STUDIES OF THE HEPATIC EXPRESSION OF HEPATITIS C VIRUS MARKERS

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Dedicated to my parents

TABLE OF CONTENTS

PAGE NO.

ABSTRACT		vii
DECLARATION		xi
ACKNOWLEDGEMENTS		xii
ABBREVIATIONS		xiv
PUBLICATIONS		xvi
CHAPTER 1	**INTRODUCTION**	
1.1	**Introduction**	1
1.2	**Historical studies**	1
1.3	**Molecular cloning of the NANB agent**	3
1.4	**HCV genome organisation and sequence diversity**	5
1.5	**Physicochemical aspects**	11
1.6	**Flavivirus replication**	12
1.7	**Culture of HCV *in vitro***	13
1.8	**HCV proteins**	14
1.9	**Serological diagnosis**	19
1.9.1	Routine assays	19
1.9.2	IgM assay	21
1.9.3	Immune response to the envelope proteins	22
1.9.4	Viral RNA detection	23
1.9.5	Serological events	24
1.10	**Transmission of HCV**	25
1.11	**HCV pathogenesis**	28
1.12	**HCV and its association with hepatocellular carcinoma**	31
1.13	**Aims of thesis**	33

CHAPTER 2 MATERIALS AND METHODS

2.1 Subcloning of HCV cDNA sequences 34

2.1.1 Vectors and plasmids 34

2.1.2 Phenol extraction and ethanol precipitation of nucleic acids 34

2.1.3 DNA modifying enzymes 35

2.1.4 Agarose gel electrophoresis 36

2.1.5 Isolation of DNA fragments from agarose gels 37

2.1.6 Preparation of competent cells 38

2.1.7 Transformation of competent cells 38

2.1.8 Mini preparation of plasmid DNA 38

2.1.9 Large scale plasmid purification from *E.coli* 39

2.2 Immunohistochemistry 40

2.2.1 Tissue preparation 40

2.2.2 Primary and secondary antibodies for antigen detection 41

2.2.3 Preparation of NHL homogenate 42

2.2.4 Adsorption of antibodies against NHL homogenate 42

2.2.5 Indirect immunofluorescence 42

2.2.6 Immunoperoxidase staining 43

2.3 Preparation of viral RNA 44

2.3.1 Extraction of RNA 44

2.3.2 RNase A digestion 45

2.3.3 Lithium chloride fractionation 45

2.3.4 Chemical modification of RNA samples 45

2.4 Amplification of HCV RNA by PCR 46

2.4.1 Synthesis of HCV cDNA 46

2.4.2 Amplification of reverse transcribed cDNA 46

2.5 Preparation of radiolabelled probes 47

2.5.1 Efficiency of radiolabelling reactions 47

2.5.2 Random prime labelling of excised plasmid DNA 47

2.5.3 RNA probes and transcripts 48

2.5.4 End-labelling of synthetic oligonucleotides 49

2.5.5 Preparation of ^{32}P-labelled molecular weight markers 49

2.5.6 Size determination of ^{125}I-, ^{32}P- and FITC-labelled probes 50

2.6 Nucleic acid hybridisation 50

2.6.1 Preparation of hybridisation membranes 50

2.6.2 Hybridisation 52

2.7 *In situ* hybridisation 52

2.7.1 Frozen liver sections and cells 52

2.7.2 Formalin-fixed, wax-embedded liver sections 53

2.7.3 Preparation of mononuclear cells from human peripheral blood 53

2.7.4 PBMC stimulation 54

2.7.5 *In situ* hybridisation 54

2.7.6 Washing 55

2.7.7 Detection of bound radiolabelled probes 56

CHAPTER 3 DETECTION OF HCV PROTEINS IN FROZEN LIVER TISSUE

3.1 Introduction 57

3.2 Experimental design 57

3.3 Results 58

3.3.1 PCR analysis of serum samples 58

3.3.2 Histological analysis 59

3.3.3 Detection of HCV antigens in liver tissue 59

3.3.4 Specificity of the indirect immunofluorescence assay for HCV antigen detection 60

3.3.5 Fixation of sections for HCV antigen detection 61

3.3.6 Detection of endogenous IgG in liver tissue 62

3.3.7 Identification of the lymphocytic population infected with HCV 62

3.4 Conclusions and discussion 64

CHAPTER 4 IMMUNOHISTOCHEMICAL DETECTION OF HCV-NS4 AND ITS RELATION TO HISTOPATHOLOGY

4.1 Introduction 68

4.2 Experimental design 68

4.3 Results 69

4.3.1 Preliminary study of HBcAg by 3 and 4 layer assays 69

4.3.2 Histological diagnosis 70

4.3.3 PCR analysis of serum samples 70

4.3.4 HCV antigen detection 70

4.3.5 Correlation between NS4 expression and histopathology 71

4.3.6 Specificity of the reaction for NS4 detection 72

4.4 Conclusions and discussion 72

CHAPTER 5 DETECTION OF THE NS4 PROTEIN IN HEPATOCELLULAR CARCINOMA AND ADJOINING NONTUMOROUS LIVER TISSUE

5.1 Introduction 75

5.2 Experimental design 75

5.3 Results 76

5.3.1 Histological diagnosis 76

5.3.2 NS4 detection 76

5.3.3 Specificity of staining for NS4 77

5.4 Conclusions and discussion 78

CHAPTER 6 **DETECTION OF HCV RNA BY *IN SITU* HYBRIDISATION**

6.1 **Introduction** 80

6.2 **Technical considerations for ISH** 81

6.3 **Experimental design** 82

6.4 **Results** 83

6.4.1 Preface 83

6.4.2 Preliminary study of HCV RNA in peripheral blood and bone marrow cells 85

6.4.3 Examination of liver autopsy samples for HCV RNA 86

6.4.4 Detection of HCV RNA in liver biopsy samples 90

6.5 **Conclusions and discussion** 93

CHAPTER 7 **PCR ANALYSIS OF HCV RNA WITHIN HCV-INFECTED LIVER SAMPLES**

7.1 **Introduction** 96

7.2 **Experimental design** 97

7.3 **Results** 98

7.3.1 Detection of HCV RNA 98

7.3.2 Detection of negative sense HCV RNA 99

7.3.3 Effect of heat on HCV RNA detection 100

7.3.4 RNase A digestion of HCV RNA 101

7.3.5 LiCl fractionation of HCV RNA 103

7.3.6 Immunohistochemical detection of double-stranded RNA 105

7.4 **Conclusions and discussion** 105

CHAPTER 8 **CONCLUDING REMARKS**

8.1 **Introduction** 108

8.2 **HCV antigen and RNA expression in hepatic and extrahepatic cells** 108

8.3	**Mechanism of RNA replication**	112
8.4	**Mechanism of liver injury**	113
8.5	**Future directions**	116

APPENDIX 118

REFERENCES 120

ABSTRACT

Persistent infection with hepatitis C virus (HCV) is associated with chronic hepatitis and cirrhosis which may eventually develop into hepatocellular carcinoma. The distribution and frequency of HCV-infected cells in the liver of patients with ongoing HCV-related liver disease is ill-defined. Hence, the work in this thesis examined HCV-specific protein and RNA expression in liver tissue from anti-HCV positive patients.

Frozen liver sections from chronic carriers were examined by indirect immunofluorescence for capsid, E2/NS1, NS3, NS4 and NS5 using polyclonal antibodies raised to synthetic peptides from these regions. HCV antigens, E2/NS1, NS3, NS4 and NS5, were found to be widely distributed within unfixed frozen liver sections, and were present as distinct granules or foci within the cytoplasm of hepatocytes and in infiltrating lymphocytes in portal tracts. The E2/NS1 and NS3 antigens were detected as intense foci, whereas a weaker signal was observed for NS4 and NS5. The capsid antigen was undetected.

The detection of viral antigens is often most successful in unfixed frozen tissues, but to preserve the histological appearance, a fixation step is often introduced. Consequently, the expression and distribution of the HCV antigens in frozen sections, following fixation in a number of commonly used fixatives, was assessed. Fixation of frozen sections with 1% formalin improved the histological appearance of the tissue sections without reducing the sensitivity of antigen detection. However, in tissue sections fixed in acetone, chloroform, carbon tetrachloride or Methyl Carnoys, detection of all hepatocyte-specific HCV antigens was reduced significantly. In contrast, the lymphocyte-specific HCV antigens were unaffected by the fixation.

To further identify the infected lymphocyte population, dual immunostaining of liver sections for lymphocyte cluster of differentiation (CD) markers, CD4, CD8 and CD20, and HCV antigens, E2/NS1 and NS3, was performed. These experiments revealed that 40-50% of CD20-positive B cells and CD4- and CD8-positive T cells, predominant in lymphoid aggregates, were positive for E2/NS1 and NS3.

