. Flow through fractions were collected and the column washed thoroughly. The sample was then eluted from the column with a 0 - 1 M NaCl stepwise gradient. Fractions were collected and analysed by SDS-PAGE and Western blotting (**Figure 3.19**). A broad elution profile was observed with NS3 eluted across almost the entire gradient range. No purification of full length NS3 from the degraded species was observed with similar proportions of full length protein to contaminating species throughout the elution range.

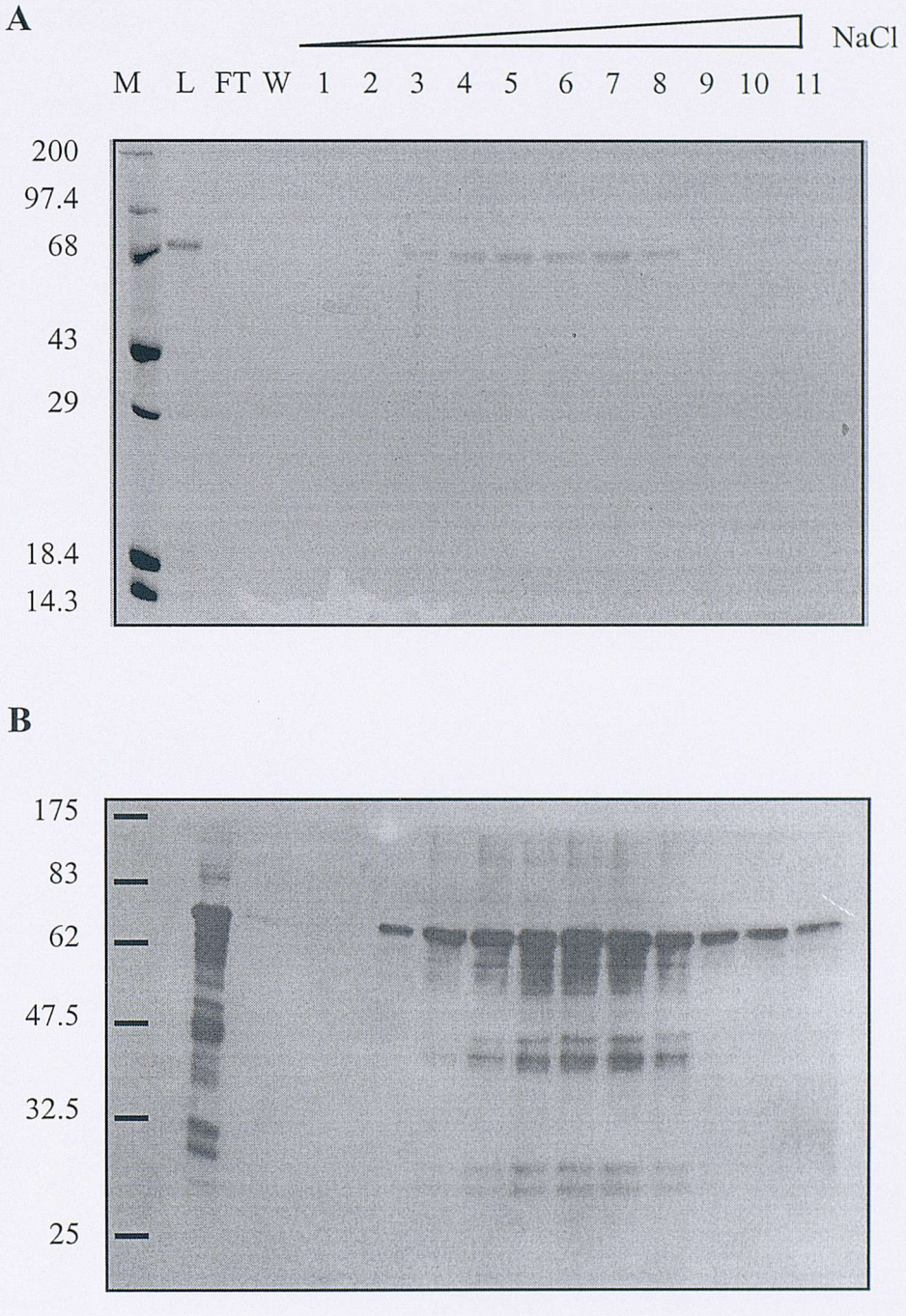
3.3 Expression of NS3 in Insect Cells

3.3.1 Construction of NS3 Expressing Recombinant Baculovirus

The NS3 coding sequence was excised from pCRNS3F using *Nde*I *Bam*HI restriction endonucleases and ligated into a similarly cut transfer vector pVL1393 (Invitrogen) to create the plasmid pVLNS3F and the sequence confirmed by DNA sequence analysis. This transfer vector was used to introduce the NS3 coding sequence into a linearised baculovirus genome by homologous recombination as described in section 2.6.7 to create the recombinant baculovirus NS3-3 which encodes the complete NS3 coding sequence (aa 1027-1657). Recombinant virus stock was prepared as described (section 2.6.9) and passaged three times to obtain a (P3) stock which was then used for expression studies. A second recombinant baculovirus, NS3-2 previously



Figure 3.19 NS3 purification using heparin sepharose affinity chromatography. Fractions were collected and analysed by SDS-PAGE. The samples were resolved using a 10% gel and stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blotting was performed with NS3-7 (1/3000) and HRP conjugated secondary detected with HRPL substrate. Lane M, molecular weight markers with sizes shown to the left; lane L, load sample; FT, flow-through; W, wash; numbered lanes refer to the number of the collected fraction.


A
NaCl
M L FT W 1 2 3 4 5 6 7 8 9 10 11
200
97.4
68
43
29
18.4
14.3
B
175
83
62
47.5
32.5
25

constructed in this laboratory by the same procedure was also used. NS3-2 was prepared before the authentic NS3 termini was known and therefore encodes the region 1007 - 1630, not the authentic 1027 - 1657. The presence of the NS3 coding sequence in the baculovirus genome was confirmed by PCR analysis of total cellular DNA extracted from Sf9 cells infected with NS3-2 or NS3-3. Primer pair P8198 and P8199 were used to amplify the full 1.9 kb NS3 coding sequence (nt 3079 - 4971) from NS3-3 (**Figure 3.20A**). This primer pair was used to amplify the original PCR fragment cloned in pCRNS3F and so were unsuitable for amplification from NS3-2. A 1.3 kb fragment corresponding to the central region of NS3 (nt 3441 - 4695) was amplified from both NS3-3 and NS3-2 using the primers A8038 and A8040 (**Figure 3.20B**).

3.3.2 Expression of NS3 Using Recombinant Baculovirus

Monolayers of Sf9 cells in a T-25 flask were infected with either NS3-2, NS3-3 or wild type baculovirus to an MOI of 10 or mock infected (i.e. no virus). Cells were harvested at 24, 48 and 72 hours post infection, protein extracts were made and these subjected to SDS-PAGE Western blot analysis (**Figure 3.21**). Full length NS3 with an expected molecular mass of 69 kDa was not detected in samples of either NS3-2 or NS3-3. Instead a truncated product of approximately 40 kDa and smaller degradation products were detected with both constructs (lanes 4 and 5, 13 and 14). Sequence analysis was performed at each stage of recombinant virus production and viral DNA extracted from NS3-3 and NS3-2 infected cells was examined and the presence of the NS3 coding sequence confirmed by PCR analysis (**Figure 3.20**). In addition a similar pattern of NS3 degradation has been observed by others when expressed in insect cells

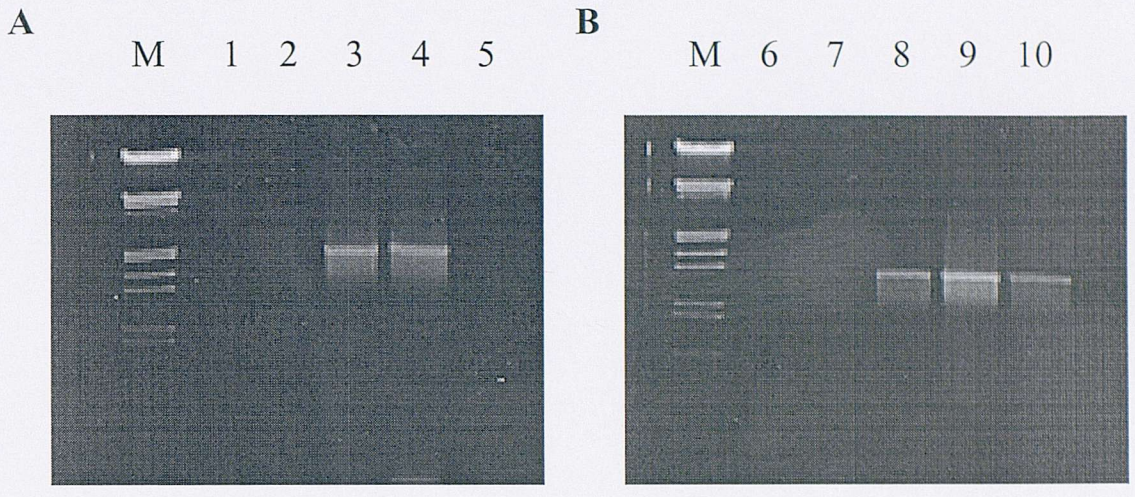
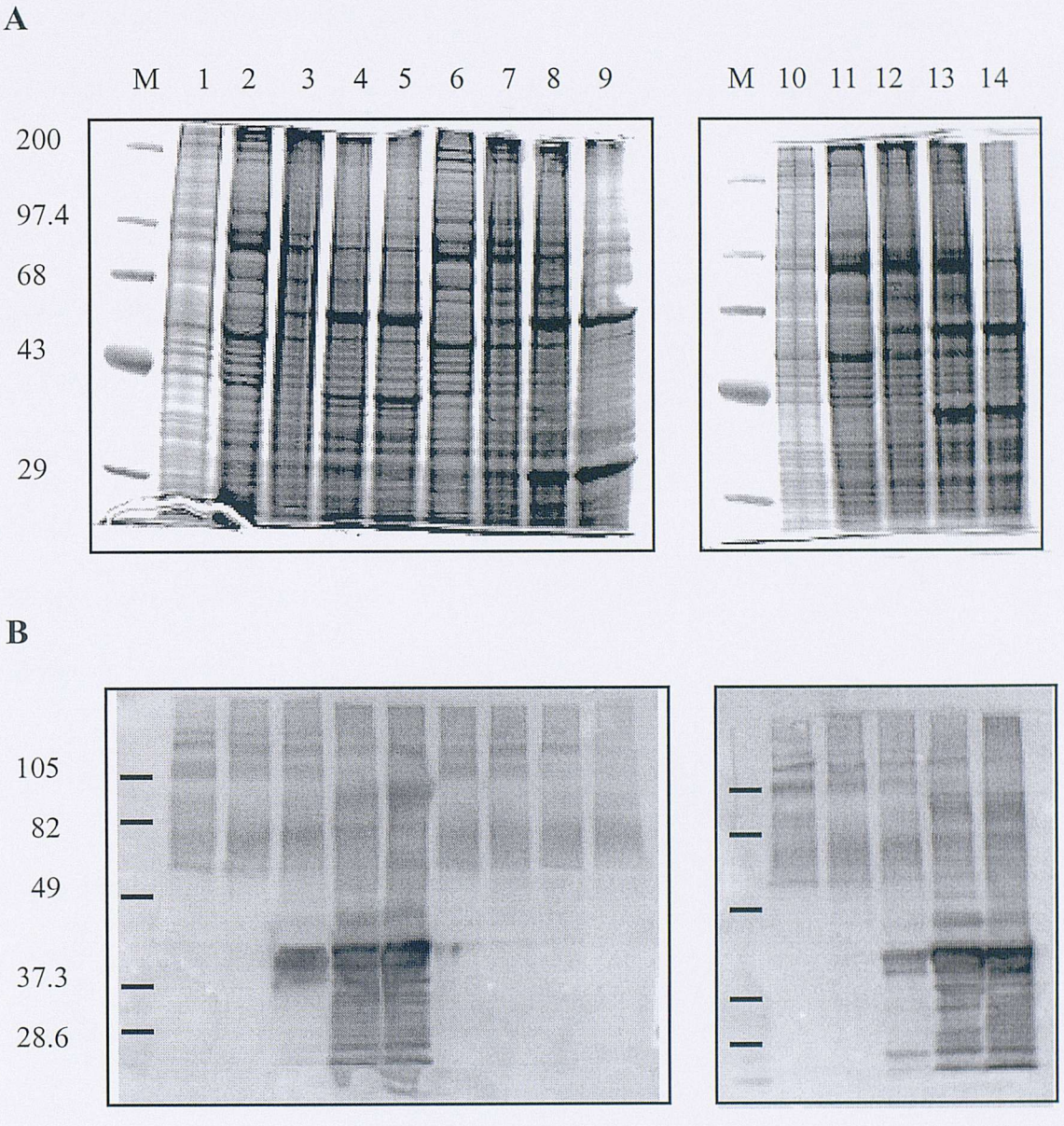


Figure 3.20 PCR analysis of recombinant baculoviruses. DNA extractions of Sf9 cells infected with either NS3-2, NS3-3, wild type virus or uninfected were used in a PCR reaction designed to amplify a 1.9 kb (P8198 and P8199 primer pair) or 1.3 kb (A8038 and A8040 primer pair) fragment corresponding to full length NS3 or an internal region of NS3 respectively. Samples were adjusted to 1 X agarose gel load buffer and DNA fragments resolved using a 1% agarose gel in 1 X TBE then stained with ethidium bromide. (A) PCR with P8198 and P8199 primer pair. M, molecular weight markers; lane 1, uninfected; 2, wild type baculovirus; 3, NS3-3; 4, pCRNS3F positive control; 5, no primers. (B) PCR with A8038 and A8040 primer pair. M, molecular weight markers; lane 6, uninfected; 7, wild type; 8, NS3-3; 9, pCRNS3F positive control; 10, NS3-2.



Figure 3.21 Expression of NS3 in insect cells. Sf9 cells infected with wild type virus, NS3-3, NS3-2 or uninfected were harvested at various time points post infection and the protein content analysed by SDS-PAGE. Samples were resolved on a 10 - 20% gradient gel and stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blot was performed with NS3-7 (1/1000) and AP conjugated secondary antibody was detected with BCIP/NBT substrate. M, molecular weight markers; lane 1, uninfected cells 72 hours post infection; 2 - 5, NS3-3 16, 24, 48, 72 hours post infection; 6 - 9, wild type 16, 24, 48, 72 hours post infection; 10, uninfected cells 72 hours post infection; 11 - 14, NS3-2 16, 24, 48, 72 hours post infection.


A
M 1 2 3 4 5 6 7 8 9
M 10 11 12 13 14
200
97.4
68
43
29
B
105
82
49
37.3
28.6

(279,281). It is therefore most likely that this 40 kDa product is the result of proteolysis by host cell enzymes.

To try to prevent or reduce this proteolysis, a variety of expression conditions were investigated. Expression was conducted at 22°C and 28°C, in suspension culture and monolayer culture using serum free (SF900II) and serum supplemented (TC100) media. Time courses were for up to 72 hours with samples taken at intervals of 24 hours. In addition extracts were prepared in the presence in protease inhibitors. The results of these expression studies are summarised in **Table 3.4**. Full length NS3 was not detected with either recombinant baculovirus with any of the culture conditions. Reduction of the temperature to 22°C delayed the appearance of NS3 by 24 hours (the protein was detected after 48 hours instead of at 24 hours at 28°C) (**Figure 3.22**) and no difference was observed between the use of serum supplemented or serum free media and between monolayer or suspension culture.

Preparation of protein extracts in the presence of PMSF or Complete™ protease inhibitor cocktail did not reduce degradation of the target protein (**Figure 3.23**). Over development, by extending the incubation with BCIP/NBT substrate (section 2.3.6) allowed the visualisation of protein with an approximate molecular mass similar to that expected for full length NS3. However, these bands were also detected in the absence of protease inhibitors (**Figure 3.22**) and were only detectable following extended incubation with BCIP/NBT substrate. Therefore this protein is likely to represent a very small proportion of the total expressed protein.

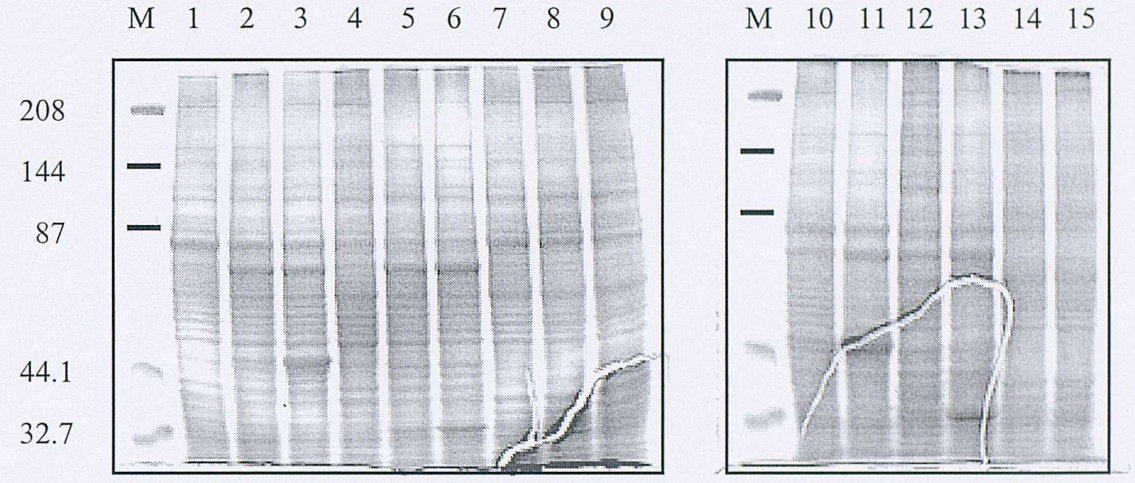
Temperature (°C)	Media	Culture	MOI	Inhibitor Cocktail	NS3 70 kDa	NS3 40 kDa
27	SFM	MONO	10	-	✗	✓
27	TC100	MONO	10	-	✗	✓
27	SFM	SUSP	10	-	✗	✓
27	TC100	SUSP	10	-	✗	✓
27	SFM	MONO	1	-	✗	✓
22	SFM	MONO	10	-	✗	✓
22	TC100	MONO	10	-	✗	✓
22	SFM	SUSP	10	-	✗	✓
22	TC100	SUSP	10	-	✗	✓
22	SFM	MONO	10	+	✗	✓

Table 3.4 Summary of attempts to express full length NS3 in insect cells. Presence or absence of full length (70 kDa) or truncated (40 kDa) NS3 indicated by ✓ or ✗ respectively. Abbreviations, SFM; SFM900II serum free media; TC100, TC100 media supplemented with 10% (v/v) FCS; MOI, multiplicity of infection; MONO, monolayer cell culture; SUSP, suspension cell culture; inhibitor cocktail, protease inhibitor cocktail.



Figure 3.22 Effect of culture temperature on NS3 recombinant baculovirus expression. Sf9 cells infected with NS3-3, wild type virus or uninfected were grown at 22°C or 27°C and harvested at 24 hour intervals post infection. Samples were resolved by SDS-PAGE on a 10 - 20% gradient gel and stained with Coomassie Blue (A) or analysed by Western blotting (B). Western blot was performed with NS3-7 (1/1000) and AP conjugated secondary detected with BCIP/NBT substrate. Lanes 1 - 9, expression at 22°C; 10 - 15, expression at 27°C. Lane M, molecular weight markers; 1 - 3, NS3-3 harvested 24, 48, 72 hours post infection; 4 - 6, wild type, 24, 48, 72 hours; 7 - 9, uninfected, 24, 48, 72 hours; 10 and 11, NS3-3, 24, 48 hours; 12 and 13, wild type, 24, 48 hours; 14 and 15, uninfected, 24, 48 hours.

A


M 1 2 3 4 5 6 7 8 9
M 10 11 12 13 14 15
208
144
87
44.1
32.7

B

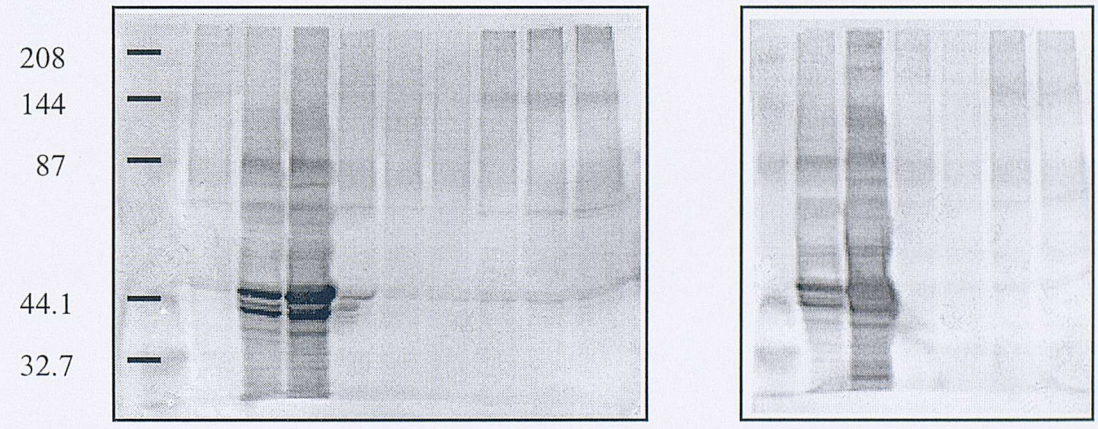
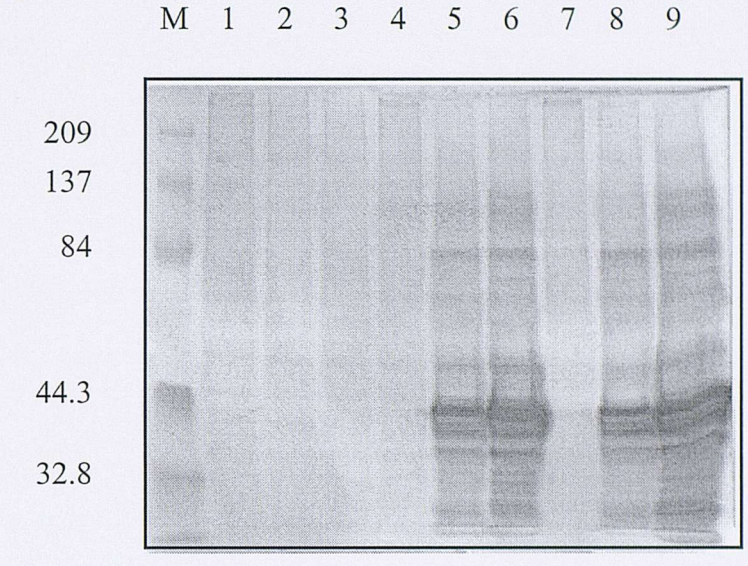

208
144
87
44.1
32.7

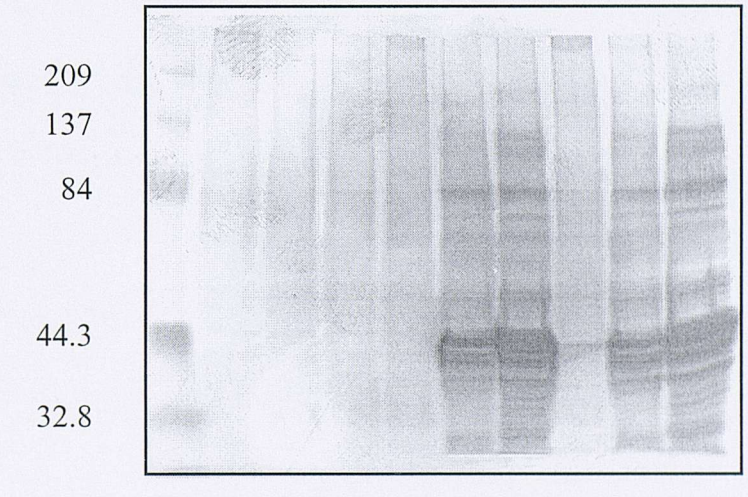


Figure 3.23 Use of protease inhibitors for the harvest of NS3 from insect cells. Sf9 cells infected with NS3-3, NS3-2 or wild type virus were grown at 27°C and protein samples harvested in the presence (A) or absence (B) of protease inhibitor cocktail. Samples were resolved by SDS-PAGE on a 10 - 20% gradient gel before Western transfer. Western blot was performed with NS3-7 (1/1000) and AP conjugated secondary detected with BCIP-NBT substrate. Lane M, molecular weight markers; 1 - 3, wild type virus, 24, 48, 72 hours; 4 - 6, NS3-3 24, 48, 72 hours; 7 - 9, NS3-2 24, 48, 72 hours.

A



B



3.4 Expression of NS5A/B Cleavage Site (NS5A/BΔ) in *E. coli*

The NS5A/B cleavage site in the HCV polyprotein is the most efficiently cleaved by the NS3 protease in the absence of NS4A cofactor (231,232). For this reason it was chosen as a substrate for assay of the protease activity of recombinant NS3.

3.4.1 Construction of NS5A/BΔ Expression Construct

A 568 bp fragment was amplified by PCR from the plasmid pBRTM/HCV 1-3011 (142) using the primers P5601 (sense) and P4837 (antisense) which introduce a 5' BglII and 3' EcoRI restriction site. This region corresponds to amino acids 2328 - 2516 of the H strain HCV polyprotein (369). The fragment was capture cloned using pCRScript vector and the resulting plasmid named pCR5HEX. The insert was the excised using BglII and EcoRI restriction enzymes and ligated into the prokaryotic expression vector pHEX which had been similarly prepared and 5' dephosphorylated. This cloning step introduced the insert downstream of, and in frame with a start codon and a sequence encoding a six histidine tag. The resulting plasmid was designated pHEX-NS5a/bΔ.

3.4.2 Expression of Recombinant NS5A/BΔ Protein

To identify the optimum time for expression of NS5A/BΔ a time course of expression was performed. Total cell and soluble protein extracts made and analysed by SDS-PAGE and Western blot. For a control, *E.coli* JM109(DE3) containing the parent vector pHEX was treated similarly.

A protein of 34 kDa was detected by Coomassie staining in total cell extracts of induced samples of JM109(DE3):pHEX-NS5A/BΔ, but absent from uninduced samples and induced samples of JM109(DE3):pHEX (**Figure 3.24**). The observed size of the protein was larger than the expected size of 24 kDa but has also been observed by other groups expressing the same region of NS5 who have suggested that this may be a characteristic of this region of NS5, possibly attributed to a high number of acidic residues (233). The identity of this protein was confirmed as NS5A/BΔ by N-terminal sequencing of the purified protein (section 2.3.7).

3.4.3 Purification of Insoluble Protein and Subsequent Solubilisation

The NS5A/BΔ was found to be entirely insoluble as was observed with the expression of NS3 (section 3.1.2). Using an identical approach used for the purification and refolding of NS3, soluble NS5A/BΔ was isolated from inclusion bodies.

A 500 ml culture of *E. coli* JM109(DE3):pHEX-NS5A/BΔ was grown and protein expression induced as before. Cells were harvested by centrifugation 3 hours post induction and expression of recombinant protein confirmed by SDS-PAGE. The inclusion bodies were then isolated using the same procedure for NS3 (section 3.1.5). Samples were taken at each stage and analysed for NS5a/bΔ and impurities by SDS-PAGE (**Figure 3.25**). The resulting denatured protein was refolded by stepwise removal of the urea by dialysis and stored under the same conditions as for NS3 (section 3.1.7). The final protein concentration was calculated by the Bradford method to be 1.2 mg/ml and a total yield of approximately 40 mg of soluble, refolded protein was obtained per litre of original culture.

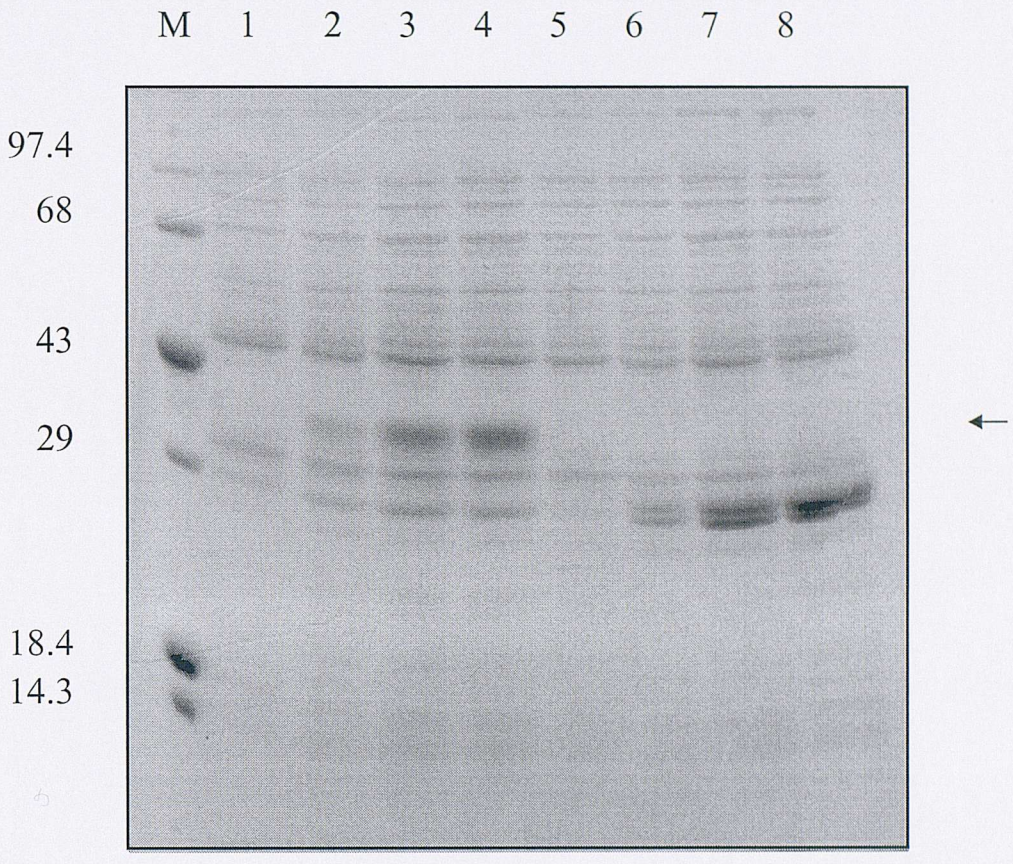


Figure 3.24 Expression of NS5A/BΔ in *E.coli* JM109 (DE3). Samples were resolved by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Lane M, molecular weight markers (sizes given in kDa); 1 - 4, pHEX NS5A/BΔ harvested 0, 1, 2, 3 hours post induction; 5 - 8, pHEX harvested 0, 1, 2, 3 hours post induction. Position of NS5A/BΔ is arrowed

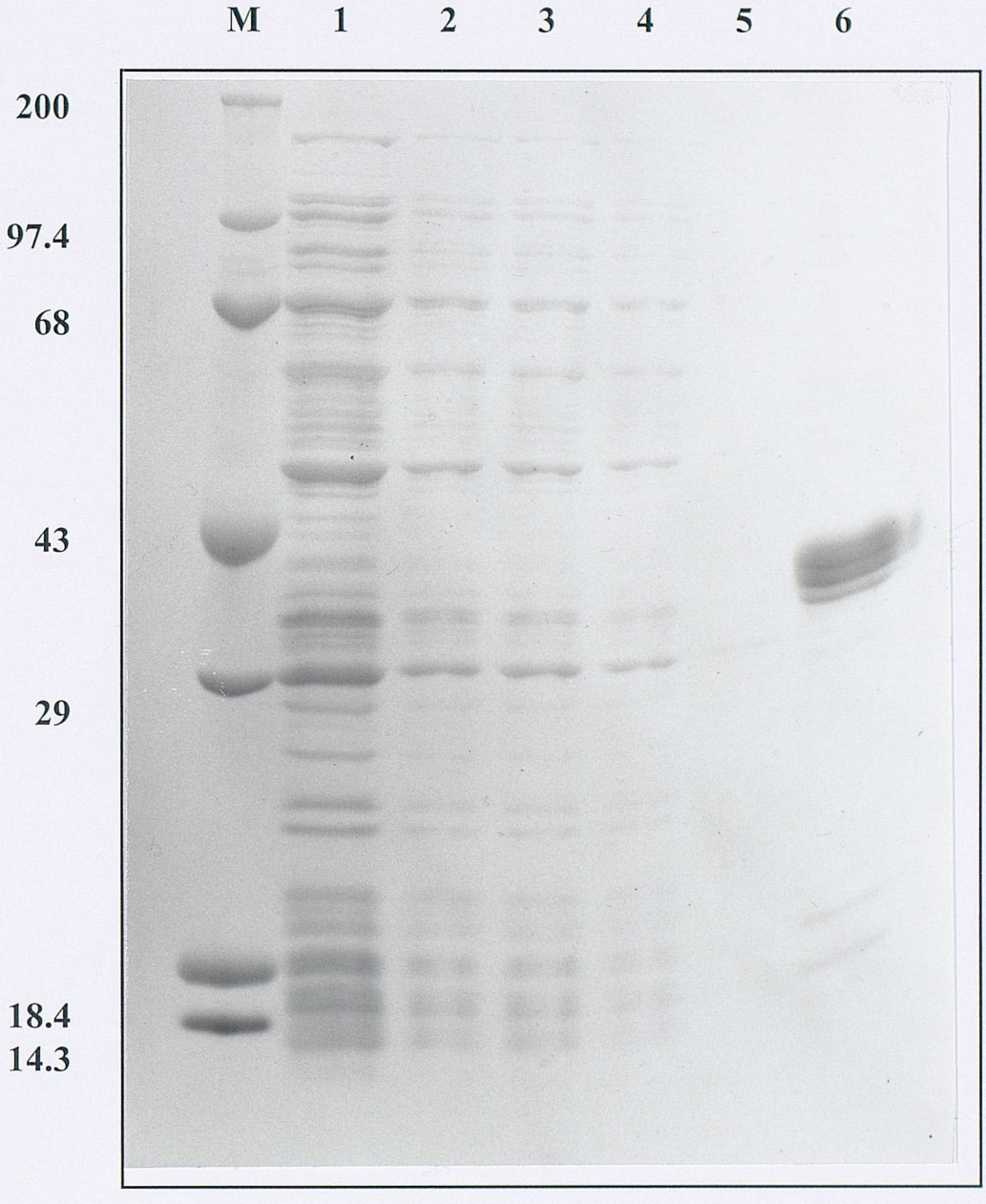


Figure 3.25 Purification of inclusion bodies containing insoluble NS5A/BΔ. Samples were resolved by SDS-PAGE on a 10% gel and stained with Coomassie Blue. Lane M; molecular weight markers (sizes given in kDa); lane 1, total cell extract; lane 2, soluble protein (wash 1); lanes 3 - 5, washes 2 - 5; lane 6, NS5A/BΔ inclusion bodies solublised with 8 M urea.

Chapter 4: Characterisation of Activities of NS3

4.1 DNA Binding Activity of HCV NS3 Protein

The helicase domain of HCV NS3 has been shown to have RNA binding activity by a number of groups (289,304). Full length NS3 prepared as described in section 3.1.6 was used to demonstrate ssDNA binding using a band shift assay. NS3 and NS5A/BΔ were incubated with ^{32}P-labelled oligonucleotide W4133 and the bound and unbound oligonucleotides were separated by TBE-PAGE. The migration of an oligonucleotide in a gel when bound by protein is retarded compared with unbound oligonucleotide and this is apparent as a 'band shift' (**Figure 4.1**). A band shift was observed with NS3 but not with NS5A/BΔ demonstrating DNA binding by NS3.

The specificity of ssDNA binding, as opposed to non-specific protein:DNA interactions was then demonstrated by a competition assay using an unlabelled oligonucleotide. The band shift assay was performed in the presence of increasing amounts of unlabelled oligonucleotide which prevented binding of the labelled oligonucleotide to NS3 in a concentration dependent manner (**Figure 4.2** lanes 1 - 5). Heat denatured NS3 was unable to bind the oligonucleotide, shown by the absence of a band shift (**Figure 4.2** lane 6), eliminating the possibility that the observed ssDNA binding activity was due to non-specific adsorption of the oligonucleotide to the protein.

4.2 NTPase Activity of HCV NS3 Protein

4.2.1 Demonstration of NTPase Activity

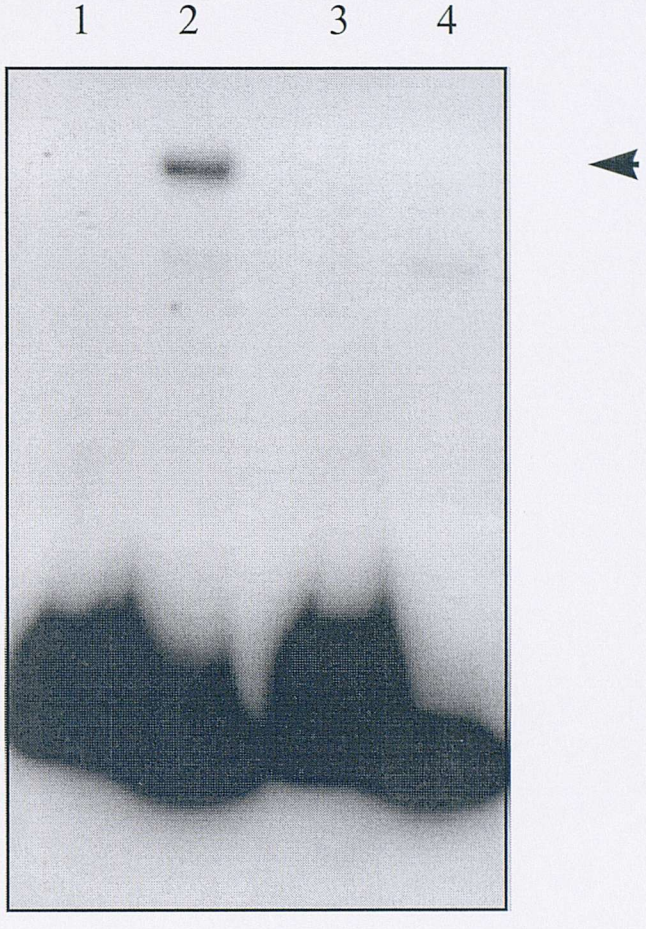


Figure 4.1 Demonstration of DNA oligonucleotide binding by NS3 using a band shift assay. 15 pmol NS3 or an equivalent quantity of NS5A/BΔ were assayed for ssDNA binding activity to 1.5 pmol of oligonucleotide W4133 (section 2.7.1). Lane 1, no protein; lane 2, NS3; lane 3, no protein; lane 4, NS5A/BΔ. The position of NS3-bound oligonucleotide is indicated with an arrow.

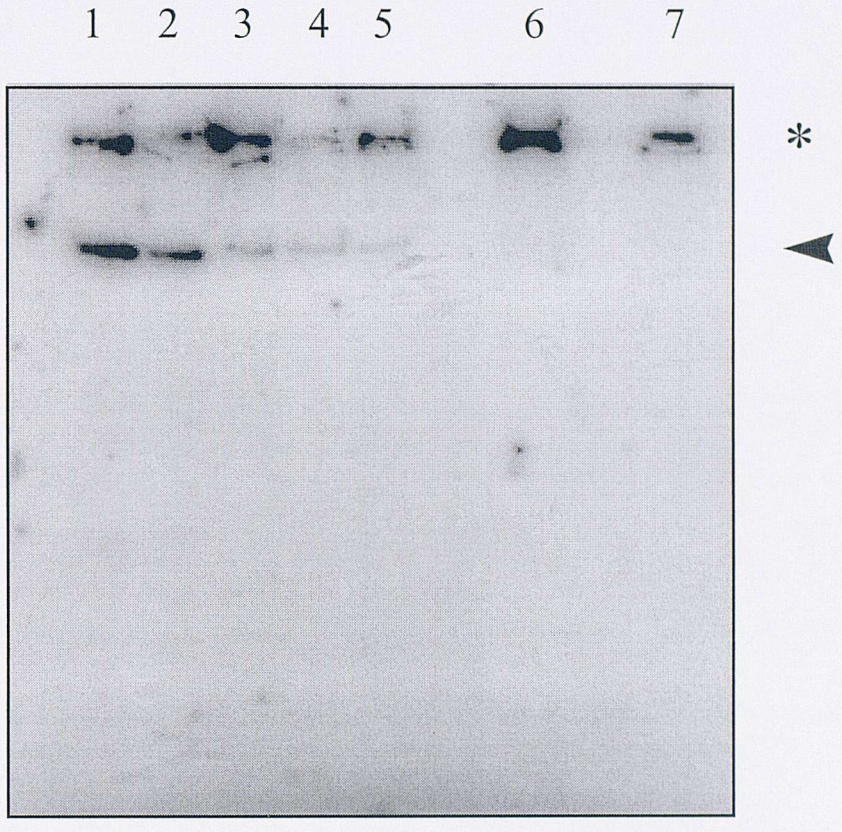


Figure 4.2 Specificity of NS3 ssDNA binding. Specificity of binding demonstrated by competition for binding with unlabelled oligonucleotide and heat denaturation of NS3. 15 pmol NS3 and 1.5 pmol of ^{32}P labelled oligonucleotide W4133 were incubated in the presence of various amounts of unlabelled W4133 and binding of labelled oligonucleotide analysed by band shift assay (section 2.7.1). Lane 1, no unlabelled W4133; lane 2, 1.5 pmol unlabelled W4133; lane 3, 15 pmol; lane 4, 150 pmol; lane 5, 750 pmol; lane 6, NS3 heated to 100°C for 2 minutes prior to assay, lane 7, no NS3. The position of the wells is marked with an asterisk and migration of the bound oligonucleotide is indicated with an arrow.

NS3 protein recovered from purified inclusion bodies was demonstrated to possess a poly(U) stimulated NTPase activity by hydrolysis of $\alpha[^{32}P]$-ATP to $\alpha[^{32}P]$-ADP and $\alpha[^{32}P]$-AMP (**Figure 4.3**). A control protein extract from *E. coli* JM109(DE3) expressing a polypeptide corresponding to the NS5A/B cleavage site (NS5A/BΔ) exhibited NTPase activity above the zero protein control suggesting the presence of contaminating non-NS3 NTPase activity, but this activity was detected only at a low level and was not stimulated by poly(U). Pre-incubation with serum from a HCV positive patient which had previously been shown to have affinity for NS3, inhibited NS3 NTPase activity, reducing the activity to basal levels (**Figure 4.4**). Serum from an HCV negative patient did not affect NS3 NTPase activity. Absence of polynucleotide stimulated NTPase activity in the control protein sample and inhibition of NTPase activity by pre-incubation with HCV positive serum indicated that NS3 was responsible for the detected polynucleotide stimulated NTPase activity. NTPase activity of NS3 was only detected in protein refolded in the absence of $ZnCl_2$ (**Figure 4.5**). NS3 prepared in the presence of $ZnCl_2$ failed to exhibit maximal NTPase activity, even though the protein had been dialysed extensively to remove any Zn^{2+} not associated with the protease domain. This suggested that NS3 does not fold correctly in the presence of Zn^{2+} possibly due to presence of the histidine tag or that the Zn^{2+} ion strongly associates with the protein in a position that inactivates the NTPase activity. This data was also used to confirm the reproducibility of the protein expression and purification procedure. Of the three batches prepared in the absence of $ZnCl_2$, activity varied by approximately 10 percent. In addition NS3 protein was stable with respect to NTPase activity over a storage period of 6 months.

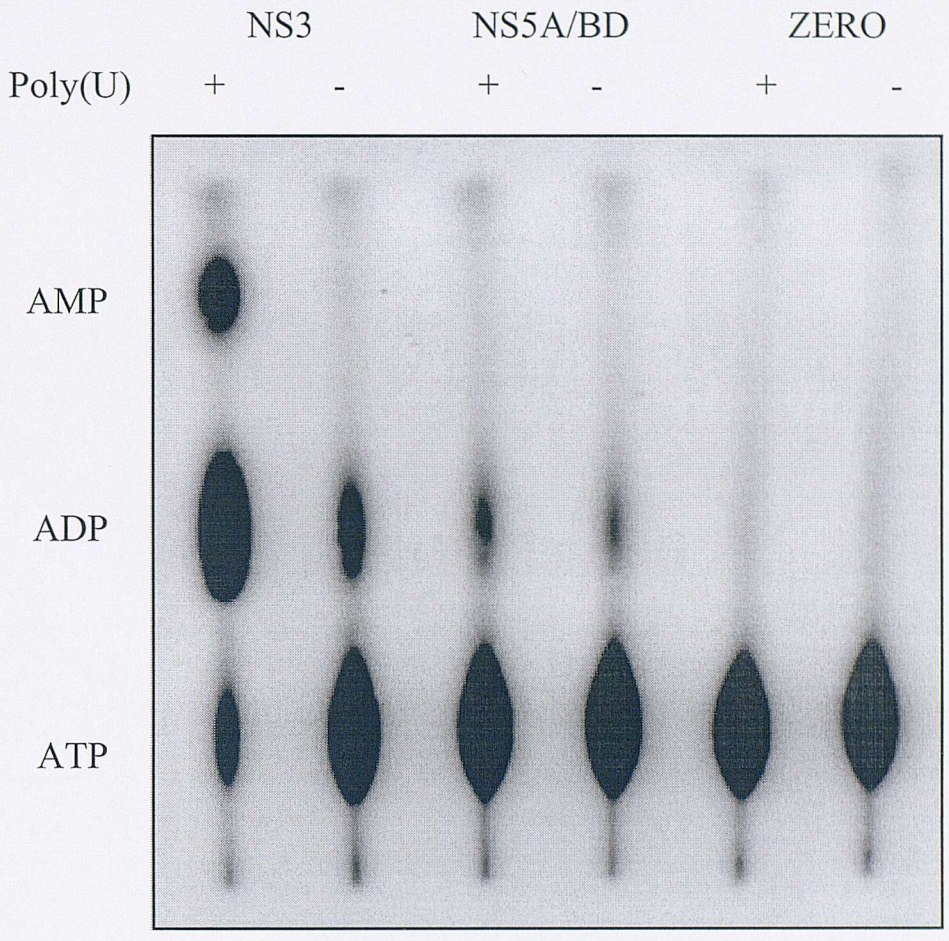


Figure 4.3 NS3 associated polynucleotide stimulated NTPase activity. The radioactive assay was performed as described in section 2.7.4 with $\alpha[^{32}P]$-ATP and either 1.5 pmol NS3, an equivalent quantity of NS5A/BΔ or no protein. Polynucleotide stimulation was shown by performing the assay ± 1.5 mM poly(U). ATPase activity is shown by the hydrolysis of $\alpha[^{32}P]$-ATP to $\alpha[^{32}P]$-ADP and $\alpha[^{32}P]$-AMP.

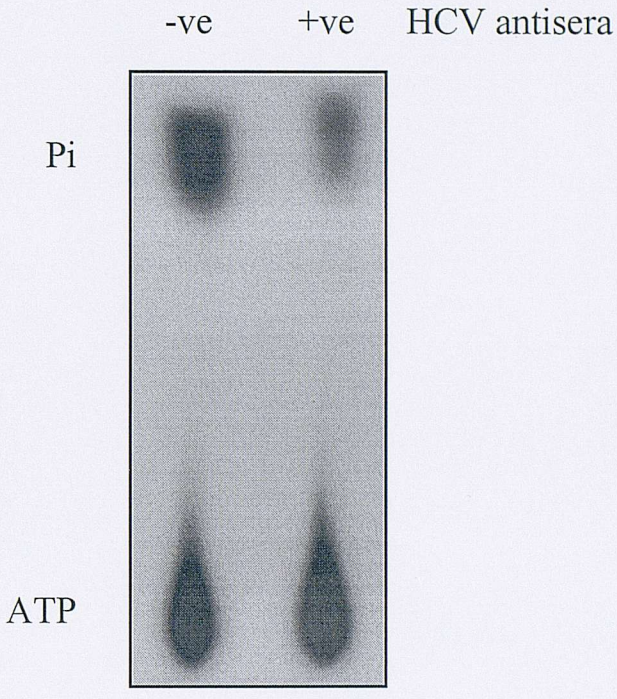


Figure 4.4 Antibody inhibition of NS3 NTPase activity. NS3 was pre-incubated with a 1/3 dilution of HCV +ve or HCV -ve human sera prior to assaying for ATPase activity. ATPase activity is shown by the hydrolysis of $\gamma[^{32}P]$-ATP to $[^{32}P]$-Pi.