In contrast to the above results, only NS4 was detected consistently in formalin-fixed, paraffin wax-embedded liver samples. Consequently, to examine the relationship between HCV antigen expression and histopathological features, a sensitive 4 layer immunoperoxidase assay was developed to detect NS4 in formalin-fixed liver biopsy samples. In 10 anti-HCV positive samples, NS4 was detected within the cytoplasm of hepatocytes, but not in the mononuclear cell infiltrates, bile duct epithelium or endothelial cells. A high proportion of hepatocytes appeared positive, but the staining intensity was variable. Following a coded histological evaluation of the liver tissue, the pattern of liver injury was shown to have no significant correlation with antigen-positive hepatocytes, and no direct relationship was observed between the distribution of antigen-positive hepatocytes and areas of hepatocyte necrosis. Furthermore, NS4 was detected in tumorous and nontumorous hepatocytes in formalin-fixed, paraffin wax-embedded liver tissue from patients diagnosed with hepatocellular carcinoma.

The staining pattern was considered to be specific because liver samples from patients chronically infected with hepatitis B virus or from uninfected individuals were negative. Furthermore, no staining was noted when either pre-immune rabbit serum or the antisera, previously adsorbed against the specific synthetic peptide, was substituted for the primary antibody.

Initially, liver tissue taken at autopsy from 2 anti-HCV positive patients was examined by *in situ* hybridisation for the presence of HCV RNA. Viral RNA was

detected by autoradiography after hybridisation with ^{32}P-labelled DNA or ^{125}I-labelled RNA probes representing 35% of the HCV genome. Only a few positive cells were identified in the HCV-infected liver samples, but not in the uninfected liver tissue. The HCV RNA-positive cells were detected with antisense, but not sense RNA probes, suggesting that they contained a high ratio of positive:negative sense RNA. The appearance and distribution of the HCV RNA-positive cells suggested that they were most likely to be lymphocytes or macrophages. In addition, HCV RNA was detected by *in situ* hybridisation within peripheral blood lymphocytes and bone marrow cells isolated from HCV-positive patients. To extend this study, attempts were made to detect HCV RNA in HCV-positive liver biopsy samples by fluorescein-labelled RNA probes representing 80% of the HCV genome. However, direct detection by FITC-labelled RNA probes failed to detect HCV RNA-positive cells. In addition, attempts to detect RNA:RNA hybrids in the liver sections by antibody layer amplification were unsuccessful.

The mechanism of HCV replication is unknown, although the classification of HCV in the Flaviviridae has led to the postulation that HCV may adopt a replication strategy similar to that of flaviviruses and/or pestiviruses. To determine if HCV double-stranded replicative forms, consistent with this strategy, were present, HCV-specific RNA was detected in total liver RNA, extracted from HCV-infected individuals, by reverse transcription followed by polymerase chain reaction. Initially, negative sense HCV RNA was detected in RNA extracted from liver tissue from naturally-infected individuals, consistent with HCV replication via a negative sense intermediate. Total liver RNA was then either: (i) fractionated in 2M LiCl (designed to precipitate single-stranded and partially double-stranded RNA); or (ii) RNase A digested in high salt conditions (designed to digest single-stranded RNA only). Amplification of positive sense HCV RNA in LiCl-insoluble fractions, but not in the RNase A-digested liver RNA, showed that HCV RNA was single-stranded in infected liver tissue. Furthermore, the yield of HCV-specific

cDNA was not increased when liver RNA was denatured prior to reverse transcription, consistent with the single-stranded nature of HCV RNA in the liver.

In conclusion, the distribution of HCV-specific RNA and proteins suggest that HCV is a hepatotropic, lymphotropic and non-cytopathic virus. Furthermore, the above data may prove useful in the choice of cells and fixation methods for *in vitro* culture experiments for HCV.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

SIGNED: **DATE:**

25/10/94

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ABBREVIATIONS

aa	amino acid
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
CAH	chronic active hepatitis
CD	cluster of differentiation
cDNA	complementary DNA
Con A	concanavalin A
CPH	chronic persistent hepatitis
CTP	cytidine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DDW	double distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dGTP	deoxyguanosine triphosphate
dsRNA	double-stranded RNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
gp	glycoprotein
GTP	guanosine triphosphate
h	hour
HBcAg	hepatitis B core antigen
IgG	immunoglobulin G
IgM	immunoglobulin M
kb	kilobase

kDa	kilodalton
LPS	lipopolysaccharide
min	minute
mRNA	messenger RNA
NHL	normal human liver
NHS	normal human serum
NSS	normal sheep serum
nt	nucleotide
OD_{260}	optical density at 260nm
OD_{600}	optical density at 600nm
PBS	phosphate buffered saline; 150mM NaCl, 6mM K_2HPO_4, 2mM KH_2PO_4, pH 7.2)
POPOP	1,4-bis[2-(5-phenyloxazolyl)] benzene
PPO	2,5-diphenyloxazole
PVP	polyvinyl pyrrolidone
RNA	ribonucleic acid
RNase A	ribonuclease A
rt	room temperature
sec	second
ssRNA	single-stranded RNA
TCA	trichloroacetic acid
TE8	10mM Tris-HCl pH 8.0, 1mM EDTA
tRNA	transfer RNA
UTP	uridine triphosphate
UV	ultraviolet
XC	xylene cyanol

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Following the development of diagnostic tests for hepatitis A virus (HAV) and hepatitis B virus (HBV) in 1975, it became apparent that 60-90% of reported post-transfusion hepatitis (PTH) cases were caused by an unknown agent(s). In addition, diagnostic evidence excluded other hepatotropic viruses including hepatitis delta virus (HDV), cytomegalovirus and Epstein-Barr virus (Feinstone *et al.*, 1975). Hence, the causative agent(s) of PTH was termed non-A, non-B hepatitis (NANBH). Although attempts were made to detect the infectious agent(s) associated with the parenteral form of NANBH, it remained elusive until 1989 when the genome was cloned and named hepatitis C virus (Choo *et al.*, 1989). In 1990, the agent mainly associated with enterically-transmitted NANBH, was cloned (Reyes *et al.*, 1990) and is now known as hepatitis E virus.

1.2 HISTORICAL STUDIES

In 1978, successful parenteral transmission of NANBH to chimpanzees by infected human serum, factor VIII concentrates, factor IX preparations or commercially prepared fibrinogen was reported (Alter *et al.*, 1978; Hollinger *et al.*, 1978; Tabor *et al.*, 1978; Bradley *et al.*, 1979; Yoshizawa *et al.*, 1980). Infected chimpanzees displayed elevated alanine aminotransferase (ALT) activity, liver histopathology consistent with viral infection and serological evidence that disease was not caused by either HAV or HBV. The development of this primate model permitted extensive studies of the disease and the infectious agent of NANBH.

Two or more distinct aetiological agents were implicated in PTH as a result of the observation that individual patients often had multiple attacks of NANBH (Mosley *et al.*, 1977; Galbraith *et al.*, 1979; Norkrans *et al.*, 1980). This assumption was supported by cross-challenge studies in chimpanzees, combined with ultrastructural examination of infected liver samples (Bradley *et al.*, 1980). In these experiments, chimpanzees were inoculated with contaminated factor VIII, factor IX or 'H' strain plasma resulting in a characteristic rise in ALT levels and histopathological evidence of NANBH infection. Sequential inoculation of chimpanzees with different pairs of proven infectious materials sometimes resulted in a second occurrence of disease, indicating the presence of at least two parenterally-transmitted NANB agents (see section 1.4 and 1.9.3 for more recent reinterpretation of this data). Electron microscopic examination of acute phase liver tissue obtained from NANB-infected chimpanzees revealed the presence of abnormal hepatocyte cytoplasmic tubular structures previously shown to be associated with NANBH (Jackson *et al.*, 1979; Shimizu *et al.*, 1979; Tsiquaye *et al.*, 1980). Additional cytoplasmic alterations included dense reticular inclusion bodies, convoluted membranes derived from smooth endoplasmic reticulum and bundles of granular microtubules. The appearance of a cytoplasmic antigen in hepatocytes of experimentally-infected chimpanzees has also demonstrated in the liver by monoclonal antibodies produced from lymphocyte-derived cell lines from chimpanzees (Shimizu *et al.*, 1985), humans (Shimizu *et al.*, 1986) and mice (Maeda *et al.*, 1989). Immunoelectron microscopy revealed that these antibodies recognised the microtubular aggregates which appear in the cytoplasm of NANB-infected (Shimizu *et al.*, 1985, 1986; Maeda *et al.*, 1989) and HDV-infected chimpanzee hepatocytes (Shimizu *et al.*, 1986; Maeda *et al.*, 1989). Maeda *et al*, (1989) and Honda *et al*, (1990) independently isolated a protein from hepatocytes of NANB-infected chimpanzees associated with these microtubular aggregates. A protein of 44kDa was detected by immunoblot analysis and later identified as a host-encoded protein in chimpanzees (Takahashi *et al.*, 1990).

Physicochemical characterisation studies of NANB-infected plasma and liver revealed two agents capable of inducing NANBH in chimpanzees (Bradley *et al.*, 1983). Type I was specifically associated with the formation of the characteristic ultrastructural alterations described above. Furthermore, the infectivity of NANB-containing plasma was destroyed by chloroform treatment, suggesting that the agent contained essential lipids as part of its structure (Bradley *et al.*, 1983; Feinstone *et al.*, 1983). In contrast, the second suspected agent was chloroform-resistant and did not induce unique changes in hepatocytes. In other studies, the NANB agent was inactivated by heat (Hollinger *et al.*, 1982; Yoshizawa *et al.*, 1982) and formalin (Tabor and Gerety, 1980; Yoshizawa *et al.*, 1982). The agent responsible for disease and persistent infection in chimpanzees (type I) was found to pass through an 80nm membrane filter (Bradley *et al.*, 1985). Subsequent work determined the approximate size of the infectious agent to be 30-60nm in diameter, by filtration of the infectious material through polycarbonate membranes (He *et al.*, 1987). These findings, in combination with the sensitivity of the agent to chloroform, suggested that the agent was a small enveloped virus.

1.3 MOLECULAR CLONING OF THE NANB AGENT

Progress in the characterisation of the NANB agent was inhibited by the consistent failure of conventional immunological methods to identify specific viral antigens and antibodies (Shih *et al.*, 1986). The inability to detect specific antibodies was thought to reflect low concentrations of viral antigens associated with NANBH infections, as the titre of the agent in plasma was low (10^3-10^5 chimpanzee infectious doses per ml). It was not until efficient nucleic acid extraction and molecular cloning techniques were employed, that the genome of the NANB agent was cloned.

In order to produce recombinant viral antigens, a cDNA library, derived from pooled infectious chimpanzee plasma, was constructed in bacteriophage λgt11.

Since the nature of the NANB genome was unknown, total extracted nucleic acid was completely denatured prior to the synthesis of cDNA from RNA and DNA with random primers for reverse transcriptase. The resultant cDNA was cloned into λgt11, to allow efficient expression of cDNA-encoded polypeptides in *Escherichia coli.* Sera from chronically-infected humans and chimpanzees were used as the source of viral antibodies to screen the recombinant cDNA library for the expression of NANB-specific proteins. Approximately 10^6 clones were screened before a positive clone (named 5-1-1), which expressed a protein specific for NANBH, was identified (Choo *et al.*, 1989).

Further characterisation of the 5-1-1 clone and overlapping clones, identified from the same library using clone 5-1-1 as a hybridisation probe, was undertaken. These clones were subsequently used as probes to establish that the target was not derived from host DNA, but from RNA extracted from infected chimpanzee liver. Furthermore, the hybridisation signal was lost when nucleic acid, extracted from chimpanzee liver and serum, was pre-digested with ribonuclease, but not with deoxyribonuclease. In addition, only one strand of the cDNA clone was found to hybridise to RNA isolated from infectious plasma liver. Based on this data, the infectious agent was determined to be a single-strand RNA virus of at least 10,000 nucleotides and was subsequently named hepatitis C virus (HCV). A number of overlapping cDNA clones were ligated and the sequence of a 7310bp region was published (Houghton *et al.*, 1988). Sequence data suggested a positive sense RNA genome with a single open reading frame (ORF). Thus, (i) the size of the virus, (ii) its sensitivity to lipid solvents, and (iii) the genome properties, were consistent with HCV being related to either Togaviridae or Flaviviridae (Choo *et al.*, 1989).

The availability of sequence data enabled other workers to design oligonucleotide primers specific for the HCV genome. Random and specific primers were used in reverse transcription (RT) followed by polymerase chain reaction (RT-PCR) on

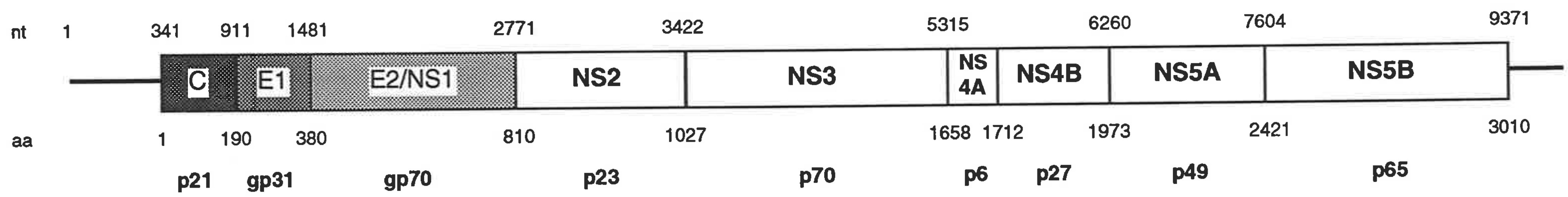
RNA extracted from infectious serum or infected liver tissue. This approach has led to the cloning and sequencing of twelve HCV isolates: (i) the original USA isolate HCV-1 (Choo *et al.*, 1991) and HCV-H (Inchauspe *et al.*, 1991); (ii) the isolates from Japan, HCV-J (Kato *et al.*, 1990), HC-J6 (Okamoto *et al.*, 1991), HCV-BK (Takamizawa *et al.*, 1991), HCV-JK1 (Honda *et al.*, 1992), HC-J1 (Okamoto *et al.*, 1992a), HC-J8 (Okamoto *et al.*, 1992b) and HCV-JT (Tanaka *et al.*, 1992); (iii) a single isolate from Taiwan HCV-T (Chen *et al.*, 1992a); (iv) an isolate from China HC-C2 (Wang Y *et al.*, 1993); and (v) an isolate from Indonesia HC-G9 (Okamoto *et al.*, 1994). In addition, partial sequences from USA isolates, HCT18, HCT23, HCT27, Th (Weiner *et al.*, 1991), from Japanese isolates, HCV-Pt-1, -K1, -K2a, -K2b (Enomoto *et al.*, 1990), C8-2 (Maeno *et al.*, 1990), HC-J1, -J2 (Okamoto *et al.*, 1990c), HCV-JH (Takeuchi *et al.*, 1990b), clone 63, 64, 168 (Hijikata *et al.*, 1991a), clone A (Tsukiyama-Kohara *et al.*, 1991), from French isolates, HCVE1 (Kremsdorf *et al.*, 1991), F1, F2 (Li *et al.*, 1991), from two Italian isolates, EC1, EC10 (Weiner *et al.*, 1991), from German isolates, GM1, GM2 (Fuchs *et al.*, 1991), HD1, HD2 (Muller *et al.*, 1993b) and from a Chinese isolate, HCV-PRC1 (Liu *et al.*, 1992), have also been reported.

1.4 HCV GENOME ORGANISATION AND SEQUENCE DIVERSITY

The HCV RNA genome is now defined as a single-stranded, positive sense molecule of approximately 9400 nucleotides consisting of one continuous ORF with the capacity to encode a polyprotein of 3010 (Kato *et al.*, 1990; Takamizawa *et al.*, 1991; Chen *et al.*, 1992a; Tanaka *et al.*, 1992), 3011 (Choo *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1992a, 1994) or 3033 (Okamoto *et al.*, 1991, 1992b) amino acids (Figure 1.1). This ORF is flanked by short untranslated regions (UTR) at the 5' and 3' ends. The length of the 5' UTR and 3' UTR vary in length from 324 to 341 and 22 to 66 nucleotides respectively, depending on the HCV isolate. Analyses of the gene organisation and deduced amino acid sequence of HCV revealed that HCV is distantly related to human flaviviruses

Figure 1.1 Organisation of the hepatitis C viral genome.

The HCV genome of approximately 9400nt consists of one continuous ORF with the capacity to encode a polyprotein of 3010-33aa. The ORF is divided into the structural region comprising the capsid (C) and 2 envelope proteins, E1 and E2/NS1, and the non-structural region comprising NS2 to NS5 proteins. The coding region is flanked by 5' and 3' UTR.