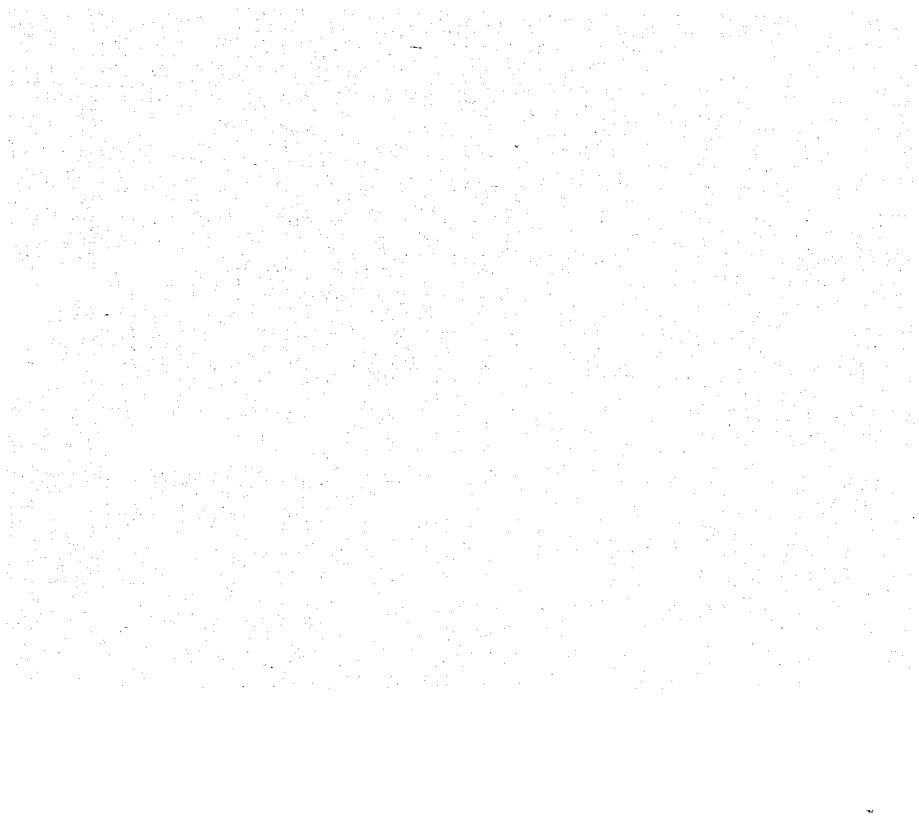
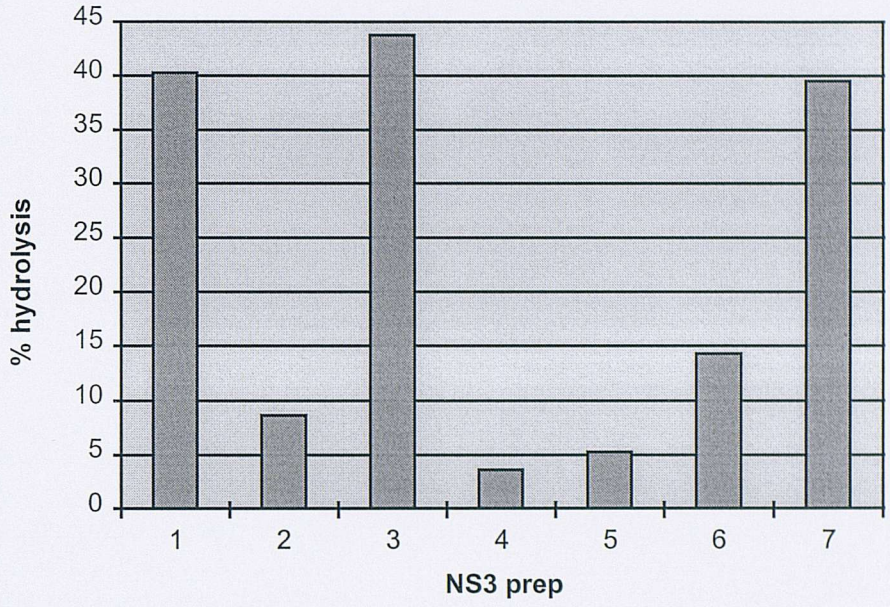
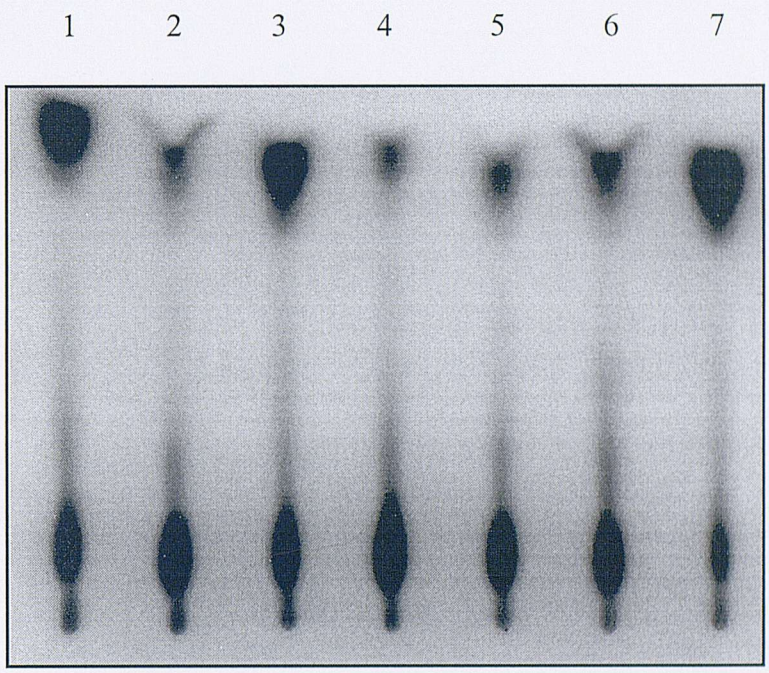


Figure 4.5 Inhibition of NS3 associated NTPase activity by the presence of Zinc during the refolding procedure and the effects of storage on NTPase activity. NS3 refolded in the presence (samples; 2, 4, 5, 6) or absence (samples; 1, 3, 7) of 100 μM $ZnCl_2$. 1.5 pmol of each protein sample was assayed for NTPase activity using γ[^{32}P]-ATP (section 2.7.4). (A) Percentage of ATP hydrolysed displayed as a histogram for each sample. (B) Autoradiograph of the chromatogram. Duration of storage (months) prior to assay is indicated below.

A


45
40
35
30
25
20
15
10
5
0
% hydrolysis
1
2
3
4
5
6
7
NS3 prep

B


1
2
3
4
5
6
7
Duration of Storage (Months)
< 1
4
6

4.2.2 Optimisation of Conditions for NTPase Activity

Conditions for NTPase activity were optimised with respect to pH, monovalent cation (NaCl), magnesium ($MgCl_2$) and polynucleotide (poly(U)) (**Figure 4.6**). NTPase activity was shown to peak at pH 7.5 and was relatively unaffected over the range pH 6.5 to 8.0. Optimum activity was dependent upon the presence of $MgCl_2$ and activity was stimulated by poly(U). Maximum polynucleotide stimulation was observed above 1.25 μM poly(U) and 5 mM $MgCl_2$ was required for optimal activity at an ATP concentration of 0.5 mM. NTPase activity was strongly inhibited by the presence of monovalent cation. Activity was reduced to less than half maximum at 100 mM NaCl and was further reduced to the basal levels observed in the absence of the polynucleotide cofactor at 200 mM NaCl. This suggests that the presence of monovalent cation may prevent correct protein-polynucleotide association.

From these experiments optimal reaction conditions were determined to be 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM ATP, 5 μM poly(U) (1.5 mM nucleotides). Activity was optimal in the absence of NaCl. These conditions were used for further analysis of NTPase activity.

4.2.3 Polynucleotide Stimulation and Kinetic Analysis of NTPase Activity

The stimulatory effects of different polynucleotides on ATPase activity were examined. NS3 ATPase activity was assayed in the absence of polynucleotide or in the presence of 1.5 mM (nucleotides) poly(U), (G), (C), (A) (dT), (dC) or (dA) and relative stimulation was expressed as a factor of activity in the absence of polynucleotide. All

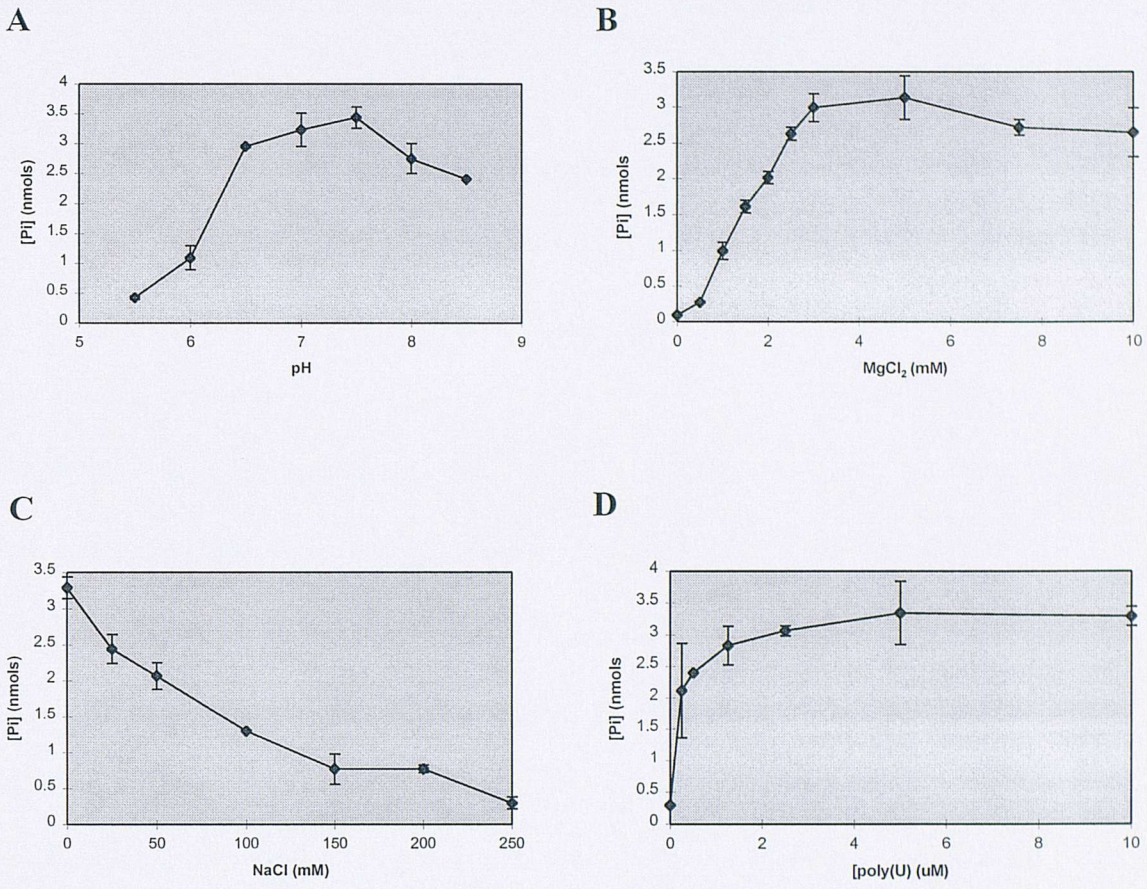


Figure 4.6 Optimisation of conditions for NS3 NTPase activity. NTPase activity was assayed using the malachite green assay (section 2.7.3). Reaction conditions were 2 pmol NS3, 25 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 0.5 mM ATP, 10 μM poly(U) except where indicated. (A) Optimisation of buffer pH. All buffers were tested at 25 mM; pH5.5 and 6.0 MES, pH 6.5 and 7.0 MOPS KOH, pH 7.5, 8.0 and 8.5 Tris-HCl. (B) Determination of optimum $MgCl_2$ concentration. (C) Effect of NaCl on NTPase activity. (D) Polynucleotide requirement of NS3 performed with indicated amounts of poly(U).

polynucleotides tested were able to stimulate ATP hydrolysis (**Figure 4.7**). Poly(U) demonstrated the greatest stimulation of ATPase activity showing an approximate seven fold increase in activity compared to activity in the absence of polynucleotide. This stimulation was twice that of poly(C), the next best cofactor. All other polynucleotides tested in this study stimulated activity between two and three fold. Poly(dG) was not tested due to difficulties in obtaining it from a commercial source.

The hydrolysis of other NTPs in addition to ATP by HCV NS3 was also studied. The kinetic parameters k_{cat} (turnover number) and K_m (Michaelis constant) were determined from Lineweaver-Burke plots of hydrolysis activity for different concentrations of substrate (**Figure 4.8**). The k_{cat}/K_m value was calculated for each NTP substrate and is a measure of the overall activity of the enzyme for that substrate (**Table 4.1**). ATP was determined to be the best substrate for NS3 showing the highest turnover (32.4 min^{-1}) and a k_{cat}/K_m of value (116 mM^{-1} min^{-1}). GTP and dGTP proved to be the poorest substrates for NS3.

4.3 Helicase Activity of HCV NS3

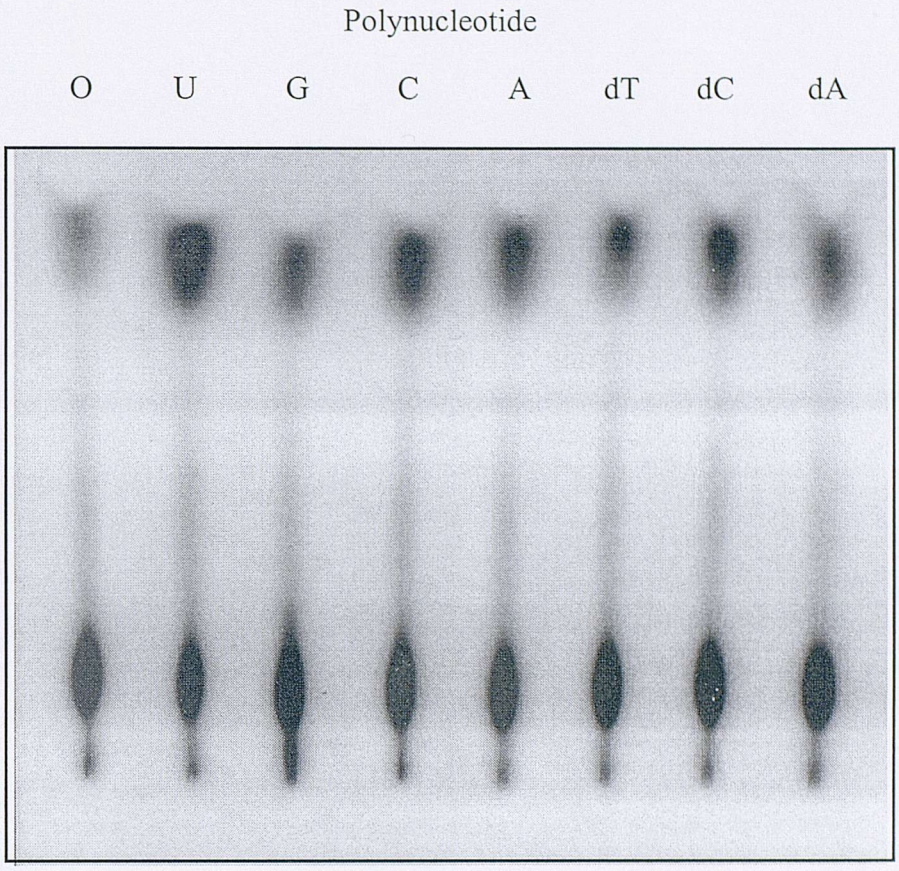
4.3.1 Demonstration of Helicase Activity

The helicase activity of NS3 was demonstrated by unwinding of a DNA duplex consisting of a 98 nucleotide template strand annealed to a ^{33}P labelled 38 nucleotide release strand (**Figure 4.9**). This unwinding was dependent on the presence of ATP; unwinding was not observed in the absence of ATP or when ATP was replaced with the non-hydrolysable analogue ATP-γS.

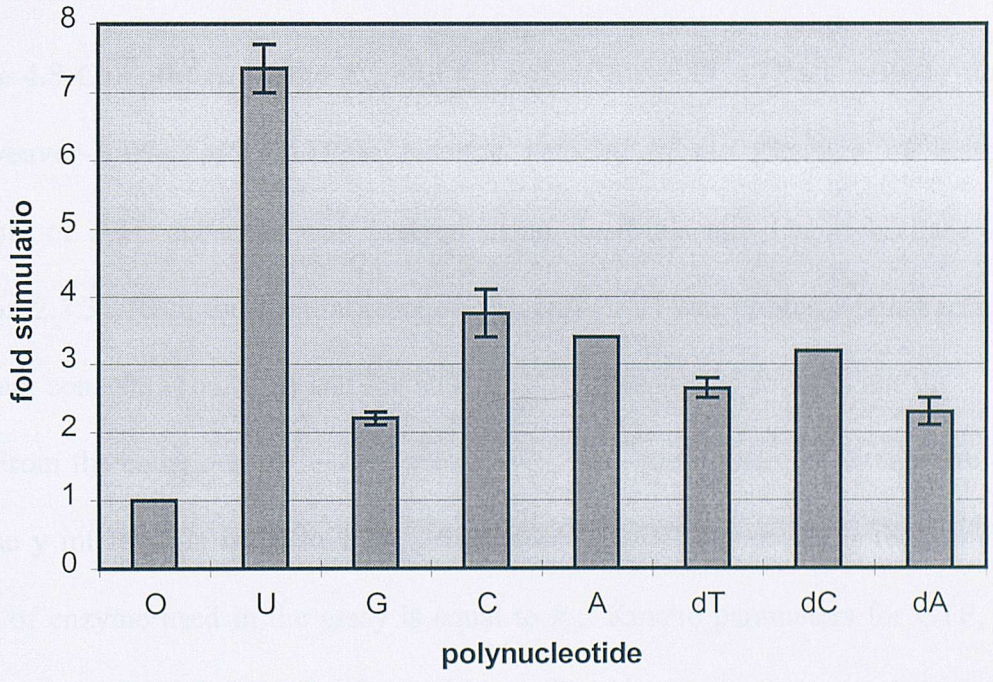


Figure 4.7 Polynucleotide stimulation of NS3 NTPase activity. NTPase activity was assayed using the radioactive method (section 2.7.4) and expressed as a factor of activity in the absence of polynucleotide which was taken to be 1.0. O; no polynucleotide, U; poly(U), G; poly(G), C; poly(C), A; poly(A), dT; poly(dT), dC; poly(dC), dA; poly(dA). All polynucleotides were used at 1.5 mM (nucleotides). (A) Autoradiograph showing $\gamma[^{32}P]$-ATP hydrolysis in the presence or absence of polynucleotides. (B) Histogram showing stimulatory effect of each polynucleotide.

A


Polynucleotide
O
U
G
C
A
dT
dC
dA

B


fold stimulatio
0
1
2
3
4
5
6
7
8
O
U
G
C
A
dT
dC
dA
polynucleotide

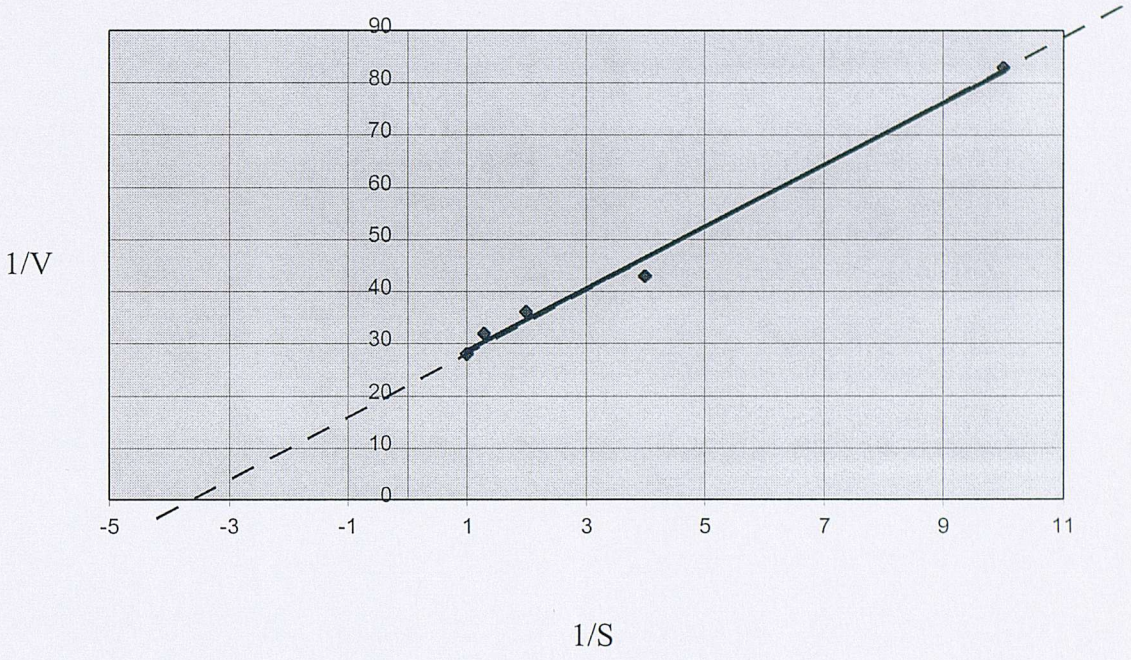


Figure 4.8 Calculation of the K_M and k_{cat} values for NS3 ATPase activity using a Lineweaver-Burke plot. ATPase reaction velocity in the presence of increasing amounts of ATP substrate was assayed using the Malachite Green NTPase Assay (section 2.7.3). Reciprocal initial reaction velocity (1/V) was plotted against reciprocal substrate concentration (1/S) and the straight line extrapolated to intercept the y and x axis. From the equation; $1/V = 1/V_{max} + K_M/V_{max}$. 1/S, the x intercept is equal to $-1/K_M$ and the y intercept is equal to $1/V_{max}$. Therefore $V_{max}/[E]$ where [E] is the number of moles of enzyme used in the assay is equal to k_{cat}. Kinetic parameters for CTP, GTP, UTP, dATP, dCTP, dGTP and dTTP hydrolysis (Table 4.1) were calculated similarly.

	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
ATP	0.28	32.4	116
GTP	0.83	23.4	28
CTP	0.36	24	67
UTP	0.59	25.2	43
dATP	0.34	22.2	65
dGTP	1.25	24	19
dCTP	0.43	29.4	68
dTTP	0.18	15.6	87

Table 4.1 Kinetic analysis of NS3 NTPase activity. K_m and k_{cat} values for NTPase substrates, calculated from Lineweaver-Burke plots determined from data which were obtained using the malachite green assay conducted over 30 minutes, with 0.1, 0.25, 0.5, 0.75, 1.0 mM NTP substrate. The $MgCl_2$ concentration was maintained at 4.5 mM above the NTP substrate concentration. All other reaction conditions were as described (Section 2.7.2).

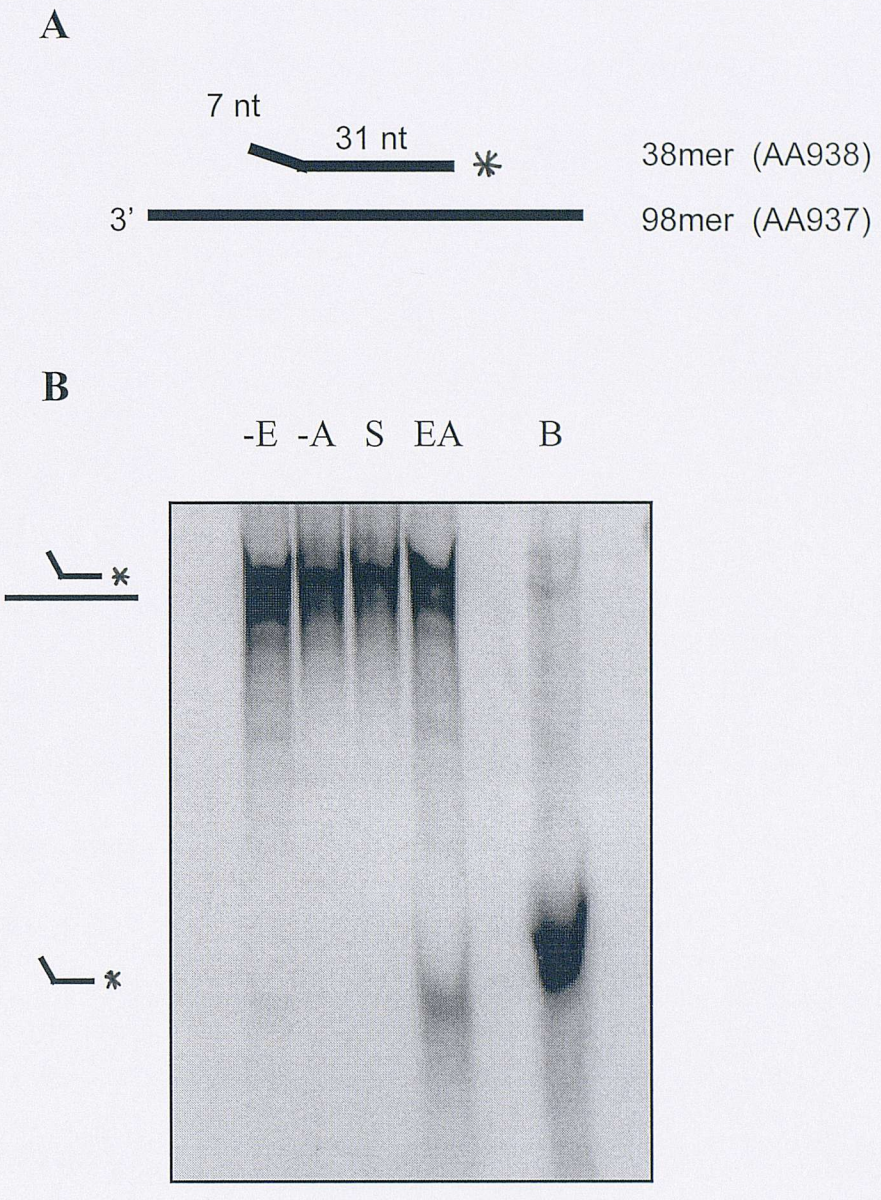


Figure 4.9 Helicase activity of NS3. (A) DNA substrate used in this assay, produced by slowly annealing the complimentary oligonucleotides A9937 and A9938 (section 2.1.5). (B) Unwinding of DNA substrate by NS3. -E, no enzyme; -A, no ATP; S, ATP replaced with ATPγS; EA, NS3 and ATP; B, heat denatured substrate. Radiolabelled strand of the helicase substrate is indicated with an asterisk.

4.3.2 Optimisation of Conditions for Helicase Activity

Helicase assay conditions were optimised with respect to pH, $MgCl_2$ and monovalent cations (NaCl and KCl). Initial experiments were carried out at 25°C however, no activity was detected for full length NS3 even though activity was detected in the HCV NS3 helicase domain prepared by the University of Virginia (Virginia helicase) (279) (**Figure 4.10**). Helicase activity of the full length NS3 protein was detected by increasing the reaction temperature to 37°C. At 37°C unwinding of the DNA duplex was observed at pH 6.5, 7.0 and 7.5 (**Figure 4.11**) indicating that NS3 helicase activity is not detectable at 25°C using this method. A titration of NS3 protein and a reaction time course were carried out at 37 °C prior to further optimise conditions for NS3 helicase activity (**Figure 4.12**). Activity was directly proportional to the amount of protein and the time course was linear between 15 and 60 minutes. Reaction velocity was greater in the initial 15 minutes but remained constant enough over the full 60 minutes to justify an extended incubation time. Further optimisation of reaction conditions was carried out at 37°C for 60 minutes with 7.5 pmol of NS3.