Structural
Non-Structural
nt
1
341
911
1481
2771
3422
5315
6260
7604
9371
C
E1
E2/NS1
NS2
NS3
NS
4A
NS4B
NS5A
NS5B
aa
1
190
380
810
1027
1658
1712
1973
2421
3010
p21
gp31
gp70
p23
p70
p6
p27
p49
p65

(eg. Yellow Fever Virus (YFV), Dengue Virus, Kunjin Virus and Japanese Encephalitis Virus) and animal pestiviruses (eg. Bovine Viral Diarrhoea Virus (BVDV) and Hog Cholera Virus) (Kato *et al.*, 1990; Miller and Purcell, 1990; Takeuchi *et al.*, 1990b; Choo *et al.*, 1991; Takamizawa *et al.*, 1991). Based on computer comparisons of the hydrophobicity profiles of the predicted amino acid sequences with published sequences of known viral genomes, the ORF of HCV was postulated to encode the structural (S) proteins at the 5' end and the non-structural (NS) proteins at the 3' end in a similar manner to that of flaviviruses and pestiviruses (Takeuchi *et al.*, 1990b; Takamizawa *et al.*, 1991). Consequently, HCV has been classified as a member of the Flaviviridae (Francki *et al.*, 1991).

Comprehensive sequence analysis of HCV genomes revealed the existence of at least six different genotypes (HCV-GI to HCV-GVI) around the world (Enomoto *et al.*, 1990; Houghton *et al.*, 1991; Tsukiyama-Kohara *et al.*, 1991; Chan *et al.*, 1992; Mori *et al.*, 1992; Okamoto *et al.*, 1992b; Wang Y *et al.*, 1993). Extensive sequence analysis of five genotypes [GI-GV; classified by Cha *et al*, (1992)] from various geographic locations identified G1-GIV to be found in USA, GII and GIII in Argentina, GI and GII in Spain, GII and GIV in Germany and GI-GIV in Italy. In this report, GV was only located in South Africa. Coexistance of these genotypes was observed in several geographic locations including USA, Japan, Germany and Italy (Cha *et al.*, 1992). The nucleotide sequences of the entire HCV genomes from HCV-GI to HCV-GIV have been determined. Group I consists of the USA prototypes, HCV-I, HCV-H, and the Japanese isolate HC-J1, Group II HCV-J, HCV-BK, Group III HC-J6 and Group IV HC-J8 (Mori *et al.*, 1992; Okamoto *et al.*, 1992b). However, group V and VI consist of partial sequences from NANBH patients in Thailand (Mori *et al.*, 1992). Overall the genotypes show differences of about 20-35% in the nucleotide sequences of their virus genomes and different isolates belonging to the same genotype showed 5-8% and 4-5% differences in nucleotide and amino acid sequences sequences, respectively.

More recently, phylogenetic analysis of nucleotide sequences (nt 7975-8196) derived from NS5 has identified 11 genetically distinct HCV populations in which six major HCV groups (1-6) comprise closely related subgroups (Simmonds *et al.*, 1993). Hence, HCV-1 and HCV-H are classified as group 1a and HCV-J, HCV-BK and HCV-T as group 1b. The most divergent genomic sequences, HC-J6 and HC-J8, have been designated as group 2a and 2b, respectively. The remaining 4 HCV groups (3-6) contain NS5 sequences from Germany, USA, Italy, Sweden, Scotland and Thailand (group 3a), Thailand (group 3b), Egypt (group 4a), South Africa (group 5a) and Hong Kong (group 6a) and in addition, allows the classification of new HCV isolates. Thus to summarise, HCV groups I, II, III, IV, V and VI, originally classified by the scheme described by Mori *et al*, (1992) and Okamoto *et al*, (1992b) correspond to groups 1a, 1b, 2a, 2b, 3a and 3b, respectively (Simmonds *et al.*, 1993). Furthermore, Cha *et al*, (1992) described five groups of HCV variants (GI-GV), where I corresponds to 1a, II to 1b, III to 2a, 2b and 2c, IV to 3a and 3b and V to 5a (Simmonds *et al.*, 1993).

Detailed sequence comparison of the individual putative genes showed the sequence diversities to be distributed along the whole genome except for the conserved 5' UTR. Within the coding region, the capsid (C) gene was the most highly conserved (81-91% nucleotide and 90-98% amino acid identity). In contrast, the region immediately downstream encoding a putative envelope protein (E1) only displayed 55-75% and 51-79% nucleotide and amino acid homology, respectively between groups. The nucleotide and amino acid identity between the E2/NS1 region in different isolates was 65-72% and 70-79%, respectively. Furthermore, two hypervariable regions, in the N-terminus of the E2/NS1 protein, have been identified in Japanese isolates; amino acid residues 384-410 and 474-480 (Hijikata *et al.*, 1991a; Kato *et al.*, 1992b) and one in an isolate from the USA, amino acid residues 384-414 (Weiner *et al.*, 1991, 1992). However, the second hypervariable domain (aa 374-480) has only been found in Japanese isolates. Within the NS genes only NS2 exhibits a greater degree of

heterogeneity at both the nucleotide and amino acid level (57-71%; 56-77%). On the other hand, NS3 (70-80%; 80-92%), NS4 (65-81%; 72-91%) and NS5 (66-79%; 70-84%) showed greater nucleotide and amino acid sequence conservation between groups.

Significant genetic heterogeneity has not only been reported among isolates from different geographic areas, but also within single isolates from the same individual (Kubo *et al.*, 1989; Kremsdorf *et al.*, 1991; Martell *et al.*, 1992; Tanaka *et al.*, 1992; Muller *et al.*, 1993b; Martell *et al.*, 1994). Furthermore, experimentally-infected chimpanzees which recovered from acute HCV infection could be reinfected with either homologous or heterologous virus (Farci *et al.*, 1992; Prince *et al.*, 1992). The observed heterogeneity in the HCV genome may be explained by multiple mutational events during RNA replication. Furthermore, the mutation rate of the HCV genome (HCV-H isolate) was reported to be 1.44-1.92x 10^{-3} base substitutions per site per year (Ogata *et al.*, 1991). Data have accumulated that suggest that the population of most RNA viruses consist of a heterogeneous mixture of related genomes that contains a master (most frequently represented) sequence and a large spectrum of mutants, referred to as quasispecies (Domingo *et al.*, 1985; Steinhauer and Holland, 1987). Martell *et al*, (1992) demonstrated a quasispecies distribution of HCV sequences in which half of the circulating RNA molecules were identical, while the remainder consisted of a spectrum of mutants. Mutations ranged from silent mutations to in-frame stop codons and included both conservative and nonconservative amino acid substitutions; 12% of the circulating particles contained defective genomes due to in-frame stop codons. The quasispecies model of mixed RNA virus populations may account for the high tendency of HCV to persist and the fluctuating course of chronic hepatitis C. The high degree of heterogeneity of the envelope-encoding region might lead to antigenic changes in the structural proteins likely to contain epitopes which induce neutralising antibody. Consequently, the virus may escape the host immune response to a previous

infection. The immune response to envelope proteins is discussed in more detail in section 1.9.3. Furthermore, the presence of defective viral particles may provide a supplementary mechanism for HCV persistence. Data are accumulating to indicate defective viral particles restrict viral replication of the homologous virus resulting in persistent infection (Holland, 1991).

The 5' UTR is highly conserved (>93% nucleotide sequence identity) among different isolates (Kato *et al.*, 1990; Choo *et al.*, 1991; Han *et al.*, 1991; Inchaupse *et al.*, 1991; Okamoto *et al.*, 1991; Takamizawa *et al.*, 1991; Chen *et al.*, 1992a; Honda *et al.*, 1992; Okamoto *et al.*, 1992a, b; Tanaka *et al.*, 1992; Wang Y *et al.*, 1993; Okamoto *et al.*, 1994) suggesting that this region has an important regulatory role during viral replication, perhaps at the level of RNA transcription or translation initiation. Computer analysis has suggested the presence of a hairpin at the extreme 5' end of the UTR (1 to 22; Han *et al.*, 1991; Inchauspe *et al.*, 1991; Okamoto *et al.*, 1991; Brown *et al.*, 1992). Similarly, hairpin structures have been located at the 5' end of the viral RNA of pestiviruses (Brown *et al.*, 1992). Sequence data demonstrate the presence within the 5' UTR region of three (Kato *et al.*, 1990; Takamizawa *et al.*, 1991; Bukh *et al.*, 1992b), four (Choo *et al.*, 1991; Han *et al.*, 1991) or five (Inchauspe *et al.*, 1991) short ORFs. In addition to the short ORFs, the 5' UTR region also shares distinct tracts of nucleotide homology with the 5' UTR of pestiviruses, but not with other viruses (Han *et al.*, 1991; Choo *et al.*, 1991; Inchauspe *et al.*, 1991; Bukh *et al.*, 1992b). The function of these ORFs is still unknown, but they have been postulated to be negative control elements, since HCV RNA translation *in vitro* was inefficient unless these ORFs were removed (Han *et al.*, 1991; Yoo *et al.*, 1992). Besides these small ORFs, an efficient *cis*-acting element, thought to be the hairpin structure, has been located at the 5' terminus that represses translation, while a highly conserved enhancer of translation has been found near the 3' terminus of the 5' UTR (Yoo *et al.*, 1992). Furthermore, primer extension analysis revealed two prominent species of HCV RNA in samples derived from HCV-infected