Helicase activity proved to be particularly sensitive to pH. Optimal activity was observed at pH 6.5 and was reduced by 50% at 0.5 pH units above or below this optimum (**Figure 4.13A**). This was in contrast to NTPase which was much less sensitive to pH. The effects of both KCl and NaCl on NS3 helicase activity were investigated and activity was shown to be sensitive to both. Activity was reduced to 15% of maximum in the presence of 50 mM NaCl and was not detectable at 150 mM NaCl (**Figure 4.13B**). KCl had an identical effect (**Figure 4.13C**). Magnesium was

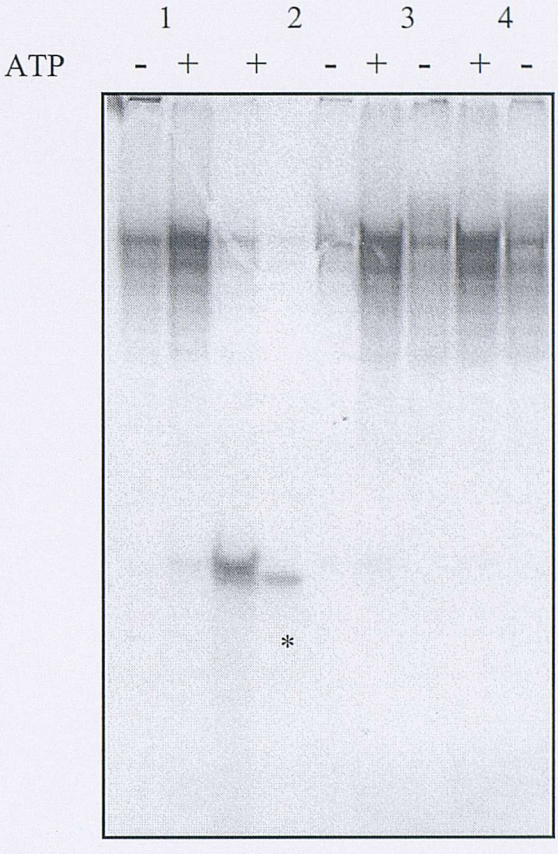


Figure 4.10 Absence of NS3 helicase activity at 25°C. Assay was performed at 25°C in the presence (+) and absence (-) of ATP, all other conditions were as described in materials and methods (section 2.7.2). Lane 1, no protein; lane 2, 1 pmol Virginia helicase; lane 3, 3 pmol (2 μl) NS3; lane 4, 100 pmol (2 μl) NS5A/BΔ. The asterisk (*) indicates overflow from lane 2+.

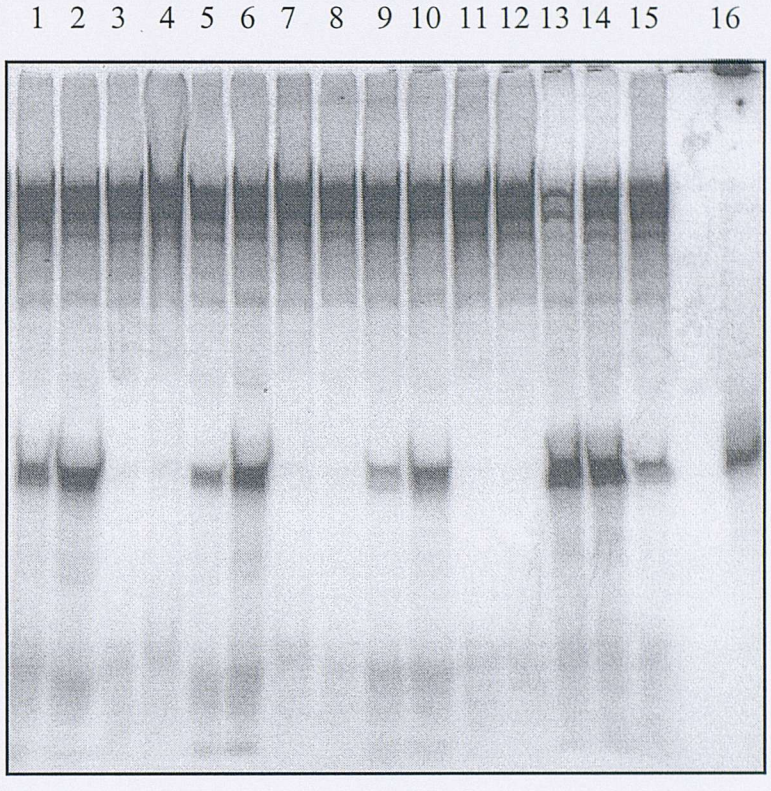


Figure 4.11 NS3 helicase activity at 37°C. Reaction conditions as described in materials and methods (section 2.7.2). Lanes 1 and 2, 1 μl and 3 μl NS3; lanes 3 and 4, 1 μl and 3 μl NS5A/BΔ - 50 mM MOPS KOH (pH 6.5). Lanes 5 and 6, 1 μl and 3 μl NS3; lanes 7 and 8, NS5A/BΔ - 50 mM MOPS KOH (pH 7.0). Lanes 9 and 10, 1 μl and 3 μl NS3; lanes 11 and 12, NS5A/BΔ - 50 mM MOPS KOH (pH 7.5). Lanes 13 - 15, 1 pmol Virginia helicase, pH 6.5, 7.0 and 7.5, respectively, buffers as before. Lane 16, heat denatured substrate. NS3 concentration, 1.5 pmol/μl; NS5A/BΔ concentration, 50 pmol/μl.

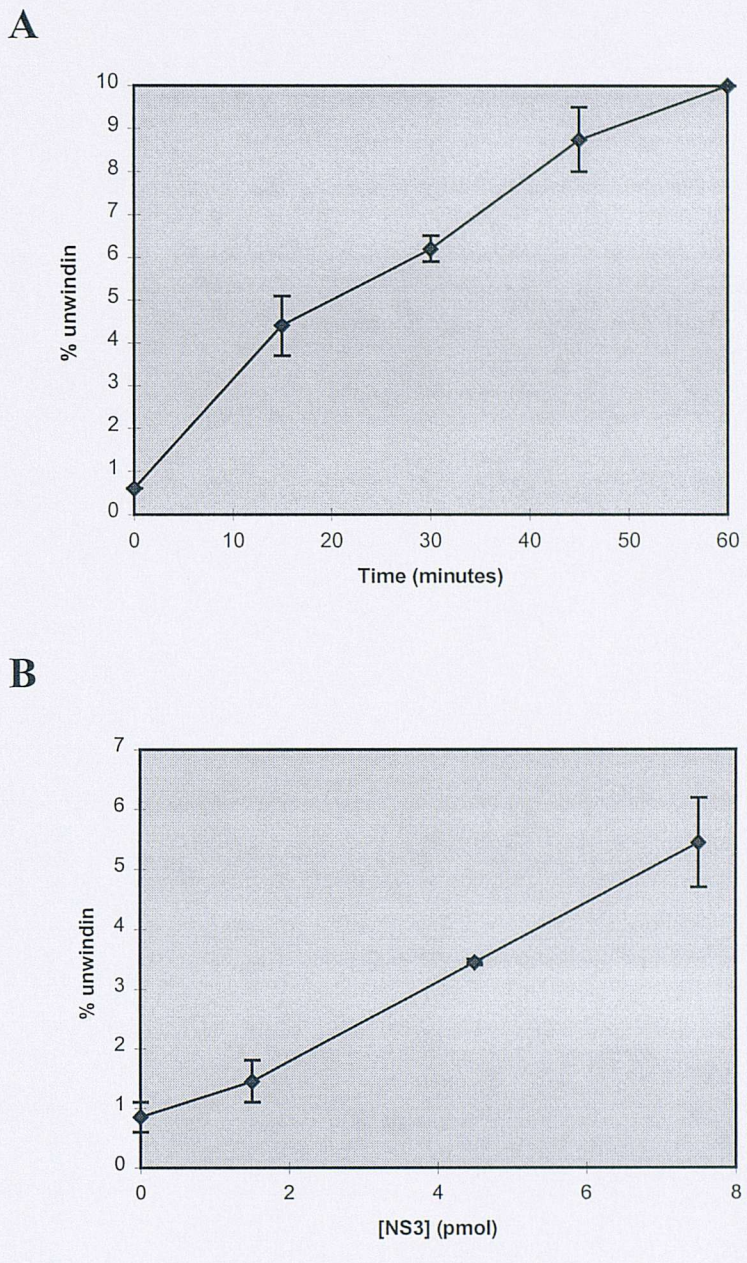
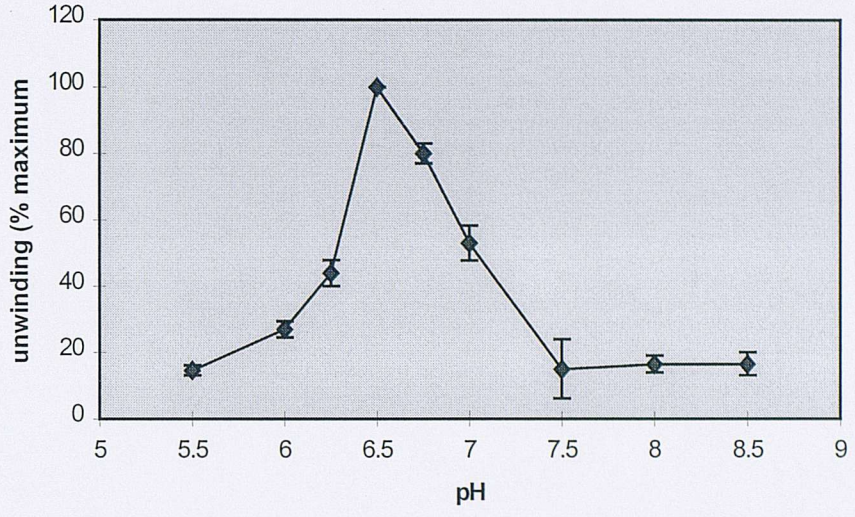


Figure 4.12 (A) Time course of NS3 helicase activity. 4.5 pmol NS3 incubated at 37°C for the length of time indicated. **(B) Helicase activity as a function of NS3 concentration**. The indicated amount of NS3 was incubated at 37°C for 30 minutes. Unwinding activity expressed as a percentage of the substrate unwound.

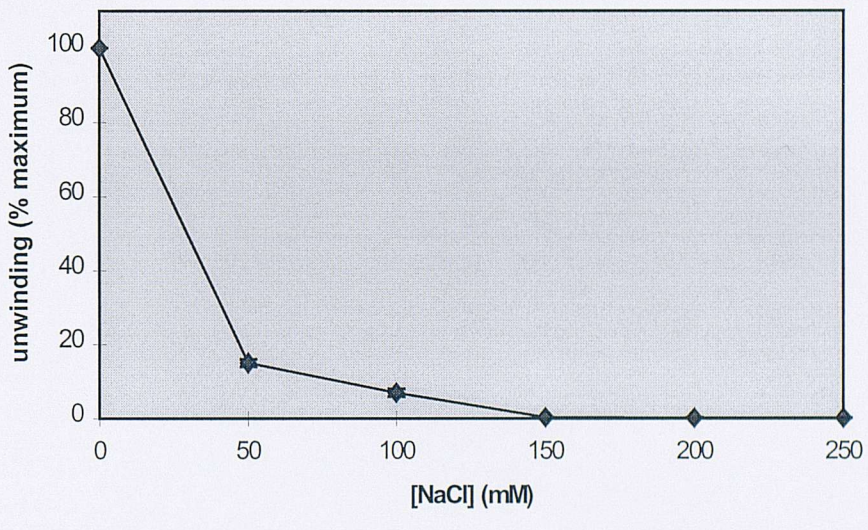


Figure 4.13 Optimisation of conditions for NS3 associated helicase activity. Helicase activity was assayed as described in materials and methods (section 2.7.2) except for the variable parameter, which is indicated on the x axis. Activity has been expressed as a percentage of the maximum observed. (A) Optimisation of buffer pH. All buffers were tested at 50 mM; pH5.5 and 6.0 MES, pH 6.5, 7.0 and 7.5 MOPS KOH, pH 8.0 and 8.5 Tris-HCl. (B) Effect of NaCl on NTPase activity. (C) Effect of KCl on NTPase activity. (D) Determination of optimum $MgCl_2$ concentration at 5 mM ATP and 10 mM ATP.

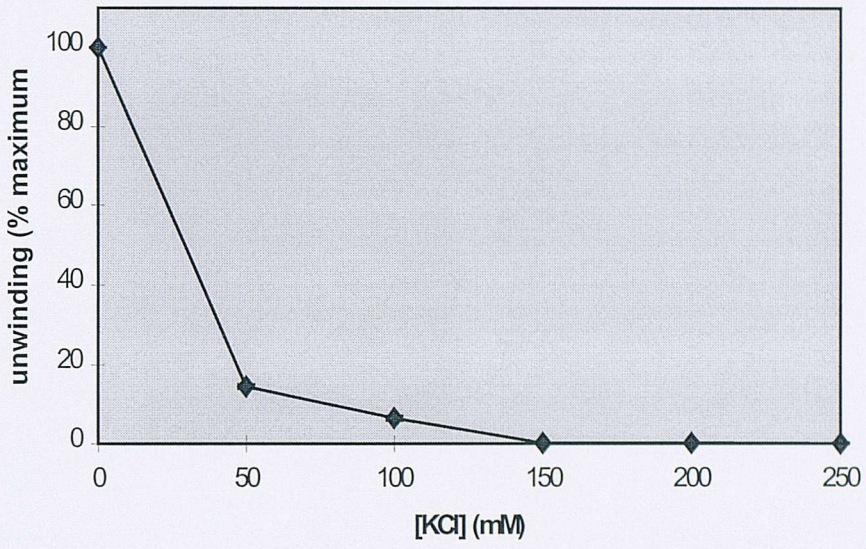
A


120
100
80
60
40
20
0
unwinding (% maximum
5
5.5
6
6.5
7
7.5
8
8.5
9
pH

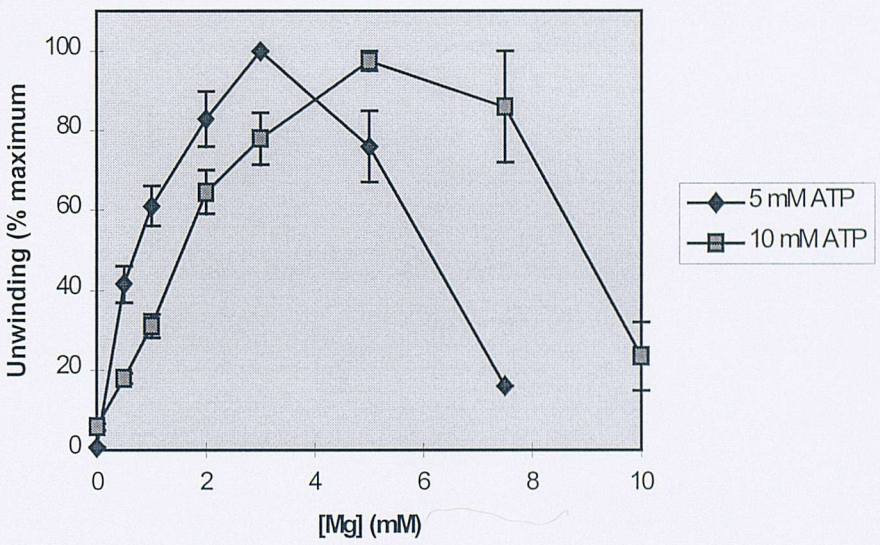
B


100
80
60
40
20
0
unwinding (% maximum)
0
50
100
150
200
250
[NaCl] (mM)

C


unwinding (% maximum
100
80
60
40
20
0
0
50
100
150
200
250
[KCl] (mM)

D


Unwinding (% maximum
100
80
60
40
20
0
0
2
4
6
8
10
[Mg] (mM)
5 mM ATP
10 mM ATP

required for helicase activity and maximum activity was observed with 3 mM $MgCl_2$ at 5 mM ATP and 5 mM $MgCl_2$ at 10 mM ATP (**Figure 4.13D**).

4.3.3 Nucleotide Triphosphate Utilisation as Energy Sources of Helicase Activity

The ability of NS3 to utilise different NTPs as energy sources for unwinding of a DNA duplex was examined. NS3 was able to unwind the substrate with all NTPs tested but to different degrees (**Figure 4.14**). Maximum unwinding was observed with ATP, CTP and dTTP. Approximately 50% of the maximum helicase activity was observed when UTP, dATP and dCTP were used as an energy source for unwinding. GTP and dGTP were the poorest source of energy for unwinding, activity was less than 25% of maximum with these substrates.

4.4 Mutational Analysis of Helicase and NTPase Activities of HCV NS3 Protein

NS3 helicase is a member of the DEAD-box family of helicases and these are characterised by highly conserved domains named motifs I - VI. Amino acid substitutions were made within these motifs as described in section 3.1.2 and the effect of these substitutions on helicase and NTPase activities was investigated.

Each mutant was prepared as for the wild type NS3 (section 3.1) then assayed for NTPase (**Figure 4.15**) and helicase (**Figure 4.16**) activity. The activities of the mutant NS3 proteins were compared to wild type NS3 activity and background activity which was determined using NS5A/BΔ. Mutants $D_{1316}A$ and $E_{1317}A$ (motif II) and $R_{1493}K$ (motif VI) showed only the basal level of each activity compared to the NS5A/BΔ

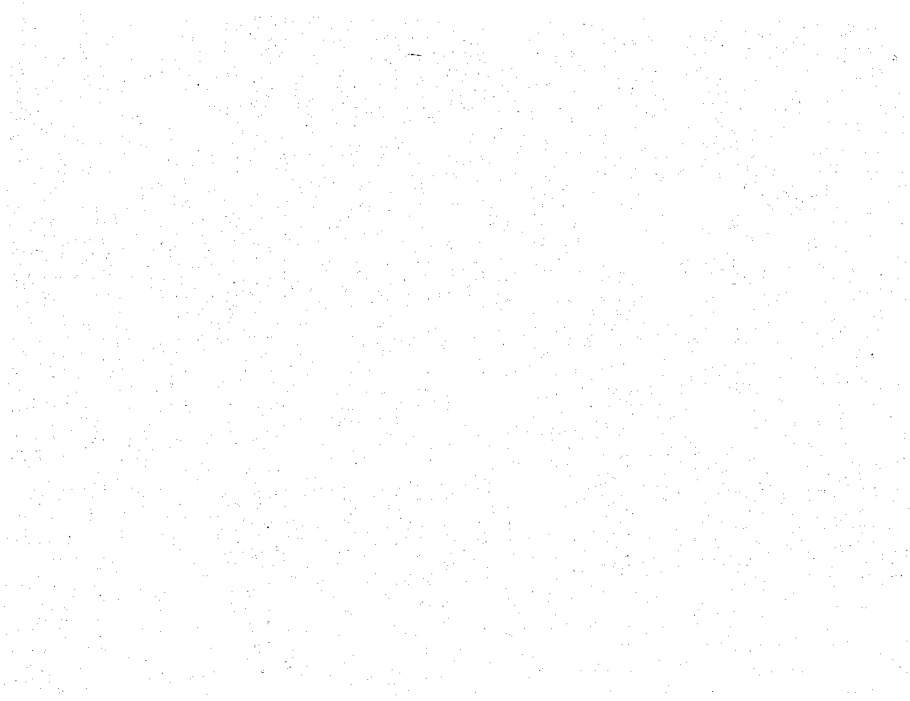
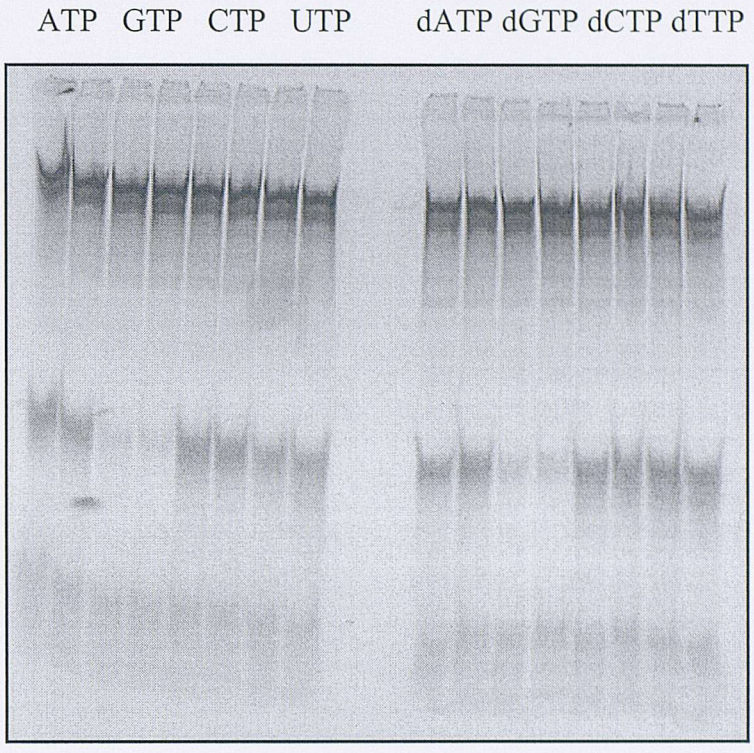


Figure 4.14 Ability of various nucleotide triphosphates to provide an energy source for NS3 helicase activity. (A) Autoradiograph demonstrating helicase activity in the presence of different NTPs (in duplicate). (B) Histogram showing helicase activity expressed as a percentage of the maximum unwinding of dsDNA substrate observed. ATP, GTP, CTP, UTP, dATP, dGTP, dCTP and dTTP were tested for their ability to act as an energy source for helicase activity. Each nucleotide was present at 5 mM and all other conditions were standard (section 2.7.2).

A


ATP GTP CTP UTP
dATP dGTP dCTP dTTP

B

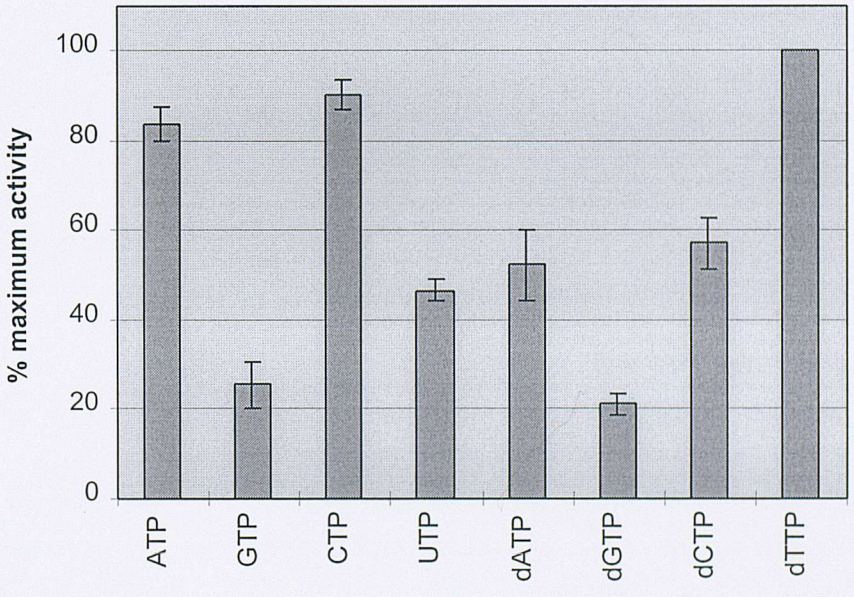
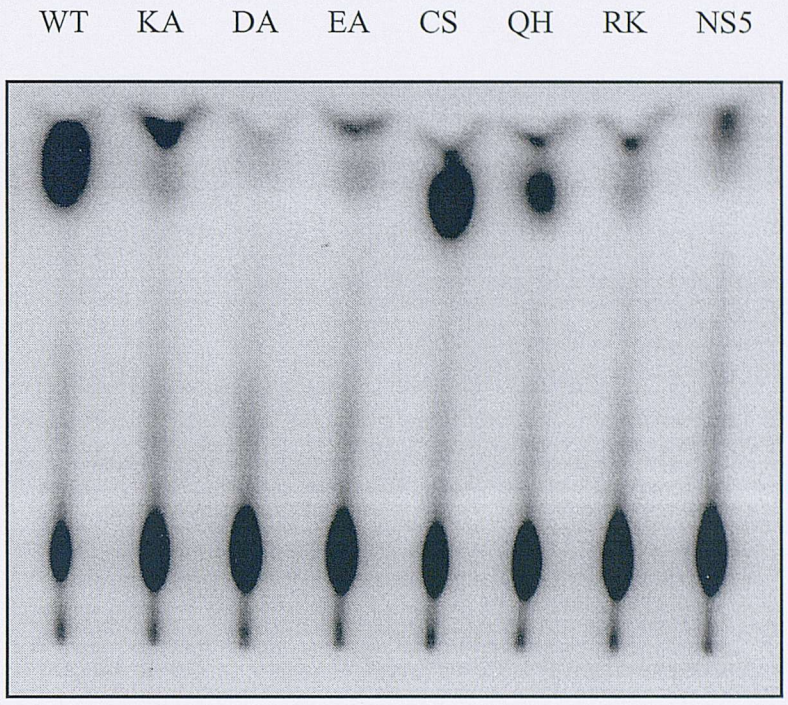

% maximum activity
100
80
60
40
20
0
ATP
GTP
CTP
UTP
dATP
dGTP
dCTP
dTTP

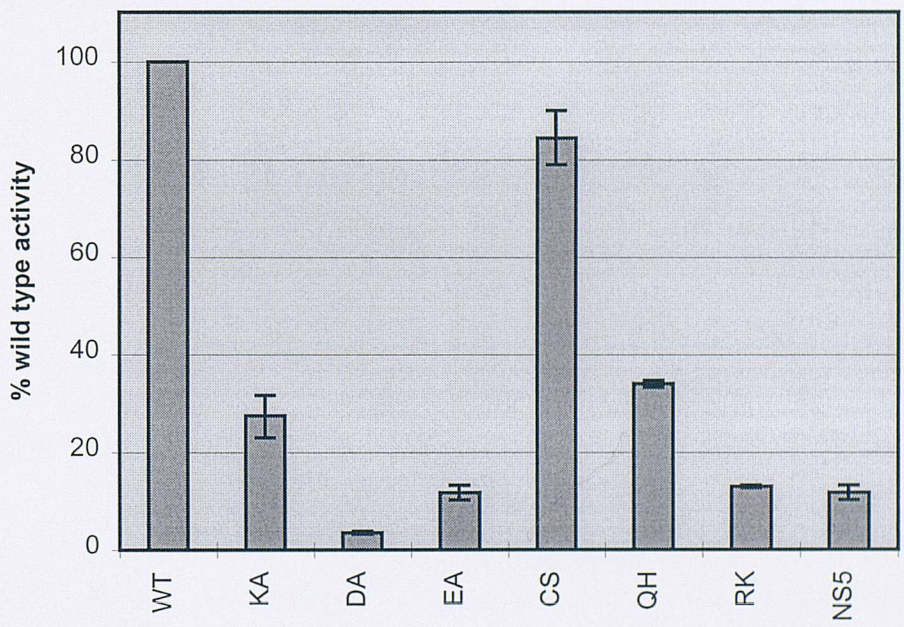


Figure 4.15 Mutational analysis of NS3 associated NTPase activity.1.5 pmol of each NS3 mutant or wild type NS3 was assayed for NTPase activity using the radioactive method. (A) Autoradiograph. (B) Histogram with activity expressed as a percentage of wild type activity. WT, wild type NS3; KA, $K_{1236}A$; DA, $D_{1316}A$; EA, $E_{1317}A$; CS, $C_{1318}S$; QH, $Q_{1486}H$; RK, $R_{1493}K$; NS5, NS5A/BΔ control.