patients; full length HCV RNA and a 5' truncated subgenomic HCV RNA species beginning 145 nucleotides downstream from the 5' terminus of the full-length RNA (Han *et al.*, 1991). However, this species has not been detected by other groups. The function and origin of the subgenomic species is unknown, but it was considered to act as the major template for the translation of the viral polyprotein (Yoo *et al.*, 1992). These observations are compatible with the scanning ribosome model for translation initiation (Kozak, 1989). However, recent evidence suggests that HCV contains an internal ribosome entry site (IRES) in the 5' UTR of HCV RNA (Tsukiyama-Kohara *et al.*, 1992; Wang C *et al.*, 1993; Fukushi *et al.*, 1994). Hence, translation initiation of HCV RNA occurs by entry of ribosomes to an internal sequence within the 5' UTR similar to that described for picornaviruses (Pelletier and Sonenberg, 1988). The IRES resides between nucleotide positions 101 and 332 (Tsukiyama-Kohara *et al.*, 1992) or 125 and 323 (Brown *et al.*, 1992) and this sequence has the potential to exhibit a secondary structure indicative of a stable stem-loop which is thought to be critical for the integrity of 5' UTR-mediated translational control. Furthermore, a short pyrimidine tract complementary to the 18S ribosomal RNA has been identified at nucleotide position 192 to 203 (Brown *et al.*, 1992). In contrast, Wang C *et al*, (1993) and Fukushi *et al*, (1994) reported that the removal of 62 to 75 nucleotides from the 5' end of the 5' UTR dramatically reduced the efficiency of translation, suggesting that the correct secondary structure formation is important for internal initiation. Finally, the 5' UTR has been shown to initiate translation in a cap-independent manner (Tsukiyama-Kohara *et al.*, 1992; Wang C *et al.*, 1993; Fukushi *et al.*, 1994).

The 3' UTR is less conserved (60-90%) between different isolates. The exact nature of the 3' terminus of the genome is not known as poly A (Choo *et al.*, 1991; Han *et al.*, 1991) or poly U (Kato *et al.*, 1990; Okamoto *et al.*, 1991; Takamizawa *et al.*, 1991; Chen *et al.*, 1992a; Okamoto *et al.*, 1992a, b; Wang Y *et al.*, 1993; Okamoto *et al.*, 1994) tails have been detected in different isolates.

Computer sequence analysis of a 300 nucleotide region at the 3' end of the genome identified 4 stem and loop structures conserved in all virus groups (Han and Houghton, 1992). Hence, it has been proposed that the poly A or U tail and the stem loop structures are recognition signals for the viral RNA polymerase similar to those found in other positive strand RNA viruses (Strauss and Strauss, 1983). Further work is necessary to establish the exact nature of the 3' terminus of the viral RNA.

1.5 PHYSICOCHEMICAL ASPECTS

The cloning of the HCV genome has allowed HCV RNA detection by RT-PCR and an indirect means to detect the virus in experiments to examine the physicochemical properties of HCV. Double-step filtration of infectious serum samples through microporous cellulose fibres with a pore size of 35nm has determined the size of the virion to be between 30 and 38nm in diameter (Yuasa *et al.*, 1991). Sucrose and potassium bromide density gradients showed that the buoyant density of HCV virions was 1.08-1.12g/ml (Bradley *et al.*, 1991; Miyamoto *et al.*, 1992; Sato *et al.*, 1993; Kanto *et al.*, 1994) and 1.11g/ml (Takahashi *et al.*, 1992a, b), respectively. Studies performed by Hijikata and coworkers found variations in virion densities in sucrose from different clinical samples: the densities were ≤1.06g/ml for some samples and ≥1.13g/ml for others. Further investigation showed that the samples with low density virus were highly infectious in chimpanzee transmission studies, compared with the samples with higher density virus (see section 1.10). Furthermore, serial serum samples from experimentally-infected chimpanzees showed a shift from low to high density HCV during the course of infection. Immunoprecipitation experiments revealed that HCV virions were precipitated with anti-human immunoglobulin only from the low infectivity serum samples that contained high density virions, suggesting that anti-HCV antibodies were bound to HCV virions as antigen-antibody complexes in chronic hepatitis C (Hijikata *et al.*, 1993c). However,

another study detected high density virions (1.22-1.25g/ml), thought to represent nucleocapsids, in the circulation of HCV-infected patients with increased liver damage (Kanto *et al.*, 1994). In addition, other studies have transformed a low density (presumed enveloped virus) into a higher density (presumed nucleocapsid) after detergent- (Miyamoto *et al.*, 1992; Kanto *et al.*, 1994) or chloroform-(Hijikata *et al.*, 1993c; Kanto *et al.*, 1994) treatment, suggesting that a lipid-rich envelope was removed. Recently, lectin-binding properties of HCV suggested that the virion surface contains asparagine-linked carbohydrates (Sato *et al.*, 1993), consistent with glycosylation of the envelope proteins (see section 1.8). Examination of detergent-treated HCV-associated particles from plasma of infected humans by electron microscopy, using a grid coated with an antibody directed to a HCV core peptide, showed a 33nm icosahedron-shaped particle. Immunoblotting studies of this preparation detected a core protein of 26kDa (Takahashi *et al.*, 1992a).

1.6 FLAVIVIRUS REPLICATION

The classification of HCV in the Flaviviridae has led to the postulation that HCV may adopt a replication strategy closely related to that of flaviviruses. The main points of the replication cycle are as follows: (i) Initially, the input viral genomic RNA is translated from a single initiation site to generate all the viral proteins by cotranslational and posttranslational processing (step 1; Figure 1.2). (ii) The incoming RNA then serves as a template for the synthesis of complementary negative RNA strands giving rise to partially dsRNA (replicative intermediates (RI)) by the viral-encoded replicase. These negative strands form replicative forms (RF) by binding to the positive RNA strands (step 2; Figure 1.2). (iii) The RF act as templates for the synthesis of multiple copies of nascent genome-length, positive and negative strand molecules (step 3; Figure 1.2). The ratio of positive to negative strands within the cytoplasm has been estimated to be 10:1. (iv) Nascent positive sense strands can be either encapsidated and released

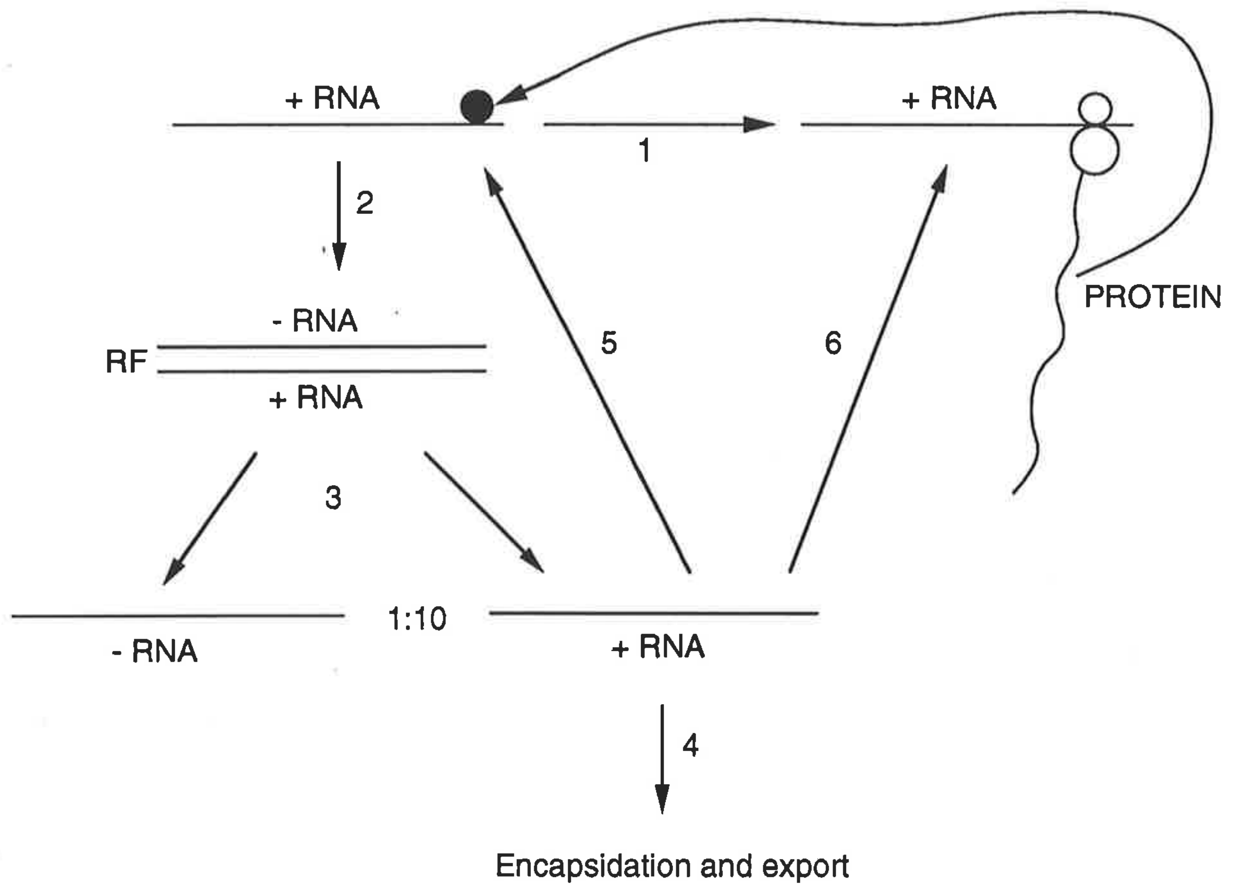
Figure 1.2 Replication strategy of Flaviviruses.