A


WT
KA
DA
EA
CS
QH
RK
NS5

B


% wild type activity
100
80
60
40
20
0
WT
KA
DA
EA
CS
QH
RK
NS5

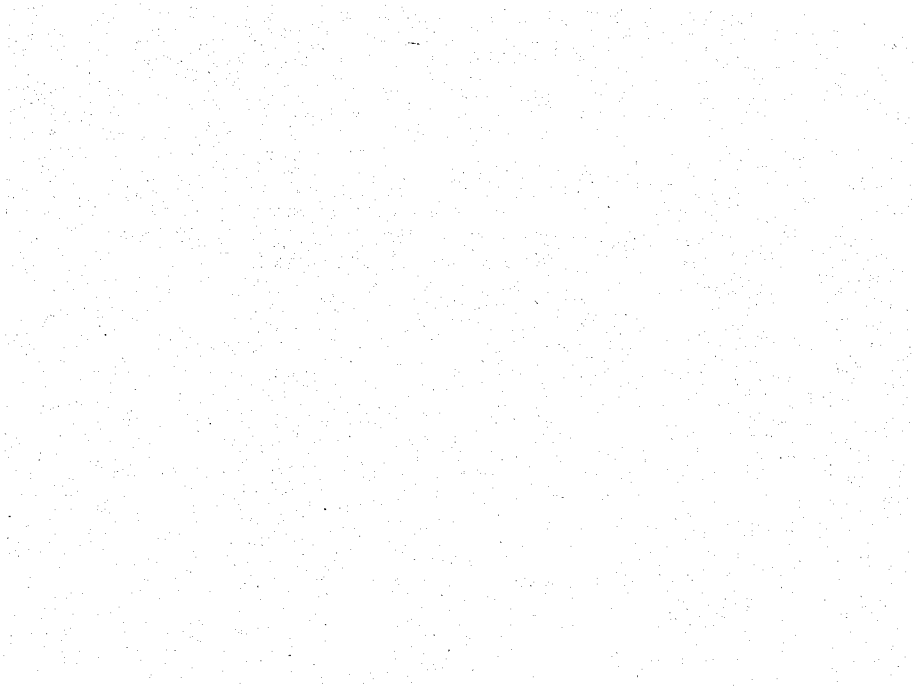
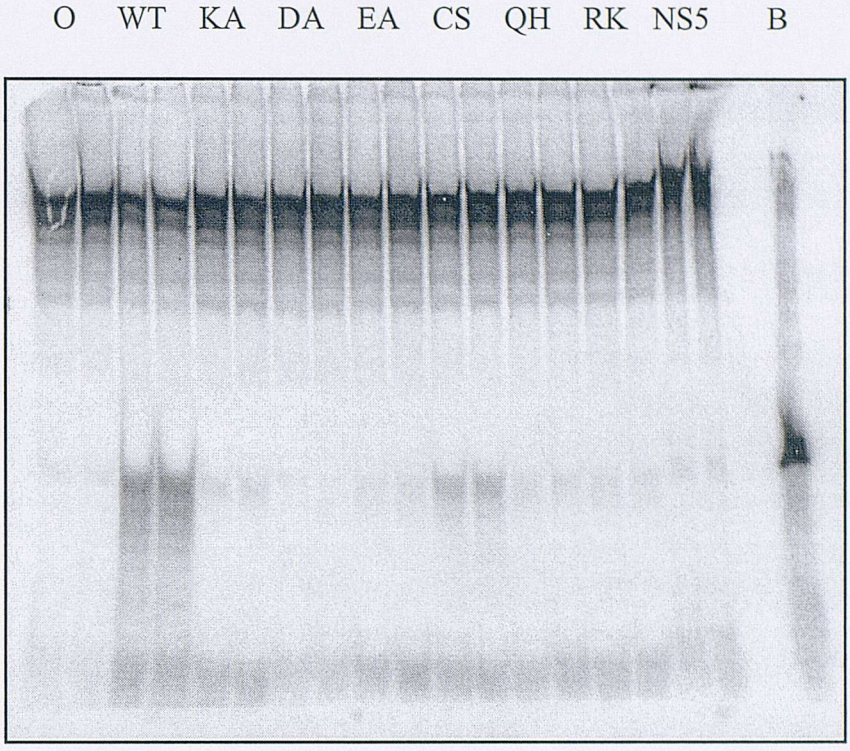
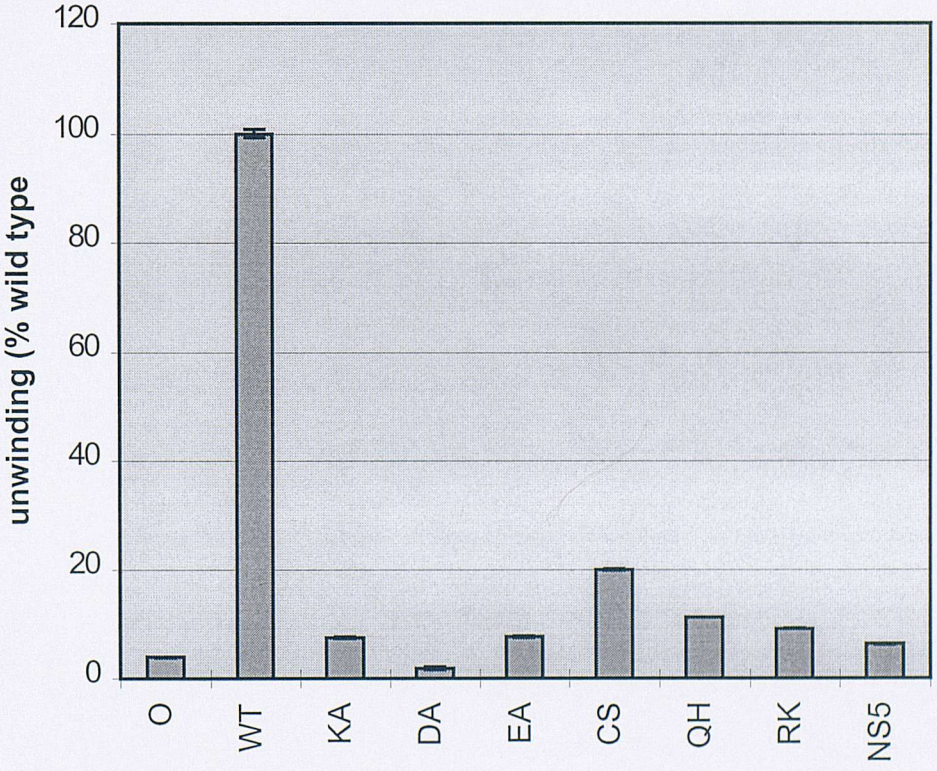


Figure 4.16 Mutational analysis of NS3 associated helicase activity. (A) Autoradiograph demonstrating unwinding activity of NS3 mutants (in duplicate). (B) Histogram with activity expressed as a percentage of wild type activity. WT; wild type NS3, KA; $K_{1236}A$, DA; $D_{1316}A$, EA; $E_{1317}A$, CS; $C_{1318}S$, QH; $Q_{1486}H$, RK; $R_{1493}K$; NS5; NS5A/BΔ control, B; heat denatured substrate.

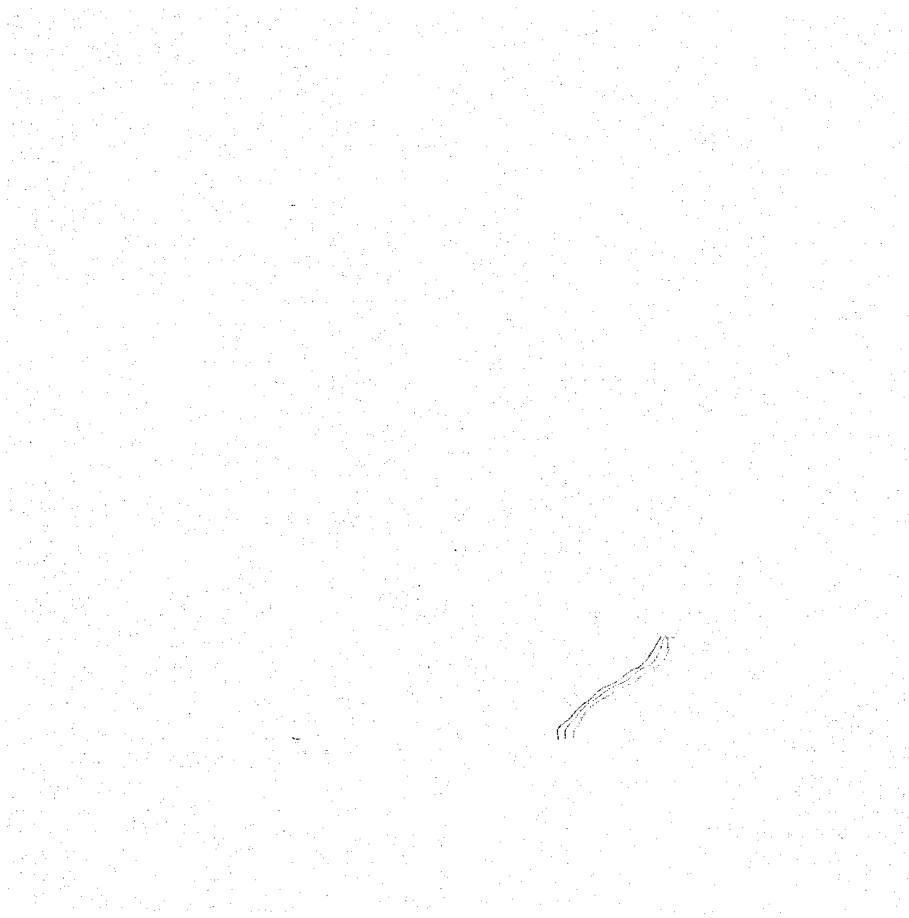
A


O
WT
KA
DA
EA
CS
QH
RK
NS5
B

B


unwinding (% wild type
120
100
80
60
40
20
0
O
WT
KA
DA
EA
CS
QH
RK
NS5

control. Substitution of the lysine residue of motif I ($K_{1236}A$) resulted in the loss of helicase activity but retention of 27 % wild type NTPase activity. Similarly the mutant $Q_{1486}H$ (motif VI) possessed 34 % wild type NTPase activity but helicase activity was detectable only at basal level. Mutation of the variable residue of motif II ($C_{1318}S$) had little effect on NTPase activity but reduced helicase activity to 20 % of wild type.



Chapter 5: Non-viral Functions of NS3

5.1 Subcellular Localisation of NS3 in Hepatocytes from Infected Patients

The subcellular localisation of NS3 has been investigated by expressing NS3 in experimental cell culture systems (320) and has been shown to localise to the nucleus in the presence of p53 (65). To investigate the localisation of NS3 in hepatocytes from infected patients, cells obtained from liver biopsies were immunostained for NS3 using an NS3-specific antibody.

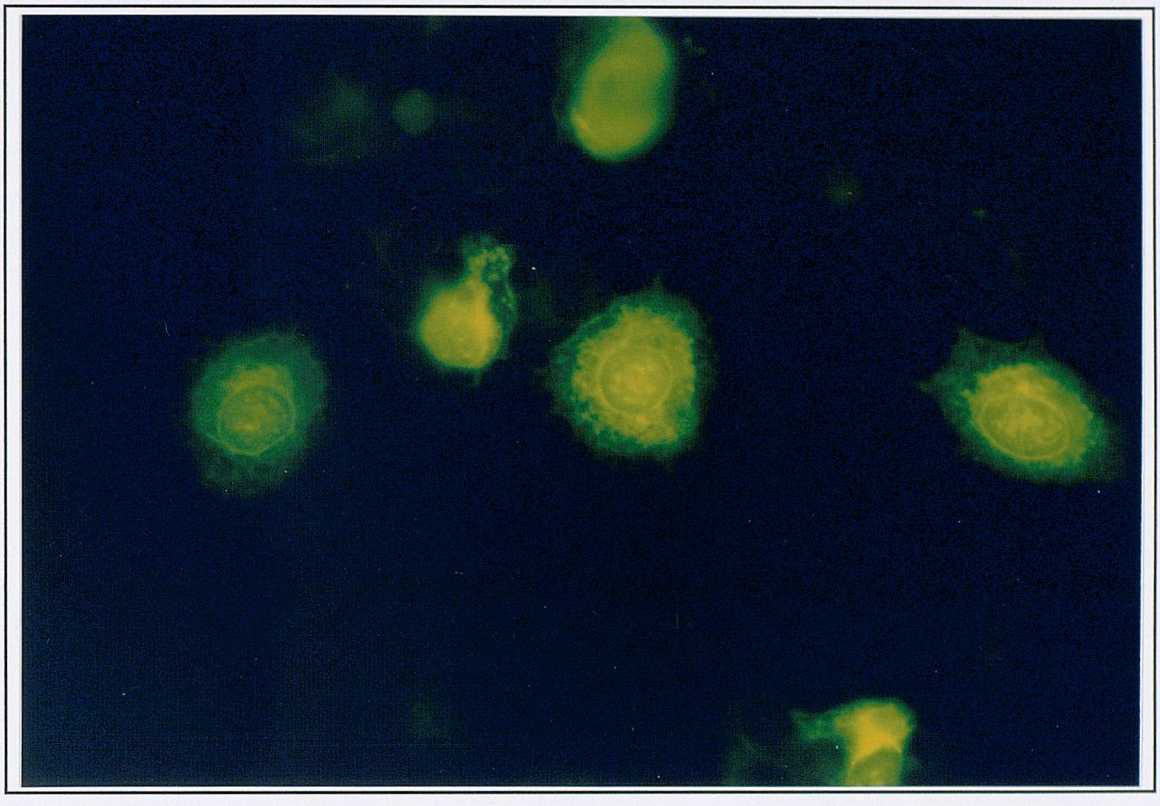
The anti-NS3 antibody NS3-7 used in this study was confirmed for NS3 specificity by Western blot (**Figure 3.3A**) and immunofluorescence of HepG2 cells transfected with pCDNS3 a mammalian expression vector containing the coding region for full length NS3 (**Figure 5.1**).

Liver biopsies were obtained from 49 patients, 30 with chronic HCV infections and 19 with other non-HCV liver diseases (e.g. alcoholic liver disease, HBV infection) and the hepatocytes were stained for NS3 using NS3-7. NS3 specific staining was observed in the cytoplasm only (**Figure 5.2A**), nucleus only (**Figure 5.2B**) or both cytoplasm and nucleus (**Figure 5.2C**) of hepatocytes from HCV infected individuals. Staining was not observed in hepatocytes of non-HCV infected patients (**Figure 5.2D**) and staining of NS3 was blocked by pre-incubation of NS3-7 with full length recombinant NS3 (**Figure 5.2E**). NS3 was predominantly located in the cytoplasm where the staining appeared granular and diffuse and the levels of staining varied from cell to cell within a single sample (**Figure 5.2A**). Occasionally dark areas of staining

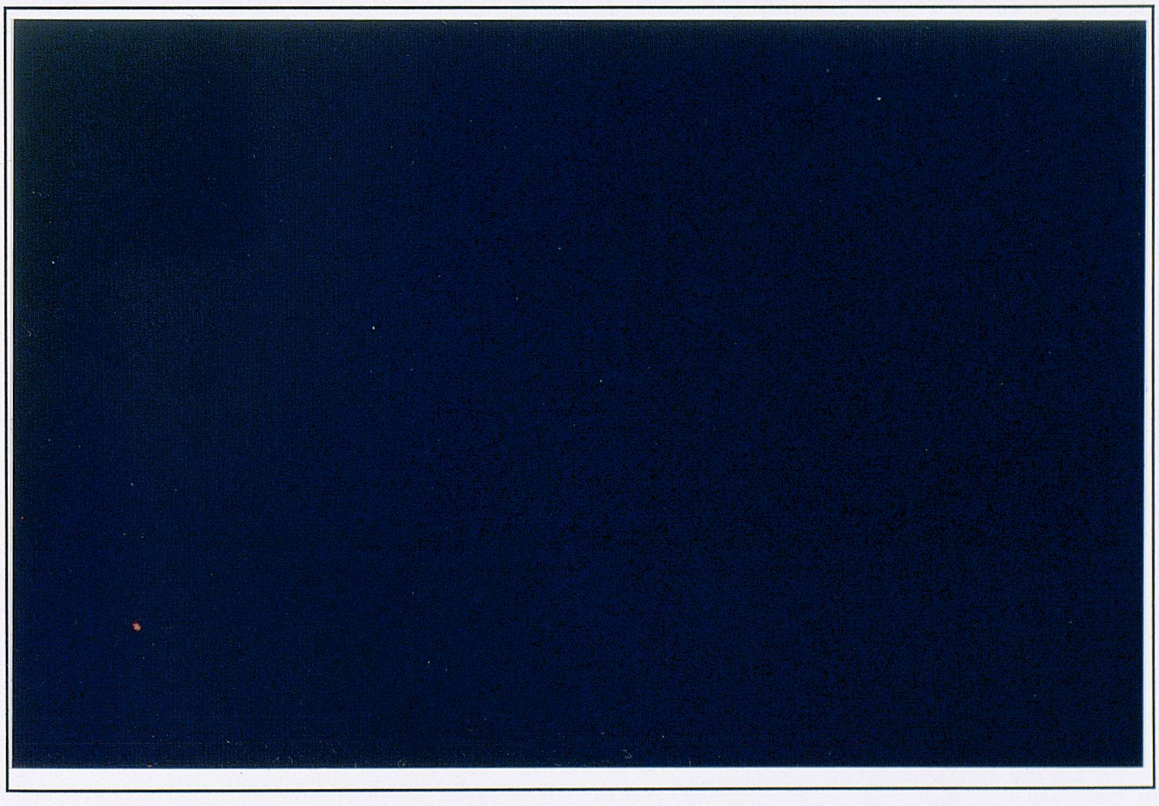


Figure 5.1 Detection of HCV NS3 in transfected HepG2 cells. A; HepG2 cells transfected with pCDNS3, B; HepG2 cells transfected with pCDN3.1. NS3 was detected with NS3-7 polyclonal antibody as described in section 2.7.6.

A



B



A

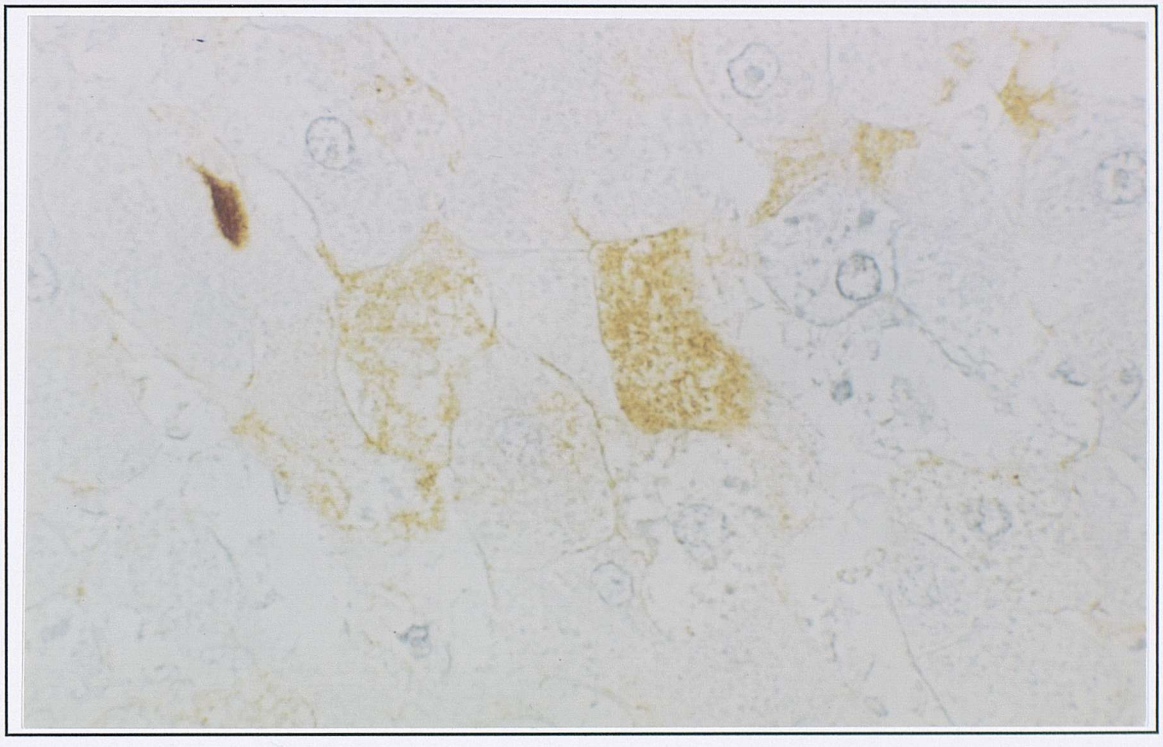
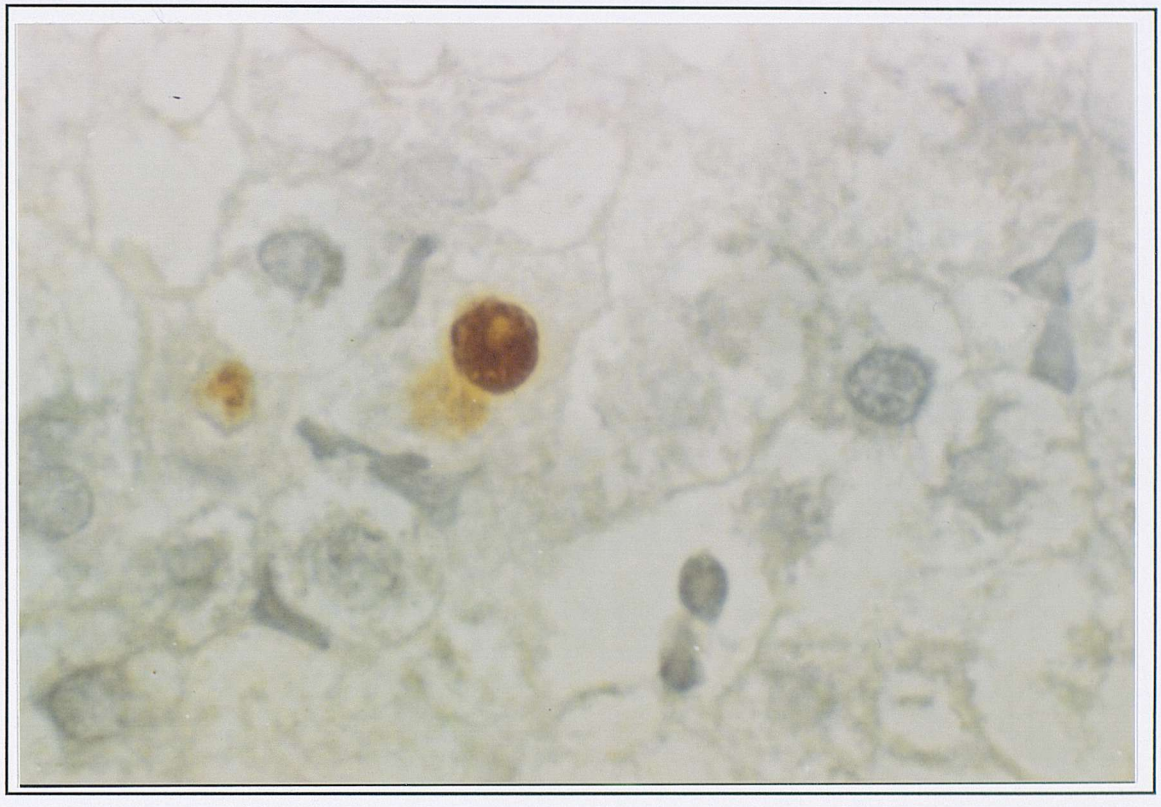
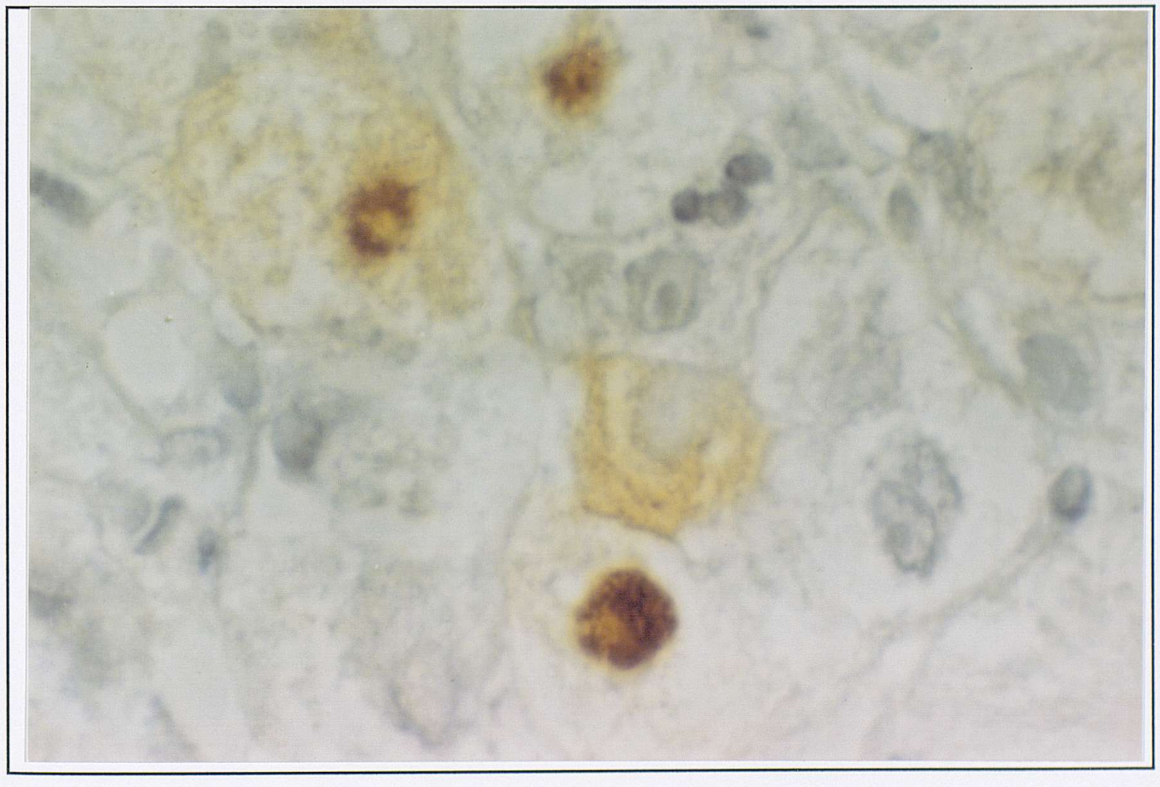


Figure 5.2 Immunostaining of HCV infected hepatocytes. Presence of NS3 was detected in HCV infected hepatocytes (that had been isolated from an infected patient) using NS3-7 polyclonal antibody as described in section 2.7.7. A - C, hepatocytes of HCV infected liver; D, hepatocytes of HBV infected liver; E, hepatocytes of HCV infected liver after preincubation of NS3-7 with recombinant HCV NS3 protein.