Step 1: Translation of input positive sense viral RNA.

Step 2: Synthesis of negative strands by the viral-encoded replicase. Positive and negative RNA strands anneal giving rise to double-stranded RF.

Step 3: RF serve as a template for positive and negative strand synthesis.

Nascent positive strands are either: encapsidated (step 4), act as templates for negative strand synthesis (step 5) or translated to produce the S and NS proteins (step 6).


+ RNA
1
+ RNA
2
PROTEIN
- RNA
RF
+ RNA
5
6
3
1:10
- RNA
+ RNA
4
Encapsidation and export

from infected cells (step 4; Figure 1.2), serve as templates for negative strand synthesis (step 5; Figure 1.2) or be translated to produce the S and NS proteins (step 6; Figure 1.2) (Westaway, 1987; Chambers *et al.*, 1990a).

1.7 CULTURE OF HCV *IN VITRO*

The lack of an *in vitro* system for the propagation of HCV has hindered the physical isolation of distinct S and NS proteins, and the subsequent identification of their functional role in the viral life cycle and disease. However, seven studies have reported *in vitro* replication of HCV. The first study used primate hepatocytes which were infected *in vivo* and cultured *in vitro*; these cells continued to synthesise HCV for up to 17 days in culture (Jacob *et al.*, 1990). A second study reported infection of Chang liver cells using HCV RNA-positive liver tissue extracts as the inoculum. After 3 days in culture, up to 10% of cells were expressing HCV antigens (Ozeki *et al.*, 1992). The third study described *de novo* infection of a continuous human T lymphocyte line (MOLT-4) using HCV-positive chimpanzee serum as the inoculum (Shimizu *et al.*, 1992). Virus replication was monitored by the presence of intracellular negative strand RNA (HCV RNA complementary to the viral genome) detected by RT-PCR. The putative replicative intermediate was detected 3 days after inoculation and the maximum signal was observed at seven days. HCV sequences were detected intermittently in the cells for 12 days and approximately 1% of cells were infected at day 7 as assessed by C and NS4 antigen detection by immunofluorescence. To extend these initial culture attempts another murine retrovirus-infected human T cell line, HPB-Ma, was infected with HCV-positive human and chimpanzee sera and shown to support HCV replication for as long as 76 days (Shimizu *et al.*, 1993). As before, virus replication was monitored by the detection of intracellular positive and negative sense HCV RNA by RT-PCR as well as the detection of the C protein. Furthermore, treatment of cells with cycloheximide, reported to increase adenovirus-specific RNA in treated cells (Craig and Raskas, 1974),

enhanced HCV RNA detection (Shimizu *et al.*, 1993). Thus, the data suggest that HCV is able to replicate in two human T cell lines. The fourth study demonstrated the susceptibility of a human bone marrow-derived B cell line to HCV infection. HCV RNA was detected after 22 days in culture while 1% of cells at day 24 were positive for C and NS3/NS4 proteins by immunofluorescence (Bertolini *et al.*, 1993). HCV RNA replication has also been demonstrated in primary human foetal hepatocyte cultures infected *in vitro* with HCV-positive human serum. RT-PCR detected intracellular positive and negative HCV RNA 12 to 24 days after infection (Iacovacci *et al.*, 1993). Finally, *in vitro* infection of primary chimpanzee hepatocytes with HCV-positive human serum was established. Strand specific RT-PCR assays detected intracellular positive and negative sense HCV RNA 4 days postinfection and for the remaining 25 days in culture (Lanford *et al.*, 1994).

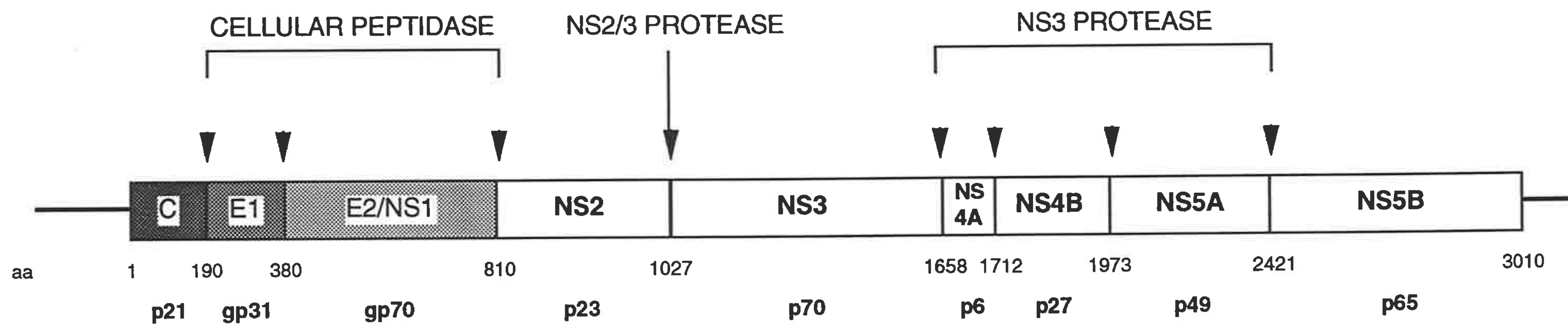
1.8 HCV PROTEINS

The low proportion of infected cells in the *in vitro* culture experiments, has led to the use of alternative approaches to study HCV-specific proteins. These include protein expression from cloned HCV cDNA sequences by: (i) *in vitro* translation systems such as rabbit reticulocyte lysate; (ii) DNA transfection into mammalian cells; and (iii) Recombinant Vaccinia Virus (rVV) infection of mammalian cells. These methods have permitted characterisation of the S and some of the NS proteins.

The polyprotein precursor is cleaved into the mature individual polypeptide products by a combination of viral-encoded and cellular proteases. The HCV-H strain and various truncated derivatives have been used in transient-expression assays to map the HCV-encoded polypeptides. Processing of the polyprotein yielded at least nine distinct proteins: 5'-C-E1-E2/NS1-NS2-NS3-NS4A-NS4B-NS5A-NS5B-3' (Figure 1.3) identified by convalescent human sera and region-specific antisera (Grakoui *et al.*, 1993c; Selby *et al.*, 1993; Tomei *et al.*, 1993).

Figure 1.3 The major cleavage sites and enzymes responsible for the cleavage of the HCV polyprotein.

The cleavage junctions are shown by arrows.