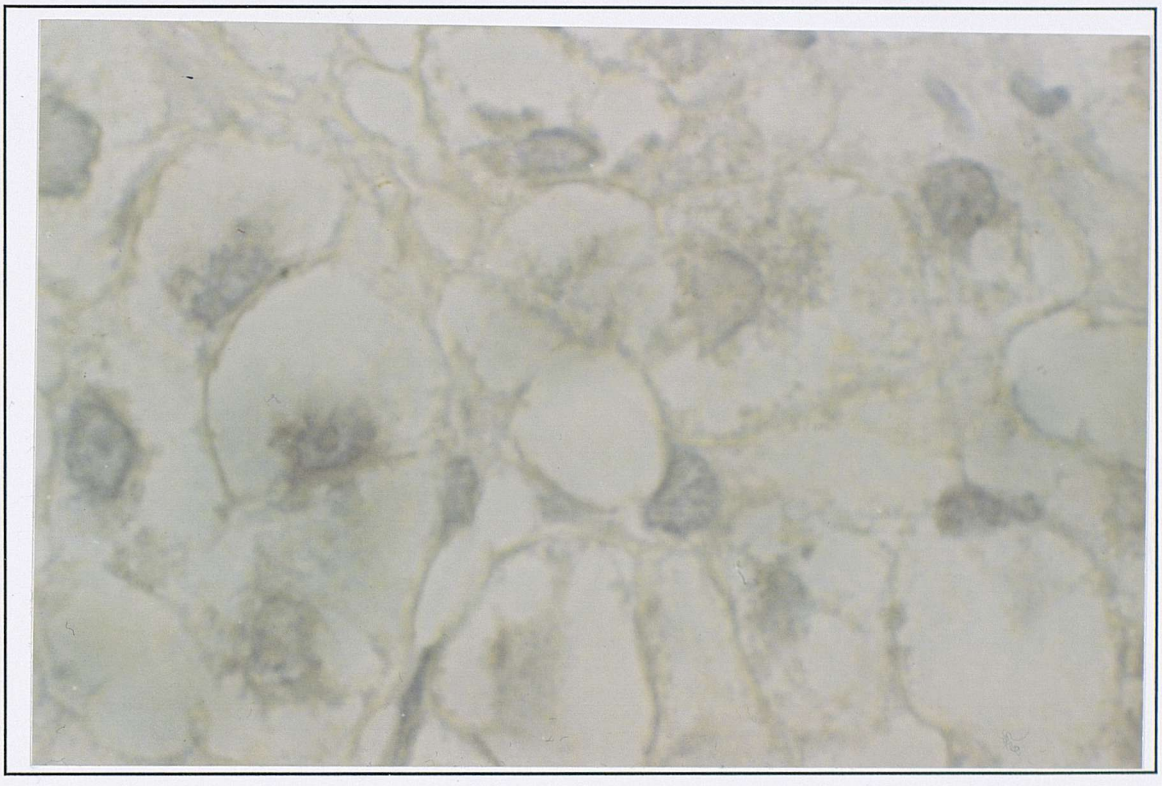
B



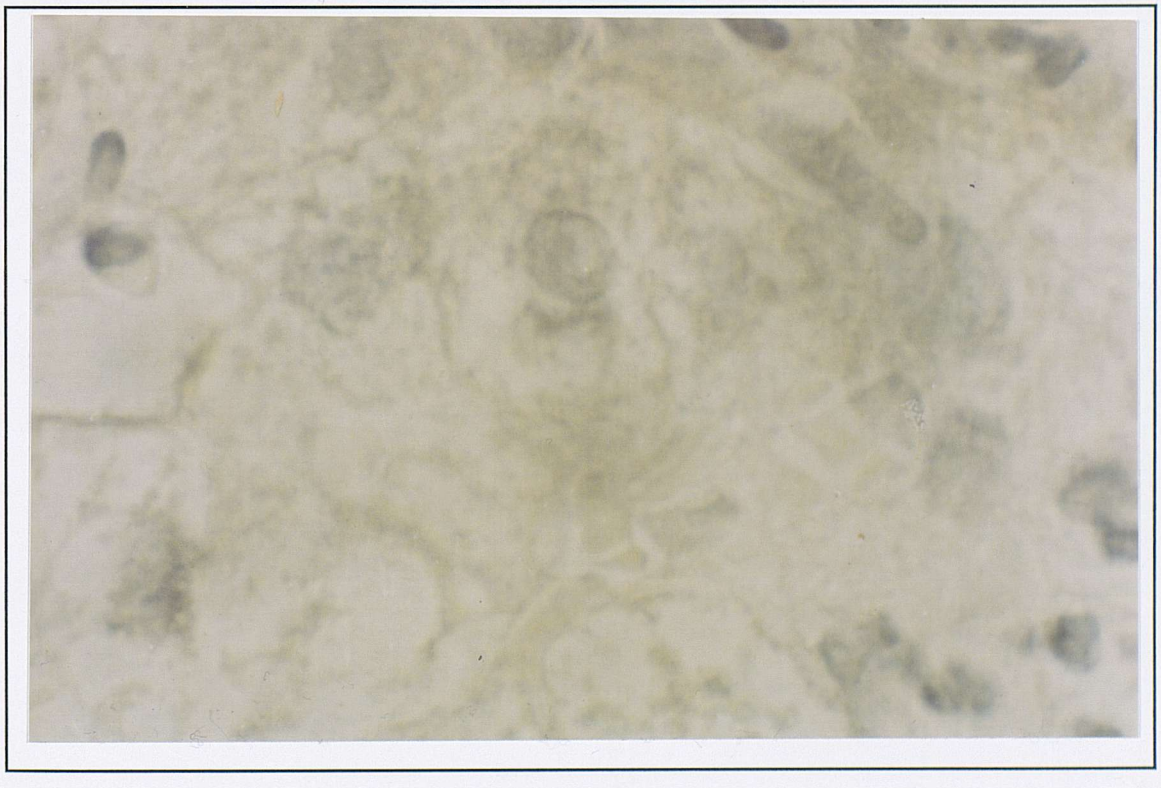
C



D



E



were present in the cytoplasm (**Figure 5.2A**) and nuclear staining was generally darker than cytoplasmic staining (**Figure 5.2B**). Between 2 and 11% of the total number of hepatocytes from HCV-infected individuals were positive for NS3 staining and greater staining was observed in cirrhotic livers (mean = 6%) than non-cirrhotic livers (mean = 3%). There appeared to be no correlation between the level of staining and grade (necro-inflammatory score) or stage (fibrosis) as determined using the modified Histological Activity Index (465).

5.2 NS3 Inhibition of cAMP-dependent Protein Kinase

Previously, Borowski *et al* have demonstrated that a recombinant protein corresponding to amino acids 1189 - 1525 of NS3 was able to inhibit protein kinase A (PKA) (321). Peptides corresponding to the region of NS3 that possessed sequence homology to the PKA inhibitor, PKI (PKI-like sequence) were also able to inhibit PKA. This region of homology mapped to amino acids 1487 - 1500 of NS3 and overlaps with helicase motif VI. This region of NS3 was compared between different HCV strains (**Figure 5.3**) and it was observed that the PKI-like sequence was not present in all HCV strains. The sequence was absent from all type 1a strains analysed and present in all 1 b sequences. Most other sequences compared contained the PKI-like sequence with the exception of types 1c, 6a, 10a (type 3) and 11a (type 6).

Full length NS3 possessing type 1b PKI-like sequence (RRGRTGRG**RR**GIYR) (pAW3-RR) was created from type 1a sequence (RRGRTGRG**KP**GIYR) by site-directed mutagenesis of pAW3(1027-1657) (section 3.1.2b). In addition peptides

HCV-JI	LPQDAVSRTQ RRGRTGRG**KP** GIYRFVAPGE	
HCV-1	LPQDAVSRTQ RRGRTGRG**KP** GIYRFVAPGE	1a
HCV-H	LPQDAVSRTQ RRGRTGRG**KP** GIYRFVAPGE	
HCV-H77	LPQDAVSRTQ RRGRTGRG**KP** GIYRFVAPGE	
HCV-UK	LPQDAVSRTQ RRGRTGRG**KP** GIYRFVAPGE	
HCV-J4	VPQDAVSRSQ RRGRTGRG**RS** GIYRFVTPGE	
HCV-C2	VPQDAVSRSQ RRGRTGRG**RR** GIYRFVTPGE	1b
HCV-JT	VPQDAVSRSQ RRGRTGRG**RG** GIYRFVTPGE	
HCV-K1	VPQDAVSRSQ RRGRTGRG**RR** GIYRFVTPGE	
HCV-J	LPQDAVSRAQ RRGRTGRG**RS** GIYRFVTPGE	
HCV-G9	VPQDAVSRSQ RRGRTGRG**KH** GIYRYVSPGE	1c
HCV-J6	VPQDAVSRSQ RRGRTGRG**RL** GIYRYVSTGE	2a
HCV-J8	VPQDACSRSQ RRGRTGRG**RL** GVYRYVSSGE	2b
HCV-BEBE1	VPQDSVSRSQ RRGRTGRG**RL** GIYRYVSSGE	2c
HCV-NZL1	APQDAVSRSQ RRGRTGRG**RL** GTYRYVASGE	
HCV-K3a	APQDAVSRSQ RRGRTGRG**RL** GTYRYVTPGE	3a
HCV-JK049	VPQDAVSRSQ RRGRTGRG**KS** GTYRYVSPGE	10a (type 3)
HCV-Tr	VPQDAVSRSQ RRGRTGRG**RP** GIYRFVTPGE	3b
HCV-ED43	VPQDAVSRSQ RRGRTGRG**RL** GTYRYVTPGE	4a
HCV-EUH1480	VPQDAVSRSQ RRGRTGRG**RH** GIYRYVSSGE	5a
HCV-EUHK2	VPQDAVSRSQ RRGRTGRG**KP** GVYRFVSQGE	6a
HCV-JK046	VPQDAVARSQ RRGRTGRG**KP** GVYRYVSQGE	11a (type 6)
PKI	SGRTGR-RN AIHD	

Figure 5.3 Sequence alignment of the PKI-like NS3 sequence from different HCV genotypes.

corresponding to 1b sequence RGRRGIYR (PEP1b) and 1a sequence RGKPGIYR (PEP1a) were obtained. This region of the PKI-like sequence had previously been demonstrated to inhibit PKA with the same efficiency as the full sequence (321). An unrelated peptide, PEPc was used as a control. NS3 expressed in *E. coli* from pAW3(1027-1657) (NS3-KP) and pAW3-RR (NS3-RR) and peptides PEP1a and PEP1b were compared for their ability to inhibit PKA activity.

In the assay employed here neither NS3-WT or NS3 RR demonstrated inhibition of PKA activity (**Figure 5.4**). Both PEP1a and PEP1b showed inhibition of PKA compared to PKA activity in the absence of peptide inhibitor or in the presence of the control peptide PEPc (**Figure 5.5**). In these experiments PEP1a was less efficient for PKA inhibition than PEP1b.

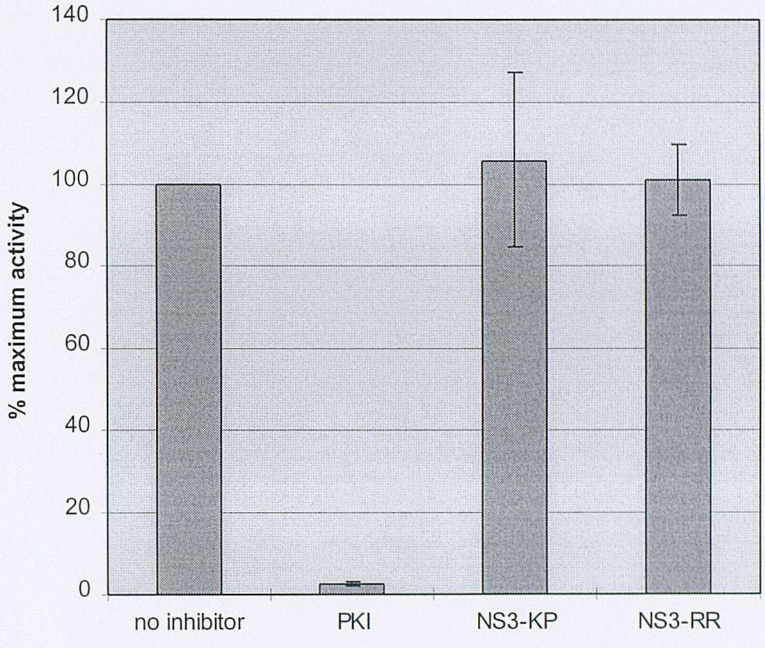


Figure 5.4 Inhibition of PKA activity with full length NS3. NS3 type 1a (NS3-KP) and NS3 type 1b (NS3-RR) were analysed for ability to inhibit PKA activity. 1.2 μM NS3 assayed.

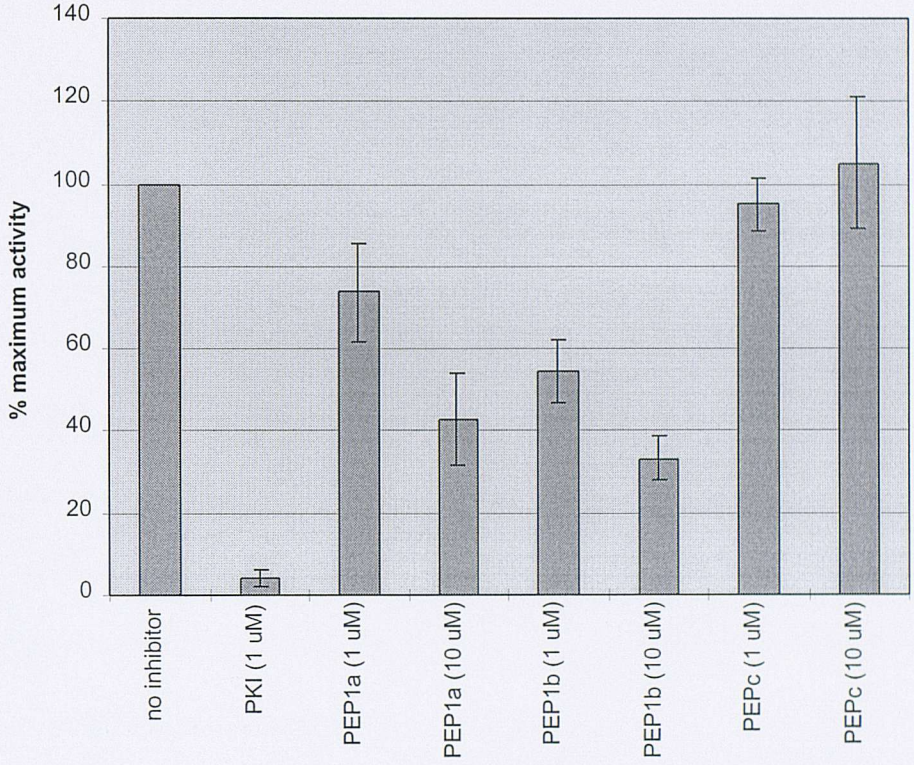


Figure 5.5 Inhibition of PKA activity with peptides corresponding to NS3 PKI-like region. Activity of PKA assayed in the presence of NS3 type 1a (PEP1a) and NS3 type 1b (PEP1c) PKI-like peptides. PEPc represents a 15'mer peptide control. Peptides were used at the indicated concentration.

Chapter 6: Discussion

6.1 HCV Replication and NS3

In the 1980's a considerable amount of research was undertaken to study the molecular biology and replication of the other members of the family Flaviviridae, the pestiviruses and flaviviruses (105,339,466). Following the discovery of HCV in 1989, the organisation of the HCV genome was proposed by comparison with that of the flavi- and pestiviruses and activities were putatively assigned to individual proteins on the basis of homologous motifs to these proteins (3,4,132). Use of the vaccinia virus T7-hybrid expression system and *in vitro* transcription and translation procedures enabled the identification of the HCV proteins, their N- and C-termini and identified essential components of the replicative machinery (142,145,224). In this way NS3 was confirmed to be essential for the processing of the viral polyprotein at several sites and it was assumed that NS3 would be essential for viral replication (149,150).

Following expression and purification of NS3, the putative helicase and NTPase activities - initially proposed on the basis of sequence comparisons and the presence of conserved amino acid motifs - were demonstrated (278,280). Since then work has progressed rapidly on the characterisation of the protease, helicase and NTPase activities, driven in the main by an urgent need for an effective antiviral therapy. However, the majority of these studies have been carried out using fragments of NS3 that correspond to either the protease or helicase/NTPase domains. Characterisation of full length NS3 protein has suggested biochemical differences compared to the

respective domains (303) and hinted at the possibility of interaction between the different domains which could in turn influence their activities (312).

The expression, purification and characterisation of full length NS3 is presented in this thesis and is discussed in comparison to studies of the individual NS3 domains. Studies to investigate the role of NS3 in altering the biochemical activity of the host cell by inhibition of cellular proteins have also been instigated in this thesis and this work and the work of others is also discussed here.

6.2 Expression of NS3 in *E. coli*

Full length NS3 was expressed from the plasmid pAW3(1027-1657) in *E. coli* JM109(DE3) as insoluble protein. Expression of the same plasmid in other *E. coli* strains failed to produce any detectable levels of NS3 protein. Comparison of the genotypes of the strains identified only one significant difference; strain JM109(DE3) carries the mutant *relA1* genotype. Wild type RelA1 protein is responsible for the accumulation of guanosine 3'5'bispyrophosphate (ppGpp) in response to amino acid starvation which inhibits initiation of transcription (467). RelA1 is also important for the control of transcription of rRNA and ribosomal protein transcription (468). This genotype, which is absent in the other strains used during the work presented here, permits RNA synthesis in the absence of protein synthesis and this may contribute to the ability of NS3 to be expressed. NS3 has been expressed in both JM109(DE3) (310) and BL21 (DE3) strains (259) however, this was achieved using HCV type 1b sequence and expression in BL21(DE3) was dependent upon tightly regulated expression conditions

(259). Thus it is likely that the genotype of the *E. coli* strain and HCV genotypes contribute to the different expression levels of NS3 observed.

Western blot analysis of *E. coli* expressed NS3 identified smaller immunoreactive proteins which were likely to correspond to degradation products of NS3. This degradation probably originated at the N-terminus since immunoblotting with an anti-histidine tag antibody identified only a single band of the expected size for full length NS3. Isolation of NS3 in the presence of protease inhibitors did not prevent degradation indicating that degradation occurred prior to cell lysis. Target protein degradation can been reduced by a reduction of the culture temperature during expression studies (451), however, when NS3 was expressed at 25°C no reduction in degradation was observed. Since the majority of the NS3 produced was full length protein and the degradation species did not contribute to NS3 activity (section 3.2.1) the protein degradation observed was tolerated.

Expression of foreign genes in *E. coli* often results in the production of insoluble recombinant protein (448,450,469) and this was observed during attempts to express NS3 in *E. coli*. Production of insoluble protein can be attributed to mis-folding of the expressed polypeptide, often involving the formation of unwanted disulphide bridges (450). The causes of inclusion body formation are not clear although it is believed that overproduction of the target protein may be sufficient (450). This could be a result of altering the relative levels of translated proteins and associated factors, for example chaperones which aid the folding of proteins (450,453). Other factors may also contribute to inclusion body formation. NS3 was expressed as a discrete polypeptide rather than as part of the complete HCV polypeptide which would occur during viral replication and this could also contribute to incorrect folding of NS3. Upstream and

downstream amino acid sequences may contribute to NS3 folding prior to release from the polyprotein. Studies of the polyprotein processing at the NS2/3 and NS3/4A cleavage sites provide evidence for this (147,148,225,234). NS3 is released from the polyprotein following cleavage by NS2 and NS3 proteases which require an active N-terminal one third of NS3 (148) and conformation of the NS2/3 cleavage site appears important for cleavage (225) so it can be assumed that at least this region will be folded before release from the polyprotein. Whether the C-terminal region of NS3 is fully folded before polyprotein release is less clear; however, it is believed that *cis* cleavage at the NS3/NS4A junction is dependent as much on 3 dimensional conformation as it is on primary amino acid sequence (234) and so this region is likely to be partially folded prior to release from the polyprotein. In addition other viral proteins such as NS4A associate closely with NS3 (239) and these may contribute to NS3 conformation. During HCV replication in its natural host, NS3 would be expressed in a eukaryotic environment and not in a prokaryotic environment as was used during this work, and this too may contribute to incorrect folding. For example, HCV replication is believed to occur at a complex associated with the endoplasmic reticulum membrane. Viral proteins NS2, present at the N-terminus of NS3 on the polyprotein and NS4A, present at the NS3 C-terminus and known to interact with NS3 (239), are believed to associate with the ER membrane (143,229) and these associations may be important for correct NS3 folding. Studies have shown that ER membrane association may aid folding of a protein corresponding to HCV NS2/3 and cleavage of the NS2/3 junction (230). Also, targeting of this protein to the membrane requires the host protein SRP and the SRP receptor (229) indicating the requirement for other host factors. It should be noted however that HCV type 1b NS3 has been expressed in *E. coli* as soluble protein (259) which implies

that *E. coli* factors may be sufficient for the correct folding of NS3 under certain culture conditions.

Expression conditions were investigated to enhance the recovery of soluble protein. A variety of strategies may be employed to improve the yield of soluble protein (448,451). As mentioned above, mis-folding of protein may be caused by upsetting the relative levels of translated proteins and associated factors (450). Reducing the culture temperature (450) or IPTG (inducer) concentration (451) can often improve yields of soluble protein possibly by reducing the levels of expressed protein. Alternatively the equilibrium between translated protein and associated factors may be restored by increasing the relative amounts of the associated factors. Rich culture media has been shown to be successful in increasing levels of soluble protein (448) possibly by increasing the relative amounts of these translation associated factors. These factors may also be co-expressed with the (target) proteins to increase their relative levels. An example of these are the chaperone proteins which are thought to bind to the nascent polypeptides to prevent unfavourable folding events from occurring (438). Recovery of soluble protein can also be improved by the presence of detergents in the lysis buffer which may prevent protein aggregation upon lysis of the cells and release membrane bound protein which would otherwise be pelleted during the isolation procedure.

All of these strategies were employed to increase the solubility of NS3 without any apparent effects. Under all expression conditions investigated NS3 was only present in the insoluble fractions.

Truncations of the target protein can be made in an attempt to stabilise the recombinant proteins by removing destabilising amino acid sequences, thus aiding protein folding and solubility. The N-terminus of NS3, sequence A_{1027} **P I** T **A** Y **A**

contains several hydrophobic residues (shown in bold) and it was considered that the removal of these residues might stabilise the protein. It has been shown previously that deletion of the 11 N-terminal amino acids which include these residues has no detrimental effect on the activity of the NS3 protease domain (256). To investigate the possibility of increased solubility of N-terminal truncated NS3, a full length NS3 construct with the 11 N-terminal amino acids removed, pET3(1038-1657) was made. Other NS3 constructs were made and expression of these constructs was investigated for the production of soluble NS3. One of these, pRS3(1027-1657) carries the same NS3 coding sequence as pAW3(1027-1657) but without an N-terminal histidine tag which may affect protein solubility by interfering with protein folding. Also, the NS3 coding sequence from a HCV type 1a infectious clone (428) was used to construct plasmid pRP3(1027-1657). Sequence analysis of this HCV clone identified three amino acid positions that differed from pAW3(1027-1657) NS3 sequence but were highly conserved in other HCV isolates (section 3.1.3h). Expression of soluble, full length NS3 in *E. coli* has been reported from constructs with the consensus amino acids at these positions (259,310). It is therefore possible that these positions may contribute to the secondary structure of NS3 and may be important for correct protein folding and thus aid the production of soluble protein.

However, soluble NS3 was not obtained from *E. coli* expression using these NS3 constructs and all attempts at producing soluble NS3 using the strategies described above were unsuccessful. NS3 was found almost exclusively in the insoluble fraction as inclusion bodies, regardless of the expression conditions or NS3 construct used.