CELLULAR PEPTIDASE
NS2/3 PROTEASE
NS3 PROTEASE
C
E1
E2/NS1
NS2
NS3
NS 4A
NS4B
NS5A
NS5B
aa
1
190
380
810
1027
1658
1712
1973
2421
3010
p21
gp31
gp70
p23
p70
p6
p27
p49
p65
Structural
Non-Structural

The S region is processed by a host cell signal peptidase of the endoplasmic reticulum (Hijikata *et al.*, 1991b; Tomei *et al.*, 1993; Santolini *et al.*, 1994), independent of the presence of the NS proteins (Hijikata *et al.*, 1991b; Grakoui *et al.*, 1993a; Tomei *et al.*, 1993), to release 3 S proteins viz. C, E1 and E2/NS1. This cleavage event is thought to be similar to that of the S proteins of flaviviruses (Markoff, 1989; Nowak *et al.*, 1989; Ruiz-Linares *et al.*, 1989). The cleavage sites between C/E1, E1/E2(NS1) and E2(NS1)/NS2 have been determined experimentally by *in vitro* translation (Hijikata *et al.*, 1991b) (Figure 1.3). A 21-22 kDa protein (p21) was identified as the HCV C protein by transient expression in cultured monkey COS cells (Harada *et al.*, 1991; Ravaggi *et al.*, 1994), chimpanzee liver cells (Manabe *et al.*, 1994), insect cells (Chiba *et al.*, 1991; Hsu *et al.*, 1993) and mammalian cells (Kumar *et al.*, 1992; Grakoui *et al.*, 1993c; Ralston *et al.*, 1993; Selby *et al.*, 1993; Tomei *et al.*, 1993; Santolini *et al.*, 1994). The C protein is a basic protein suggesting it may interact with viral RNA (Takeuchi *et al.*, 1990b; Hijikata *et al.*, 1991b), and a recent study demonstrated RNA binding activity in the N-terminal region of the C protein (Santolini *et al.*, 1994). Furthermore, the same region was shown to bind ribosomes, suggesting the potential role of the C protein as a structural component of the virion involved in RNA packaging and virion assembly (Santolini *et al.*, 1994). *In vitro* translation assays and site directed mutagenesis revealed that an arginine residue at codon 9 led to the synthesis of p21, whereas a 16kDa protein (p16) was produced when codon 9 was a lysine residue. Further analysis indicated that p16 was likely to be co-N-terminal with p21, however the mechanism of p16 synthesis remains unclear (Lo *et al.*, 1994).

E1 and E2/NS1 have been identified as glycosylated proteins of 31-37kDa (gp31) and 61-72kDa (gp70) respectively, in cell-free translation systems (Hijikata *et al.*, 1991b; Tomei *et al.*, 1993) or mammalian (Kohara *et al.*, 1992; Matsuura *et al.*, 1992; Spaete *et al.*, 1992; Grakoui *et al.*, 1993c; Ralston *et al.*, 1993; Selby *et al.*, 1993; Tomei *et al.*, 1993; Manabe *et al.*, 1994) or insect cells (Matsuura *et al.*,

1992; Hsu *et al.*, 1993), and are believed to represent virion envelope glycoproteins. Both envelope proteins are heavily modified by N-linked glycosylation, and E1 and E2/NS1 contain 5-6 and 9-11 potential glycosylation site motifs [Asn-X-Thr(Ser)], respectively (see section 1.5). Endoglycosidase F digestion of these polypeptides resulted in a decrease in the electrophoretic size consistent with the loss of the sugar residues (Hijikata *et al.*, 1991b; Matsuura *et al.*, 1992; Grakoui *et al.*, 1993c; Hsu *et al.*, 1993). Furthermore, there are hydrophobic regions (aa residues 174-191 and residues 370-383, respectively) upstream of the N-termini of gp31 and gp70 that may act as signal sequences to direct the protein to the endoplasmic reticulum for cleavage (Hijikata *et al.*, 1991b). Consistent with this suggestion is the finding that E1 and E2/NS1, expressed by rVV-infected cells, were found localised in endoplasmic reticulum membranes as core-glycosylated species (Ralston *et al.*, 1993; Santolini *et al.*, 1994). In addition to the putative envelope proteins, an 88kDa glycoprotein reactive with both E2/NS1 and NS2 region-specific antisera was identified, suggesting that this E2/NS1-NS2 protein may represent a precursor to E2/NS1 (Grakoui *et al.*, 1993c; Tomei *et al.*, 1993). This observation is not unique to HCV, but has been reported in flavivirus polyprotein processing (Mason, 1989; Chambers *et al.*, 1990b), suggesting that the maturation of E2/NS1 and NS2 proteins is not a cotranslational event. Furthermore, preliminary studies have led to the suggestion that a proportion of E1 is associated with E2/NS1 and E2/NS1-NS2 via disulphide bonds (Grakoui *et al.*, 1993c). In contrast, Ralston *et al*, (1993) found no evidence of intermolecular disulphide bonding between E1 and E2/NS1. Recently, a tenth HCV-encoded cleavage product, p7, was identified. The p7 protein (7kDa), located between the E2/NS1 and NS2 proteins, is preceded by a hydrophobic sequence which may direct its translocation into the endoplasmic reticulum. Moreover, cell free translation analyses indicate the host signal peptidase is responsible for cleavage at E2(NS1)/p7 and p7/NS2. However, cleavage between E2/p7 is incomplete (Lin C *et al.*, 1994), supporting additional posttranslational cleavage.

Studies to examine the polyprotein cleavage sites of the NS proteins have been performed *in vitro*. Based on the analogy with pestiviruses and flaviviruses and the presence of characteristic motifs, the location and potential functions of these HCV proteins have been predicted (Table 1.1). Recently, the NS region has been expressed *in vitro* from a rVV and the polyprotein shown to be cleaved by a serine proteinase encoded by the N-terminal domain of the NS3 region that is responsible for cleavage of the HCV polyprotein at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (Bartenschlager *et al.*, 1993; Eckart *et al.*, 1993; Grakoui *et al.*, 1993a; Hijikata *et al.*, 1993b; Tomei *et al.*, 1993; Bartenschlager *et al.*, 1994; Failla *et al.*, 1994; Manabe *et al.*, 1994) (Figure 1.3). Substitution of the histidine-1083 and/or serine-1165 residue of the putative catalytic triad (His-1083, Asp-1107, Ser-1165) by alanine or glycine prevented cleavage. However, these substitutions caused no observable effect on cleavages in the structural region and at the NS2/NS3 junction (Bartenschlager *et al.*, 1993; Eckart *et al.*, 1993; Grakoui *et al.*, 1993a; Tomei *et al.*, 1993; Bartenschlager *et al.*, 1994; Manabe *et al.*, 1994). Furthermore, cleavage at the N-termini of NS4B, NS5A and NS5B could be mediated *in trans* by NS3, whereas processing at the N-terminus of NS4A could not, suggesting that this cleavage event occurs *in cis* (Tomei *et al.*, 1993; Bartenschlager *et al.*, 1994). Cleavage between NS2 and NS3 required the C-terminal portion of the NS2 protein and the serine proteinase domain of NS3 (Grakoui *et al.*, 1993b; Hijikata *et al.*, 1993a), and it has been proposed that the NS2/NS3 protease (aa residues 827-1207) is a zinc-dependent metalloproteinase (Hijikata *et al.*, 1993a) (Figure 1.3). Alanine substitutions of the NS2 histidine-952 or cystidine-993 abolished cleavage at the NS2/NS3 site, but not at the remaining sites of the polyprotein. In addition, mutations previously shown to inactivate the NS3 serine proteinase function did not block cleavage at the NS2/NS3 junction (Grakoui *et al.*, 1993b) (Figure 1.3). More recently, in addition to NS3, a C-terminal 33 amino acids of the NS4A protein was required for cleavage at the NS3/NS4A and NS4B/NS5A junctions and for accelerated cleavage at the NS5A/NS5B junction (Bartenschlager *et al.*,

Table 1.1 Predicted HCV proteins.

Protein	Size (kDa)	Putative function
Structural		
C	p21	Capsid
E1	gp31	Envelope
E2/NS1	gp70	Envelope*
Non-structural		
NS2	p23	Zn^{2+}-dependent protease component
NS3	p70	Serine proteinase/NTPase/helicase*
NS4A	p6	Protease co-factor
NS4B	p27	?
NS5A	p49	?
NS5B	p65	replicase*

Protein sizes Grakoui *et al.*, 1993 a,c
* Predicted functions
gp = glycosylated protein

1994; Failla *et al.*, 1994) and processing at these sites could be restored when NS4 was provided *in trans* (Bartenschlager *et al.*, 1994; Failla *et al.*, 1994) or *in cis* (Bartenschlager *et al.*, 1994).

The putative NS proteins include NS2 (23-24kDa), NS3 (68-70kDa), NS4A (6-8kDa), NS4B (26-27kDa), NS5A (49-58kDa) and NS5B (65-68kDa) (Grakoui *et al.*, 1993c; Selby *et al.*, 1993; Tomei *et al.*, 1993; Manabe *et al.*, 1994) (Figure 1.3). In addition to the protease activity, characteristic sequence motifs within the C-terminus of NS3 suggested that NS3 possesses nucleoside triphosphatase (NTPase) and RNA helicase activity (Choo *et al.*, 1991; Selby *et al.*, 1993; Suzich *et al.*, 1993; Manabe *et al.*, 1994). Recently, Suzich *et al*, (1993) demonstrated a polynucleotide-stimulated NTPase activity associated with the purified C-terminal region of NS3 (aa residues 1193-1657) expressed in *E. coli.* The NTPase activity was similar to flavivirus and pestivirus enzymes with respect to pH, $MgCl_2$ and salt concentration required for optimum activity *in vitro*. However, the HCV NTPase was enhanced by polyribonucleotides and deoxyribonucleotides (Suzich *et al.*, 1993). At present, a RNA helicase activity has still not been demonstrated experimentally.