Recently the NS3 protein of GBV-C (HGV) has been expressed and characterised in *E. coli* (470). The authors reported similar solubility problems using the

pET system and were only able to overcome these problems using an MBP fusion protein to stabilise NS3. Production of soluble, full length HCV NS3 in *E. coli* has also been described (233,259,310). This has been achieved using different culture conditions, HCV genotypes and fusion partners which are all likely to affect protein production and solubility (471). X-ray crystallography studies of the NS3 protease domain has identified differences in the 3 dimensional structure of the N-terminus between HCV strains which may also contribute to protein solubility (266,267).

Inclusion bodies are protein aggregates composed almost entirely of the target protein and enable a quick and simple method for protein purification (450). Inclusion bodies have a high density and can be pelleted by low speed centrifugation and this enabled separation of NS3 as inclusion bodies from the soluble fraction. Contaminating soluble proteins were removed by a series of washes consisting of sonication in a 'wash buffer' followed centrifugation and separation of the inclusion body pellet from the supernatant. The final protein pellet which contained only the inclusion body material was solubilised with 8 M urea containing the reducing agent DTT at a concentration of 1 mM.

To generate soluble, active NS3 from the denatured protein, refolding strategies were investigated. Urea is a strong denaturant containing two amino groups capable of forming hydrogen bonds which interfere with intramolecular hydrogen bonds of proteins, thus denaturing and solubilising the insoluble protein. DTT was also present to reduce any disulphide bridges that may have contributed to the aggregation of NS3 as disulphide bond formation between proteins is thought to occur during the formation of inclusion bodies (450). To refold proteins solubilised in urea, it is necessary to remove the urea which then allows the proteins to reform intramolecular hydrogen bonds and

reform their tertiary structure. The denaturant can be removed by dialysis or dilution or a combination of the two. Dialysis was preferred as this did not involve dilution of the protein sample which would have made assays for NS3 activity more difficult. To encourage refolding to a native state, it is advantageous to include reagents expected to enhance the refolding of the protein in to the native conformation. To this end urea was removed from the sample buffer by stepwise dialysis against Buffer R, which was designed to optimise refolding potential. Sodium chloride was included in the refolding buffer to provide a low ionic strength to prevent non-specific protein interactions that may cause mis-folding and aggregation of proteins. DTT, a reducing agent, mimics the reducing environment of the cytoplasm preventing the formation of disulphide bonds between cysteine amino acid residues. A pH of 7.6 was used as this represents the physiological pH of the mammalian cell. Glycerol was included in the buffer to mimic the conditions of relatively low water activity observed in the cell cytoplasm thus stabilising the proteins (472). The detergent CHAPS was included since it had been shown to be required for optimal NS3 protease activity (233,255) possibly by preventing protein aggregation. In addition CHAPS has been shown to aid refolding of other proteins expressed in *E. coli*, for example transforming growth factor β2 (450).

As much as 80% of the NS3 protein precipitated during refolding and the typical yield of soluble protein using this method was 1.25 - 1.5 mg protein per litre of culture at a concentration of 25 - 30 μg/ml. To improve the yield and concentration of soluble protein recovered, alternate refolding conditions were investigated.

Recovery of soluble NS3 during refolding was not improved by the presence of detergents, zinc, the absence of DTT or by using dilution or single step dialysis to remove the urea. Aggregation of protein occurred regardless of buffer composition and

method of removal of urea. The yield of soluble protein was only improved by first diluting NS3 in 8 M urea. This did not improve the concentration of protein recovered but by first diluting the protein, the overall loss of protein was reduced.

The causes of aggregation during *in vitro* refolding of NS3 may be, in part due to the absence of eukaryotic factors such as chaperones and cell organelles such as the ER which would be present during viral RNA translation. Also, other viral proteins which would normally be present during HCV replication such as NS4A which is known to associate strongly with NS3 (239,312) may be required for NS3 folding. In addition, during viral replication NS3 is expressed as part of a polyprotein flanked by protein sequences which may contribute to NS3 folding. Requirement of these factors for correct folding of NS3 have been discussed previously in this chapter.

6.3 Expression of NS3 in Insect Cells

As discussed above, one of the factors contributing to the expression of insoluble NS3 may be the absence of eukaryotic factors during expression in *E. coli*. In an attempt to overcome this, expression of NS3 was performed in insect cells using the baculovirus system. The use of insect cells for protein expression has many advantages compared to other eukaryotic cell lines; they can be grown easily in suspension culture to relatively high densities and media requirements are relatively simple, for example serum and CO_2 are not required. In addition use of the baculovirus system enables high levels of protein production from the strong polyhedrin promoter and simple efficient infection of insect cells.

Expression of NS3 using two recombinant baculoviruses (NS3-2 and NS3-3) resulted in the production of truncated NS3 specific proteins with the major species a protein of approximately 40 kDa. The presence of the full length NS3 coding sequence in the transfer vector had been confirmed by sequence analysis and in the recombinant baculovirus by PCR. Therefore, possible explanations for the appearance of a truncated product are that it was due to the introduction of a frameshift or premature stop codon by PCR error or proteolysis by host enzymes. NS3-2 and NS3-3 were prepared from different PCR reactions and both sequences confirmed by sequencing, therefore the presence of stop codons or frameshifts due to errors introduced by PCR was discounted. The introduction of a premature stop codon by recombination errors during production of the recombinant virus or subsequent genome replication was considered unlikely to have occurred in two independently produce recombinant viruses. The cause of the truncated NS3 proteins was most likely to be a result of proteolysis by host cell enzymes.

A number of strategies were used in an attempt to reduce the level of NS3 degradation. Protease inhibitors introduced to the cell lysis buffer had no significant effect on protein degradation indicating that degradation was occurring in the intact cell during culture. Various culture conditions were investigated to improve the yield of full length protein without success and this mirrored results experienced previously using a baculovirus construct composed of NS2/NS3 coding sequences with demonstrated active NS2/3 protease (A. Wardell, sandwich year project, SKB Pharmaceuticals) and the expression of NS3 alone by others (281). Though not investigated further here, this observation may have implications for the role of NS3 in the virus life cycle. Shoji *et al* have recently reported the specific cleavage of HCV NS3 between amino acids

R_{1488}/G_{1489} within motif VI of the helicase domain when expressed in insect and mammalian cell lines (315). In addition, the NS3 protein of dengue virus 2 has also been shown to be cleaved specifically at the homologous residues (314). Data presented here (section 4.4) demonstrates that motif VI is important for helicase and NTPase activity and therefore cleavage of NS3 at this site is expected to abrogate helicase activity but have no effect upon protease activity. Cleavage at this site may provide a means of regulation of the NS3 protein though it remains to be shown that this cleavage occurs *in vivo*.

6.4 NS3 has DNA Binding, Helicase and Polynucleotide Stimulated NTPase Activity

Using a bandshift assay, NS3 was shown to bind single stranded DNA. Specificity of binding, as opposed to random adsorption of DNA to the protein, was demonstrated by competition with unlabelled oligonucleotide and by the inability of heat denatured NS3 to bind DNA. It has previously been shown that NS3 requires between 7 and 21 nucleotides for binding (304) although X-ray crystallography has been performed for the NS3 helicase domain bound to an eight nucleotide poly(U) single stranded RNA molecule (306) which indicates an ability to bind smaller polynucleotides. However, analysis of the polynucleotide stimulation of NS3 showed that poly(U) oligomers less than 10 nucleotides in length were unable to stimulate activity and greatest stimulation was with oligonucleotides [poly(dU)/(U)] of 15 - 25 nucleotides (313). This suggests that the size of nucleic acid molecule bound by NS3 may be larger than 8 nucleotides under normal conditions or that although NS3 is able

to bind smaller molecules, larger molecules are required for stimulation of NTPase activity.

NTPase activity of full length NS3 was demonstrated and characterised. Optimal activity was observed in the absence of monovalent cations at pH 6.5 - 7.5, was dependent on magnesium and was stimulated by the presence of polynucleotides. NS3 NTPase activity was stimulated up to 7-fold by a range of single stranded RNA and DNA homopolymers unlike the corresponding proteins of flaviviruses and pestiviruses which are stimulated by a limited range of polynucleotides. The NS3 NTPase activity of the flavivirus YFV showed greatest stimulation with poly(A) and inhibited by poly(G) (301) whilst NS3 NTPase activity of the flavivirus JEV showed greatest stimulation with poly(U) or poly(C) and inhibited by poly(G), (dG) and (dC) (473). Like HCV NS3, the corresponding protein of the pestivirus BVDV showed greatest stimulation with poly(U) but the BVDV NTPase activity was not stimulated by either poly(A), (G), (dA) or (dC) (302).

It is apparent that HCV NS3 is able to bind to a range of polynucleotide substrates which suggests that polynucleotide binding is not dependent upon nucleotide sequence. In support of this, structural analysis has shown that NS3:oligonucleotide interactions occur at the oligonucleotide backbone rather than with the bases (306). However, poly(U) was shown to have the greatest stimulatory effect upon NTPase activity, stimulating activity by at least seven fold and this has also been shown by others (278). In addition NS3 has been shown to bind poly(U) with the strongest affinity (303,304) and these data suggest a preference for binding to poly(U). Since polynucleotide binding by NS3 has no apparent sequence specificity this preference is likely to be due to inherent properties of the poly(U) molecule such as persistence length

(P_∞) which is a measure of the flexibility of a linear polymer (474). Stiffer molecules have longer persistence lengths for example poly(U) is a flexible polynucleotide (P_∞ = ~ 10) compared to poly(A) (P_∞ = ~ 60) (474). The difference in flexibility of polynucleotides may go some way to explaining the preference of NS3 for poly(U) over other polynucleotides. These observations may have implications for HCV replication as a poly(U) rich tract is present at the HCV 3' UTR (141). NS3 helicase activity is non-specific i.e. it is able to catalyse the unwinding of both DNA and RNA substrates regardless of nucleotide sequence (277,279,280,297), therefore a preference for poly(U) binding may provide a binding pad for NS3 and target NS3 helicase activity to an origin for viral genome replication in the 3' UTR of the HCV genome.

NS3 was able to hydrolyse a variety of NTPs and dNTPs and showed a preference for ATP. ATP is the most abundant energy source in the cell and it is therefore unsurprising that this is the preferred substrate for NS3 NTPase activity. GTP and dGTP were the least favoured substrates, the k_{cat}/K_m values for GTP and dGTP respectively were 4 and 6 - fold lower than for ATP. This was mainly attributed to a higher K_m and therefore lower affinity of NS3 for dGTP and GTP. Similar results were obtained from studies of the NTPase activity of the NS3 helicase domain (278). In the work presented here, the ability of NS3 to utilise different NTPs as an energy source for helicase activity was also investigated. All NTPs assayed were able to be utilised by NS3 for helicase activity with the degree of efficiency (percentage unwinding) reflecting the affinity of NS3 for the NTP (K_m). In support of the NTPase data, helicase activity was least efficient using GTP and dGTP and this observation has also been made by others using the NS3 helicase domain (297). The substrate preferences of GBV-C (HGV) NS3 (470) and pestivirus NS3 protein (302,475) were shown to be identical to

HCV NS3 whereas GTP and dGTP are preferred substrates of flavivirus NS3 (301,473,476). These differences in substrate preference may be in part determined by the secondary structure of the different proteins and structural changes which occur during binding of different polynucleotides.

The NTPase activity of NS3 was inhibited by zinc and nickel metal ions. The presence of zinc during the refolding procedure produced NS3 which was inactive for NTPase activity and addition of nickel to NS3 abrogated NTPase activity. The mode of inhibition was not investigated further but may be due to competition for binding with magnesium ions which are required for NTPase activity or the inhibition may be non-competitive with the inhibiting ion binding to a site distal to the magnesium binding site. Inhibition of NTPase activity by metal ions has been described for other enzymes such as MutT and MTH1 8-oxo-dGTPases and it was suggested that inhibition of these enzymes may be non-competitive (464). Inhibition of NS3 NTPase activity had implications for the refolding and purification of NS3. Zinc, which is required for structural integrity of the protease domain (223,265) had to be omitted from the refolding procedure and metal chelate chromatography was not used during purification to avoid nickel contamination of NS3.

Optimal conditions for helicase activity were similar to the conditions required for optimal NTPase activity. Maximal activity was observed in the absence of monovalent cations and at pH of 6.5, however, helicase activity was greatly reduced above pH 6.5 whilst NTPase activity was optimal up to pH 7.5. Activity was dependent on the presence of ATP and $MgCl_2$. Reaction conditions for helicase and NTPase of full length NS3 reported here were similar to those previously reported for the helicase domain expressed alone (278,280). However, the NTPase activity of full length NS3

was relatively higher than the helicase domain at a pH greater than 7.0 (278) and this has also been shown using full length NS3-NS4A (312). In contrast helicase activity was optimal at pH 6.5 both in our study and for the helicase domain alone (280). The pH requirements of helicase activity of the NS3-NS4A protein were not shown (312). The difference between the pH optima of NTPase activity in full length NS3 and the helicase domain alone may reflect the stabilisation of the structure of the helicase domain in the presence of the N-terminal protease domain.

Helicase activity was almost linear throughout a 60 minute reaction. The high levels of unconverted substrate visible on the helicase activity gels (**Figure 4.11**) indicates that this may be due to lack of substrate exhaustion. This is likely to be a result of the low activity of NS3 protein used during this study. A similar profile has been reported for BVDV NS3 using a sub-optimal substrate which apparently reduced the efficiency of unwinding to maintain a linear reaction profile (475). It is therefore possible to explain the linear reaction observed for HCV NS3 unwinding as a result of low enzyme activity, either because this reflects the actual activity of full length HCV NS3 or it may be due to the substrate design or method of enzyme preparation.

The helicase and NTPase activities of NS3 were inhibited by monovalent cations as has been observed previously for the HCV NS3 helicase domain (278), GBV-C NS3 (470) and for the NS3 proteins of the flaviviruses and pestiviruses (301,302,473). These studies demonstrated that the polynucleotide stimulated NTPase activity was affected by monovalent cations whilst NTPase activity in the absence of polynucleotide was not or only slightly affected by monovalent cations. This suggests that monovalent cations do not significantly affect NTP hydrolysis *per se*, but do interfere with polynucleotide binding and consequently prevent polynucleotide stimulation of NTPase activity.

Inhibition of HCV NS3 polynucleotide binding by monovalent cations has been demonstrated previously using the helicase domain alone (297) and so it is evident that monovalent cations inhibit polynucleotide stimulated NTPase activity by abrogating polynucleotide binding. As expected, inhibition of HCV NS3 NTPase activity was associated with a loss of helicase activity which appeared to be more sensitive to monovalent cation than polynucleotide stimulated NTPase activity. At 100 mM NaCl, helicase activity is reduce to less than 10% of the maximum observed whilst NTPase activity is reduced to approximately 50% of the maximum observed. GBV-C NS3 helicase activity is also more sensitive to monovalent cations than the associated NTPase activity (470). This may reflect the increased affinity of NS3 for poly(U) compared to single stranded region of the DNA helicase substrate which may partially offset the inhibition of binding by monovalent cations. It may also indicate an additional effect of monovalent cations on either NS3 translocation, duplex separation or release of unwound DNA.

The biochemical characteristics of HCV NS3 helicase and NTPase activity were similar to those of other viral and non-viral RNA helicases, for example the yeast protein PRP22 (292) and the bluetongue virus VP6 protein (477), which both exhibited polynucleotide stimulated ATPase activity. The degree of similarity appeared to correlate with the relatedness of the proteins. The NS3 protein of the hepacivirus GBV-C was biochemically, almost identical to HCV NS3 showing only a slightly increased optimal pH for helicase activity (470). In addition, like HCV NS3, GBV-C NS3 is able to unwind RNA, RNA/DNA and DNA duplex substrates (470) unlike other members of the family *Flaviviridae* (475). Of the other members of the family, HCV NS3 more closely resembles the NS3 proteins of pestiviruses (302,475) which by sequence

comparison are more closely related to HCV than the flaviviruses (139) whose NS3 proteins show several differences to HCV NS3 activities for example substrate preferences and polynucleotide stimulation (301,473,476).

6.5 Mutational Analysis of NS3 Helicase and NTPase Activity

The effects of mutating the conserved motifs I, II and VI of DEAD-box NTPase/helicases have been studied and related to the proposed mechanism of ATP hydrolysis and helicase activity of HCV NS3 (306) (1.7 and **Figure 1.4**). According to this model, the ATP molecule lies in an interdomain cleft where the β- and γ-phosphates interact with the phosphate binding loop of motif I and with the acidic residues (D_{1316} and E_{1317}) of motif II via Mg^{2+}. The interaction of arginine residues of motif VI to the β- and γ-phosphates closes the domain resulting in translocation of NS3 and unwinding of the bound duplex (**Figure 1.5**). It is suggested that H_{1319} and Q_{1486} of motifs II and VI interact to assist opening and closing of this domain, thus coupling NTPase activity to unwinding.

Motif I

Substitution of the invariant lysine of motif I (K_{1236}) for alanine in mutant $K_{1236}A$ resulted in a reduction of NTPase activity to 30% of the wild type activity and abrogation of helicase activity as was observed in studies of the helicase domain (289). However, this lysine residue is present in all members of the DEAD-box family and has been shown to be essential for NTP hydrolysis in the closely related helicases of vaccinia virus, NPH-I (478) and NPH-II (287) and the eukaryotic DEAD box protein eIF4A (288). Therefore residue K_{1236} was expected to be essential for NTP hydrolysis by

HCV NS3. Mutational studies of the NTPase activity of *E. coli* rho protein, another member of this group of enzymes, have suggested that an alternate lysine residue is able to compensate for mutation of this conserved residue (479) and it is possible that the proximal lysine (K_{1239}) of HCV NS3 is also able to compensate for this mutation. It is interesting to note that rho protein was recognised by the anti-NS3 antibody FIZ-3 (**Figure 3.5**) which was raised against a peptide encompassing motif I, further demonstrating the similarity of the two proteins in this region. The requirement for lysine at, or close to the phosphate binding loop of motif I is consistent with the proposed mechanism of helicase activity.

Motif II

In this study it has been demonstrated that D_{1316} and E_{1317} of motif II are required for NTPase activity and helicase activity. This is consistent with the proposed mechanism of helicase activity where these residues are required for interaction with NTPs. Mutational analysis of the NS3 helicase domain also identified these residues as essential for NTPase activity (289) and these residues are also required for NTPase activity of related proteins NPH-I (478), NPH-II (287) and eIF4A (288). The residue corresponding to HCV D_{1316} can be replaced with a glutamic acid residue in eIF4A with no loss of NTPase activity (288) indicating that it is the acidic side chain, proposed to be required for binding the γ phosphate of the NTP via a magnesium ion, that is required for activity.

Mutant $C_{1318}S$ was demonstrated to have almost full NTPase activity and retained 20% of the wild type helicase activity, yet structural data has demonstrated that the cysteine residue is buried within the secondary structure (306) and therefore presumably not involved in catalysis. This suggests that though the cysteine residue

may not be involved in the catalysis of hydrolysis or duplex unwinding, this residue may be required, for example in the formation of disulphide bonds that are important to maintain structural integrity of the enzyme or that the introduction of novel side chains causes a reduction in activity. Studies of the HCV NS3 helicase domain alone have shown that substitution of C_{1318} with serine abolished both helicase and NTPase activities whilst substitution with glycine reduced NTPase activity but had little effect on helicase activity (289). This supports the hypothesis that it is the introduction of novel amino acid side chains that affect activity rather than the loss of essential side chains. The amino acid serine possesses a polar side chain containing a hydroxyl group, it is possible that in mutant $C_{1318}S$ this side chain is solvent exposed and interferes with the opening and closing of the two domains (**Figure 1.5**), thus affecting protein translocation and therefore unwinding. The limited effect of this mutation on the NTPase activity of full length NS3 may be a result of serine side chains interfering with nucleotide binding and or release, or restriction of translocation along the single stranded poly(U) cofactor.

The $C_{1318}S$ mutation had different effects in full length NS3 and NS3 helicase domain, this mutation abolished NTPase and helicase activity in helicase domain (289) whereas partial activities remained in full length NS3. This implies that there are structural differences between NS3 helicase domain and full length NS3 that stabilise the active conformation in full length NS3 and partially overcome the effect of this mutation. This has implications in the use of NS3 domains for the study of activity and in particular the design of antiviral compounds since the activities of the domains may differ from the full length NS3 protein.

Motif VI

Studies of the HCV NS3 helicase domain (289) and the mechanism of NTPase/helicase activity proposed by Kim *et al* (306) indicate that residues Q_{1486} and R_{1493} of motif VI are both required for NTPase and helicase activities. Here it has been shown by the substitution of Q_{1486} with histidine ($Q_{1486}H$) and R_{1493} with lysine ($R_{1493}K$) in full length NS3 that these two residues are required for full NTPase and helicase activity. For mutant $R_{1493}K$, NTPase and helicase activities are reduced to basal levels and mutant $Q_{1486}H$ retains one third NTPase activity and helicase activity is reduced to basal levels. This is consistent with the proposal that Q_{1486} is involved in the coupling of NTPase to helicase activity but not directly required for NTP hydrolysis. Mutation of the residue of eIF4A corresponding to R_{1493} (480) has shown that it is not required for NTP binding but is required for NTPase activity. This further confirms the hypothesis that this residue has a role in the catalysis of NTP hydrolysis which, in the context of the mechanism of Kim et al (306), requires opening and closing of the interdomain which contributes to duplex unwinding.

Though it is not investigated here, there is some debate as to whether or not this region plays a role in RNA binding and it is appropriate that recent work by other groups is reviewed and discussed here. Motif VI of eIF4A has been shown to be required for RNA binding (480) and Yao *et al* have proposed that this region of HCV NS3 is involved in RNA binding from X-ray crystallography data (305). However, the structure proposed by Kim *et al* indicates that motif VI is not involved in RNA binding and mutations to motif VI of vaccinia virus NPH-II had no affect on RNA binding (290). Recently Gwack *et al* have reported the characterisation of GBV-C (HGV) NS3 protein (470). HCV and GBV-C are members of the hepacivirus genus and NS3 of GBV-C is the most closely related to HCV NS3 of the helicases characterised to date. In

the report of Gwack *et al* (470), analysis of both GBV-C and HCV NS3 proteins showed that the deletion of motif VI abrogated RNA binding activity, suggesting that this region is in fact required for binding of RNA substrates. No data was presented for the effects of individual amino acid substitutions. In contrast to this, Kanai *et al* demonstrated that deletion of this region did not effect RNA binding and that truncated proteins which retained this region were inefficient at binding RNA (303). In addition, amino acid substitutions to motif VI of HCV NS3 helicase domain (289) showed only a reduction and not abrogation of RNA binding. This was also observed for all other mutations (excluding Q_{1486}) to residues of the conserved motifs residing in the proposed interdomain cleft. Motif VI may therefore be required for correct protein conformation for RNA binding but not directly involved in RNA:protein contact.

In light of these conflicting reports the precise region of NS3 required for RNA binding cannot be accurately defined. It is evident that motif VI is important for RNA binding but it cannot be stated for certain that this region actually binds RNA and equally it cannot be stated for certain that this region does not bind RNA. It is also possible that more than one region of NS3 may be capable of binding RNA as has been shown for RNA helicase CI from plum pox potyvirus (481,482). A 50 amino acid region containing motif VI was demonstrated to be essential for RNA binding (482) though a specific requirement for DEAD-box motif VI of this protein was not determined. In addition, a protein fragment corresponding to a region of the CI helicase which contains the DEAD-box motif I, required for NTP binding, has also been shown to be able to bind RNA (481). It is therefore possible that a region other than motif VI is present to facilitate RNA binding by HCV NS3 and that whilst motif IV is able to bind RNA, binding at this position may not be required for helicase/NTPase activity. The

availability of active full length HCV NS3 protein developed during this work will be a valuable tool for further analysis of RNA binding by this protein.

6.6 Subcellular Localisation of NS3

Previously NS3 has been detected in the cytoplasm of HCV infected hepatocytes (483,484) and more recently two groups have demonstrated nuclear accumulation of NS3 that was dependent upon the presence of the N-terminus in HeLa and BHK-21 cell lines expressing NS3 (320,485).

Using the anti-NS3 antibody NS3-7 which was raised against a peptide corresponding to the NS3 protease/helicase domain 'junction', NS3 has been detected in both the nucleus and cytoplasm of hepatocytes taken from HCV infected individuals. Though predominantly found in the cytoplasm only, NS3 was also detected in both the cytoplasm and nucleus, or solely in the nucleus. The specificity of this detection was confirmed by the negative results obtained from non-HCV infected hepatocytes and by blocking NS3 detection by pre-incubation of the antibody with recombinant NS3 protein prior to detection.

NS3 is a multifunctional protein with protease and helicase/NTPase activities. These activities are important for viral replication and polyprotein processing, which is believed to take place in a replication complex located on the cytosolic side of the ER (143,336,337). NS3 is therefore expected to locate to the cytoplasm. The significance of nuclear NS3 is unknown. However, NS3 has been shown to inhibit cAMP-dependent protein kinase (321) which, under certain conditions is present in the nucleus. NS3 has also been implicated in the suppression of p53-mediated apoptosis (319) and p53

appears to augment nuclear localisation of NS3 (65,320). In the absence of p53 NS3 is present in the nucleus and cytoplasm and when co-expressed with wild type p53, levels of nuclear NS3 are greatly increased (65,320).

Enhanced nuclear localisation of NS3 is probably a result of transport of by direct or indirect interaction with p53 or by p53-induced expression of a nuclear transporter protein. The observation that NS3 and wild type p53 co-localise in the nucleus whilst NS3 and mutant p53 co-localise to the cytoplasm (65,320) suggests the former to be correct. In addition many other viruses bind and inactivate p53, preventing apoptosis. In SV40 transformed cells, most p53 is complexed with SV40 Large T antigen and this is thought to be a key event in transformation (486-488). Adenovirus E1B protein (489), human papilloma virus E6 protein (318) and the hepatitis B virus transactivator protein HBx (490,491) all bind and inactivate p53 and lead to transformation. It is therefore reasonable to consider that HCV associated HCC may also be attributed to NS3:p53 mediated interactions, at least in part.

In infected hepatocytes, NS3 was detected either mainly in the cytoplasm, in the cytoplasm and nucleus or predominantly in the nucleus. The role of NS3 in the HCV replication complex and the higher proportion of cells with cytoplasmic NS3 suggest that this is the more common function of NS3. What causes the switch from cytoplasmic to nuclear location? As discussed above and in section 1.7.2 NS3 has been implicated in the inhibition of p53-mediated apoptosis and other viral proteins do this by binding and inactivating the p53 protein itself. NS3 has also been shown to form a complex with p53 (67) and it is therefore possible that NS3 may simply bind to and inactivate p53 and translocate to the nucleus by a passive mechanism. Under normal cell conditions and low basal levels of p53, NS3 would be found predominantly in the cytoplasm. Under

apoptic stimuli, for example as a response to viral infection, levels of p53 are greatly increased and therefore the nuclear location of NS3 may also be increased, possibly to the extent that almost all NS3 is present in the nucleus. Nuclear localisation of NS3 may therefore be a consequence of NS3 inhibition of p53 function although further nuclear functions of NS3 can not be ruled out.

6.7 Inhibition of cAMP-dependent Protein Kinase by NS3

cAMP-dependent protein kinase (PKA) is a protein kinase activated by cAMP which is produced in response to extracellular stimuli and is involved in the control of a range of cellular processes [reviewed in references (326-328,492)]. PKA is involved in the regulation of gene expression (329,493) for example PKA is believed to regulate expression of the somatostatin gene via phosphorylation of the CREB transcription factor (329). In addition intracellular motility and the cellular cytoskeleton (330,331,494) and the cell division cycle (495) may be regulated by PKA. Studies of human serum and blood platelets have shown the specific release of PKA following physiological stimulation and cAMP dependent phosphorylation of vitronectin, a protein associated with platelet activation (496). PKA may therefore also have extracellular functions. HCV NS3 has been demonstrated to interfere with PKA activity (321,322) and this may have important implications in the pathogenesis of HCV since PKA is an important component of the cell signal pathway.

HCV NS3 contains a sequence in its C-terminus (PKI-like sequence) which is highly homologous to the inhibitor region of PKI, a potent inhibitor of PKA (321,332). Various polypeptides encompassing this region of NS3 have been shown to inhibit PKA

activity *in vitro* (321) and block migration of PKA to the nucleus *in vivo* (322). Synthetic peptides corresponding to the PKI-like sequence inhibited PKA in a competitive manner whereas an NS3 polypeptide (aa 1189 - 1525) expressed in *E. coli* showed mixed type inhibition. It was proposed that regions of NS3 other than the PKI-like sequence would interact with and modulate the PKA substrate to prevent phosphorylation. Conversely NS3 (aa 1189 - 1525) was also able to enhance autophosphorylation of the R subunit and therefore increase the level of active C subunit (321). The possible implications of this are discussed later.

Comparison of the region of NS3 comprising the PKI-like sequence of different HCV genotypes showed that this sequence was absent from HCV type 1a but present in HCV type 1b and most other genotypes. It was therefore desirable to investigate the PKA inhibition of both type 1a and 1b NS3. To avoid any possible effect of NS3 on the autophosphorylation of the regulatory subunit of PKA, purified PKA C subunit was used as a PKA source in the assay. Comparison of the abilities of peptides corresponding to type 1a and 1b sequences showed that although the PKI-like sequence was not complete in type 1a, this peptide was still able to inhibit PKA activity but with apparently less effect than 1b.

Full length NS3 proteins containing either type 1a or 1b PKI-like sequence were unable to inhibit PKA activity. One explanation for this is that full length NS3 and therefore NS3 in an infected cell does not normally inhibit PKA and that viral replication and disease pathogenesis may not involve PKA inhibition. The most likely cause of this would be NS3 folding in such a way as to hide the inhibitory domain. However, a polypeptide encompassing most of the NS3 coding sequence (aa 1189 - 1525), but which lacked the amino and carboxyl termini was able to inhibit PKA (321)

and so other factors must also be considered. Full length NS3 used in this study was prepared using a denaturation then refolding protocol. It is therefore possible that not all of the resulting protein was folded correctly. It can be assumed that at least some protein was correctly folded since the PKI-like sequence overlaps with motif VI of the DEAD-box helicases and helicase activity was demonstrated for NS3 produced in this way. Another complication was that this denaturation and refolding method yielded protein samples at low concentrations (~ 0.1 mg/ml) and therefore the maximum amount of protein that could be introduced to the PKA reaction was limited to 1.2 μM. This, and the fact that a proportion of NS3 in the reaction may be incorrectly folded may mean that the level of PKA inhibition by full length NS3 may be reduced to below the detectable limits of the assay.

It can be concluded therefore that the PKI-like sequences of NS3 types 1a and 1b are able to inhibit PKA activity. It is apparent that type 1a NS3 is able to inhibit PKA even in the absence of the full PKA-like sequence. However, the initial experiments described here indicate that NS3 type 1b may have a greater inhibitory effect. Attempts to confirm this inhibition using full length NS3 were unsuccessful, though due to the method of preparation of NS3 it cannot be determined if PKA inhibition is, or is not an activity of full length NS3. Interestingly, processing of NS3 has been observed when expressed in insect and mammalian cell lines and this processing event is predicted to reveal the proposed PKA inhibiting domain (315). It is reasonable to speculate that full length NS3 may be unable to inhibit PKA but that inhibition of PKA by the truncated NS3 protein may occur following the proposed cleavage event. It has yet to be determined if this processing occurs *in vivo* and so it is still to be determined whether or not PKA inhibition is an activity of NS3 during infection.

It is apparent from the literature and the results presented in this thesis that NS3 has the ability to inhibit PKA activity by direct competition for the PKA active site, modulation of the substrate and by blocking movement of PKA to the nucleus. As discussed above PKA is an important component of the cell signalling pathway, controlling a variety of cellular processes. Inhibition of PKA is therefore likely to have a wide range of effects. Gene expression and protein transport may be affected which may in turn contribute to the success of viral replication, for example by shutting off or modifying the translation of host cell mRNA or apoptosis pathways.

NS3 may also activate PKA by enhancing autophosphorylation of the regulatory subunit and therefore increase the cytoplasmic levels of PKA (321). Type II R subunit (R_{II}) contains a autophosphorylable serine at the inhibitory site and upon phosphorylation of this residue affinity for C is greatly reduced so that a lower level of cAMP is required for activation. By increasing cytoplasmic levels of PKA and preventing accumulation of nuclear PKA, regulation of certain cellular processes may be specifically targeted by NS3 for the advantage of the virus replication/persistence.

Evidence suggests that PKA can modulate the Jak/Stat signalling cascade by inhibiting the activation of the mitogen-activated protein kinase (MAPK) kinase, ERK2 (497). By increasing the cytoplasmic levels of active PKA and therefore reducing the levels of active ERK2 it is possible for interferon-mediated antiviral responses to be regulated by NS3. IFN-alpha mediates its antiviral effects partly through the PKR mediated pathway (498) and the Jak/Stat pathway (499,500). The Jak/Stat pathway is dependent upon phosphorylation by ERK2 (501) and therefore PKA inhibition of ERK2 would block this arm of the IFN-alpha antiviral response. This is in addition to the proposed inhibition of the PKR IFN-alpha response by NS5A (353,355). ERK2 activity

is also required to maintain a steady state level of p53 (502) and so increased levels of cytoplasmic PKA may also result in a reduction of p53 levels and suppression of p53-mediated apoptosis. In accord with this Gopalbhai *et al* noted that transformation of rat fibroblasts was associated with repression of ERK2 (503). Another potential target for regulation by NS3 is the Ras/Raf pathway involved in the regulation of cell growth and development (495). Interaction between Ras and Raf initiates a signal cascade involving a number of kinases including ERK2. Phosphorylation of Raf by PKA inhibits formation of the Ras/Raf complex and prevents initiation of the signal cascade involving activation of ERK2 which can also be inhibited directly by PKA (497).

In addition to the effects upon cellular processes, NS3 may also be able to regulate the activity of viral proteins through modulation of PKA activity. It has been demonstrated that NS5A can be phosphorylated by PKA (342) and whilst the significance of the phosphorylation states of NS5A is unclear it is possible that NS5A function can be regulated by PKA and therefore by NS3.

One of the least understood areas of HCV infection is the mode of chronicity. Here it has been discussed how NS3 inhibition of PKA may contribute to prevention of apoptosis and evasion of viral clearance through different cellular pathways. The NS3-mediated effects are likely to work in concert with other viral proteins such as core and NS5A which have also been implicated in suppression of apoptosis and viral clearance through other cellular pathways (64,186,353,355). As yet there are no functions assigned to NS4B an so it cannot be ruled out that this protein too may play a role.

NS3 belongs to the DEAD box family of proteins and this group of proteins are involved in RNA replication and maturation at many levels. DEAD box proteins have been shown to be important for mRNA transcription (295), splicing (292,504), transport

(505,506) and stability (291) and for rRNA maturation and ribosome biogenesis (293,294). These proteins have also been implicated in mRNA translation (480,507) and the control of cell growth and differentiation (508,509). NS3 may perform these functions in an HCV infected hepatocytes to control viral RNA replication, protect viral RNA and alter cell growth and in doing so contribute to disease chronicity. However, there is no chronic infection stage for other viruses of the family *Flaviviridae* for example yellow fever virus even though the NS3 proteins of this family of viruses share considerable homology with HCV NS3 (3,4,132). It is therefore likely that if NS3 has a role in disease chronicity it requires the presence of either other HCV proteins, proteins unique to hepatocytes or both. Recently HCV core protein has been shown to interact with, and alter the activity of, human DEAD-box proteins present in hepatocytes (197-199). Interaction was shown to be between the core N-terminus and a region of the DEAD-box proteins encompassing conserved DEAD-box motifs which are present in HCV NS3 (197,199). It is therefore possible that core interacts with NS3 to modulate NS3 activity and disrupt normal host cell functions. It is also possible that core interaction with NS3 may alter the role of NS3 in HCV replication, for example providing a switch between protease and helicase/NTPase activities, and interactions between core, NS3 and viral RNA may aid viral RNA packaging and virus particle maturation.

Future research into the non-replicative functions of NS3 may enable the identification of new antiviral targets - and since many viruses evade host detection and viral clearance - the potential for development of novel, broad range, antiviral therapies.

6.8 Future Work

Detailed kinetic analysis of the NTPase activities of the NS3 mutants prepared during this work should be investigated. K_m and k_{cat} data can be used to identify which residues are required for ATP binding and which are required for catalysis.

Further NS3 mutants should be constructed to enable identification of the precise residues required for RNA binding, an area where there is currently conflicting published data.

The full length NS3 protein described in this thesis should now be used to demonstrate NS3 protease activity. The relationship between helicase/NTPase and protease activities should then be investigated for example by studying the effect of polynucleotides upon protease activity and NS4A upon helicase activity to identify the potential for self-regulation of activity.

Subcellular localisation of NS3 should be investigated using confocal and immunoelectron microscopy to confirm nuclear localisation of NS3.

The ability of NS3 to inhibit PKA has been demonstrated using peptides corresponding to the active region of NS3. However, PKA inhibition by full length NS3 was not demonstrated. This problem may have been a result of the preparation method of NS3 and so alternate methods should be applied to overcome this. NS3 may be expressed in hepatocyte cell culture and then extracts from these cells can be assayed for PKA activity *in vitro* using the PKA assay (Life Technologies). Inhibition by type 1a

and 1b HCV NS3 can be compared in this way and the effects of cofactors such as NS4A can also be investigated by using a construct that expresses NS3/4A. Also the truncated NS3 protein produced in insect cells infected with NS3 expressing recombinant baculovirus should be investigated for inhibition of PKA activity to determine if the cleavage observed induces PKA inhibition.

The effects of PKA inhibition by NS3 on cell metabolism may also be studied;

- Immunoprecipitation of PKA substrate proteins from hepatocyte cell lines (e.g. Huh7, HepG2) that express NS3 cultured in the presence radiolabelled ATP can be used to identify alterations in phosphorylation pattern by PKA due to the presence of NS3.
- NS3 may affect many host cell processes by interfering with PKA or other cellular proteins which may manifest themselves by affecting gene expression. By comparing the mRNA species present in NS3 expressing and non-NS3 expressing cells by using the subtractive hybridisation technique, alterations in gene expression might be identified. Data generated from these experiments could be used to provide insight into any NS3 mediated mechanisms e.g. to prevent the clearance of HCV by the host and possibly in the development of carcinomas.

Finally, NS3 may interact with and modulate the activities of other cellular proteins. The yeast two-hybrid system could be employed to screen hepatocyte cDNA

libraries for these potential NS3 target proteins or to screen a library composed of viral DNA sequences to identify the viral proteins that NS3 may interact with.

6.9 Conclusions

The expression of full length NS3 and the demonstration and characterisation of the helicase and NTPase activities of this protein have been described in this thesis. The characteristics of full length NS3 were essentially the same as those described for NS3 helicase domain. However, full length NS3 was apparently more stable than the helicase domain as demonstrated by the enzyme activity pH range and by differences in the tolerance of amino acid substitutions to the helicase motif II. This may have implications for the use of NS3 helicase domain in the study of NS3 activity and design of antiviral agents.

Preliminary studies on the localisation of NS3 in hepatocytes from infected individuals indicates that NS3 is present in the cytoplasm and nucleus of cells. HCV replication is believed to occur in the cytoplasm explaining the presence of NS3 in this compartment, nuclear localisation of NS3 suggests further roles for NS3 possibly in the control of host cell biochemistry.

Sequence comparison of NS3 demonstrated that the PKI-like sequence implicated in the inhibition of PKA was not typical of all HCV subtypes, an observation that has not previously been published. This sequence was absent from HCV type 1a but present in most other types including type 1b. Peptides corresponding to both type 1a and 1b PKI-like regions were both able to inhibit PKA but initial results suggested that type 1b may be a more potent inhibitor.

The work presented in this thesis provides a method for the recovery of full length NS3 from *E. coli* and the characterisation of full length NS3. The studies of PKA inhibition instigated here should provide a springboard for future work into the role of NS3 in PKA regulation and cell signalling and ultimately a greater understanding of the role of NS3 in HCV replication and disease pathogenesis. A greater understanding of the mechanisms of HCV replication and persistence should enable the identification of new targets for antiviral therapy and the discovery of therapeutic agents.

Chapter 7: References

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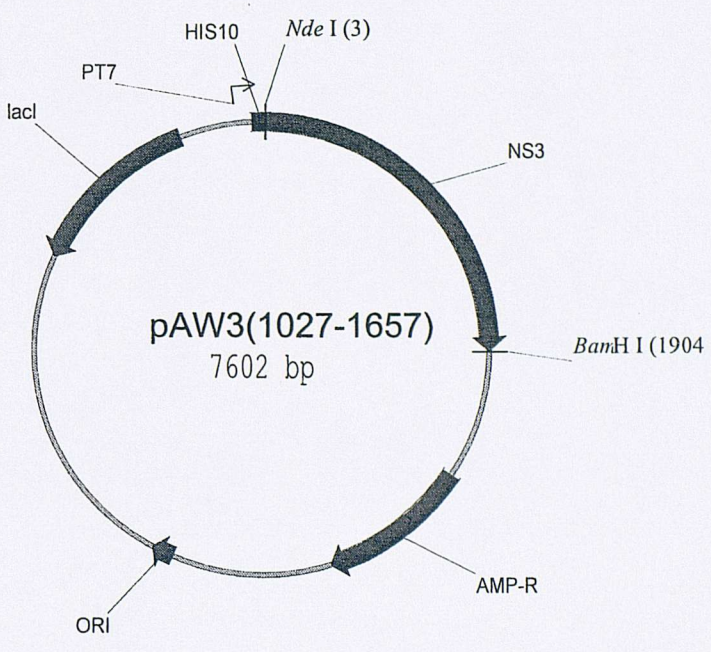
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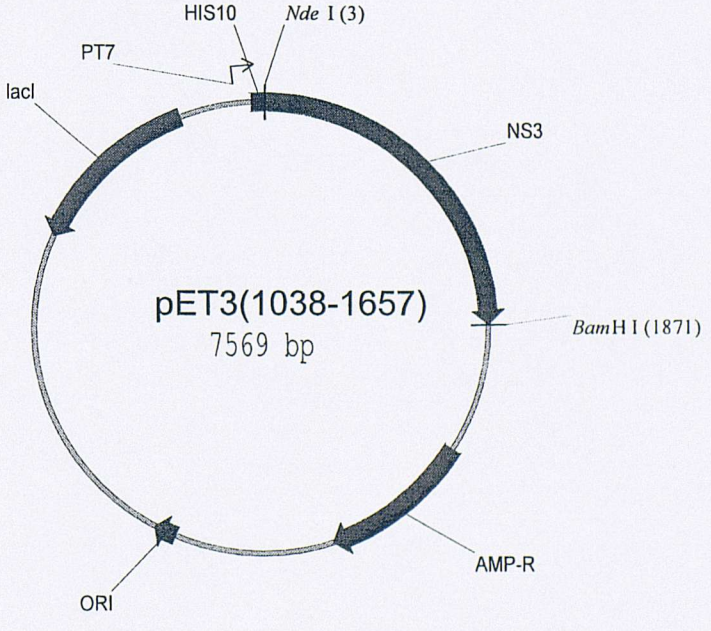
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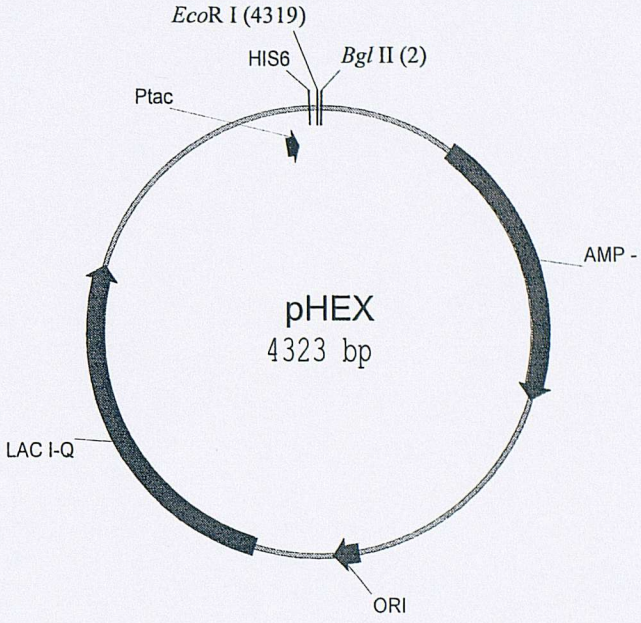
509. R. J. Stevenson, S. J. Hamilton, D. E. MacCallum, P. A. Hall, F. V. Fuller-Pace. Expression of the 'dead box' RNA helicase p68 is developmentally and growth regulated and correlates with organ differentiation/maturation in the fetus. *J. Pathol.* **184**, 351-359.(1998).

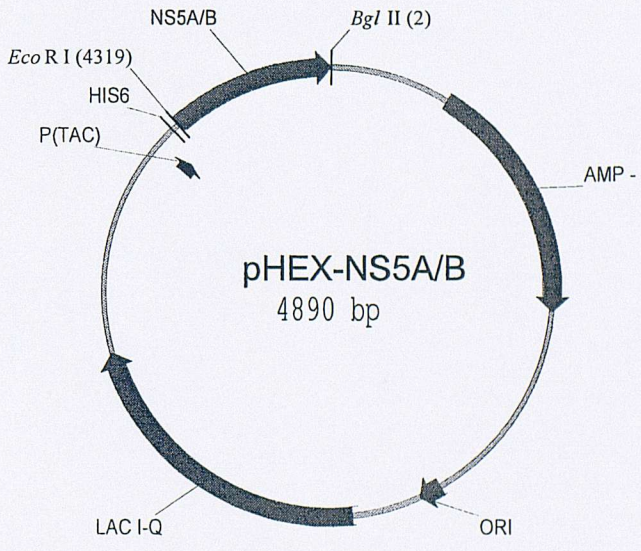
Appendix I: Plasmids

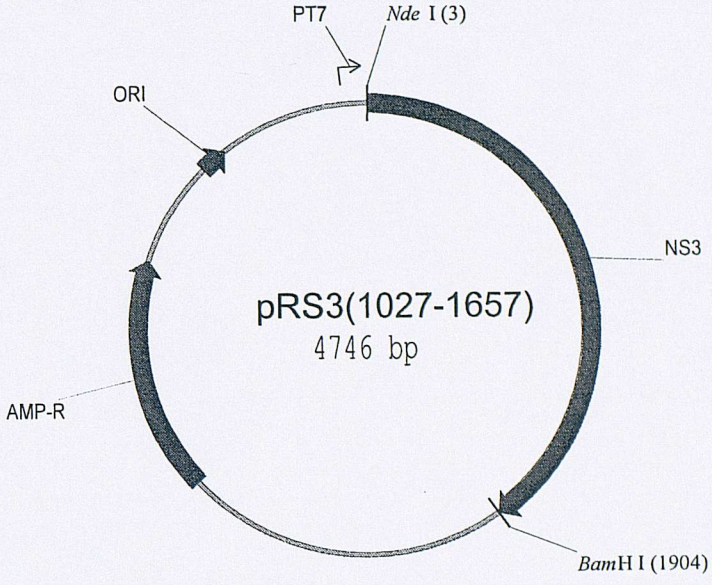
Plasmids shown here are those constructed in the process of the work presented in this manuscript or those that have not previously been published.

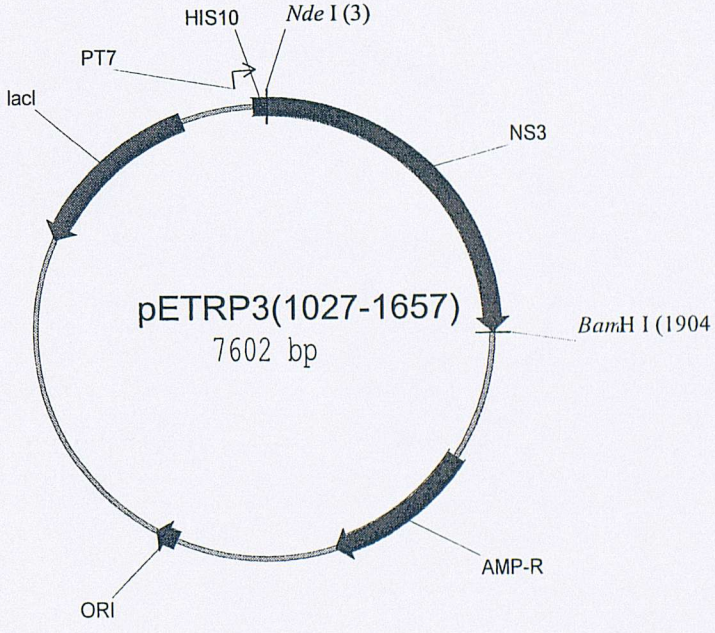


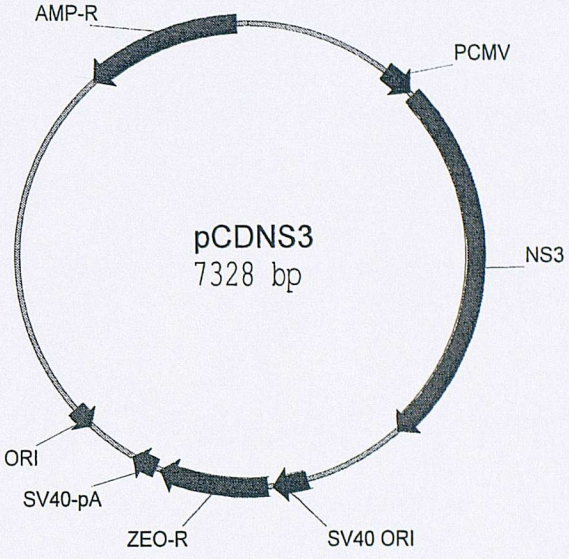


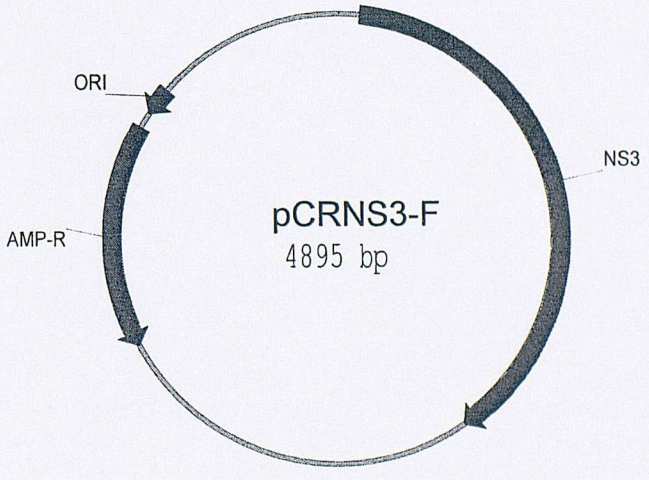

*Eco*R I (4319)
HIS6
Bgl II (2)
Ptac
AMP -
pHEX
4323 bp
LAC I-Q
ORI


NS5A/B
Bgl II (2)
Eco R I (4319)
HIS6
P(TAC)
AMP -
pHEX-NS5A/B
4890 bp
LAC I-Q
ORI


PT7
Nde I (3)
ORI
NS3
pRS3(1027-1657)
4746 bp
AMP-R
BamH I (1904)


HIS10
Nde I (3)
PT7
lacI
NS3
pETRP3(1027-1657)
7602 bp
BamH I (1904
AMP-R
ORI


AMP-R
PCMV
pCDNS3
7328 bp
NS3
ORI
SV40-pA
ZEO-R
SV40 ORI


ORI
NS3
pCRNS3-F
4895 bp
AMP-R

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