The NS4 region is cleaved into two distinct products, NS4A and NS4B, consistent with the processing profile of the NS region of flaviviruses (Chambers *et al.*, 1990b). The NS4A protein of 6-8kDa represents the N-terminal part of the NS4 gene, whereas the NS4B protein is encoded by the C-terminal domain (Grakoui *et al.*, 1993a; Tomei *et al.*, 1993). Similarly, NS5 is processed into NS5A and NS5B, a feature shared by the pestiviruses. The RNA-dependent RNA polymerase, necessary for virus RNA replication, is thought to be encoded by the NS5B domain because this region contains a gly-asp-asp motif (aa residues 2736-2738) (Okamoto *et al.*, 1991; Takamizawa *et al.*, 1991) present within the RNA-dependent-RNA-polymerase of well-characterised RNA viruses, including flaviviruses and polioviruses (Kamer and Argos, 1984). However, the RNA

polymerase is probably only one component of the replication complex (replicase). To date, NS4B and NS5A have not been assigned functions, although computer analysis indicates they are hydrophobic and are probably membrane bound (Choo *et al.*, 1991). These proteins could also have a function in the replication of the viral genome.

1.9 SEROLOGICAL DIAGNOSIS

1.9.1 Routine assays

The cloning of the HCV genome has permitted the expression of recombinant HCV antigens which have subsequently been used to develop immunoassays to detect antibodies to HCV (Kuo *et al.*, 1989). Three overlapping clones, identified from the cDNA library by using 5-1-1 as a hybridisation probe, were ligated to construct a continuous ORF. The resultant protein, termed C100-3, was expressed in yeast as a fusion polypeptide with human superoxide dismutase (SOD) to facilitate the efficient expression of the foreign protein. The SOD-HCV fusion polypeptide, contains 363 HCV-specific amino acids spanning a portion of the NS3 and NS4 proteins of HCV. Purified and solubilised C100-3 was used to coat the wells of microtitre plates and used to detect circulating anti-HCV in chronic carriers by an enzyme linked immunosorbent assay (ELISA). This constituted the first generation commercial HCV ELISA. Screening of seven sera, from known NANB-infected patients, by this first generation ELISA detected antibody in six samples (Kuo *et al.*, 1989). The negative sample was obtained from an individual with acute phase NANBH. Hence, the C100-3 antigen based immunoassay preferentially detects antibodies from chronically-infected patients (Kuo *et al.*, 1989; Alter HJ *et al.*, 1989; Esteban *et al.*, 1990; Miyamura *et al.*, 1990; Mosley *et al.*, 1990; Weiner *et al.*, 1990). Anti-C100-3 has been suggested to be an indicator of potential infectivity (Esteban *et al.*, 1990; Katayama *et al.*, 1990; Miyamura *et al.*, 1990; Weiner *et al.*, 1990). Seroconversion to the C100-3

protein in patients with HCV infection occurs approximately 6 weeks after infection, although some studies have reported earlier (4 weeks) and later (52 weeks) conversion (Alter HJ *et al.*, 1989). Thus, there is a long 'window' period after the patient is infected before antibody to C100-3 is detected.

The availability of full length HCV RNA sequences allowed the production of other HCV antigens in order to improve serodiagnosis. Nasoff *et al*, (1991) demonstrated that an immunodominant epitope located within the N-terminal portion of the C protein was preferentially recognised by antibodies in both human and chimpanzee sera. Antibodies directed to this epitope appeared 2-21 weeks after the onset of infection (Nasoff *et al.*, 1991). In efforts to overcome the limited sensitivity of the first generation anti-HCV ELISA, synthetic peptides from the C region (Okamoto *et al.*, 1990a; Hosein *et al.*, 1991; Bresters *et al.*, 1992; Kotwal *et al.*, 1992; Okamoto *et al.*, 1992c) and recombinant C protein produced in the baculovirus expression system (Chiba *et al.*, 1991) or expressed in *E. coli* (Vallari et al., 1992; Yoshikawa *et al.*, 1992) were used to examine the serological response to this region. The C protein (C22) was shown to elicit antibody production earlier in HCV infection than C100-3 thus reducing the 'window' period before the appearance of antibody. Interestingly, a number of patients who were anti-C100-3 negative were anti-C positive while the converse was true for other patients. Thus, it was considered that an ELISA containing both S and NS proteins, would increase the sensitivity and consequently improve the serological diagnosis of infection. This approach was used in the second generation commercial test in which C22 and NS3 (C33c) have been added, with the original C100-3 antigen, to the solid phase. More recently, NS5 has been added to a third generation assay (Breschkin *et al.*, 1993). The time lag of seroconversion was decreased from 6 weeks after the onset of hepatitis with the first generation test to 2 weeks with the second generation assay (Mattsson *et al.*, 1992). Additional advantages of the second generation assay are the improved specificity and sensitivity of HCV diagnosis, although the assay is

unable to distinguish acute, chronic or past infection. However, the specificity and sensitivity of the third generation assay remains to be determined.

The additional antigens utilised in the second generation ELISA have been deposited onto nitrocellulose to develop a 4-antigen recombinant immunoblot assay (RIBA), whereby the sensitivity in diagnosing chronic hepatitis C was substantially increased (Van Der Poel *et al.*, 1991). The results of this assay suggests that antibodies to 2 or more HCV proteins confirms viraemia and potential infectivity (Van Der Poel *et al.*, 1991). RIBA currently represents a confirmatory assay.

1.9.2 IgM assay

Measurement of IgG in the above serological assays does not distinguish between ongoing or past infection and it was considered that the detection of IgM would indicate acute phase infection. Furthermore, assays to detect IgM may reduce the 'window' period seen with the first and second generation ELISA assays. Recently, it has been suggested that an IgM antibody response against the C antigen in patients with HCV infection, may serve as a marker of acute phase HCV in which a transient IgM response was noted in 88% of HCV-positive patients (Chen *et al.*, 1992b). The IgM antibody appeared shortly after the onset of hepatitis (3-4 weeks) and persisted for approximately 18 weeks and then disappeared, whereas the IgG antibody persisted. However, it has been demonstrated that IgM anti-HCV is not always limited to the acute phase of the disease and some chronic carriers have protracted periods of IgM anti-HCV positivity (Quiroga *et al.*, 1991; Clemens *et al.*, 1992). A number of studies suggest that persisting titres of IgM anti-HCV may serve as a marker to indicate active HCV infection (Brillanti *et al.*, 1992; Chen *et al.*, 1992b; Clemens *et al.*, 1992; Sansonno and Dammacco, 1992; Hellstrom *et al.*, 1993). In contrast, a recent report concluded that the IgM response in HCV infection may be absent,

late or persistent after HCV infection (Zaaijer *et al.*, 1993). Thus, specific IgM assays, which in other virus infections are usually able to identify recent or acute infection, do not appear helpful in the diagnosis of acute phase HCV.

1.9.3 Immune response to the envelope proteins

The envelope region, encoding gp31 and gp70, shows marked sequence diversity (section 1.4) and a number of studies have identified a hypervariable domain (aa 384-414) in the N-terminus of E2/NS1 (Hijikata *et al.*, 1991a; Weiner *et al.*, 1991; Kato *et al.*, 1992b; Weiner *et al.*, 1992). Sequence data suggest that each hypervariable domain contains an immunological epitope specific for an individual virus (Weiner *et al.*, 1992; Kato *et al.*, 1993a). Recombinant envelope proteins and synthetic peptides from the envelope region have been used for the detection of antibody against E1 (Matsuura *et al.*, 1992; Chien *et al.*, 1993; Lok *et al.*, 1993; Ray *et al.*, 1994) and E2/NS1 (Chien *et al.*, 1993; Lesniewski *et al.*, 1993; Lok *et al.*, 1993) in serum samples from HCV-infected individuals. Antibody to E1 was detected in 10-20% of patients with HCV at various stages of infection and in a proportion (1-2%) of convalescent cases, considered to be rare (Matsuura *et al.*, 1992). Despite the presence of the hypervariable domain within E2/NS1, antibody to E2/NS1 was found at high frequency (58-97%) among hepatitis C carriers (Chien *et al.*, 1993; Lesniewski *et al.*, 1993; Lok *et al.*, 1993). Furthermore, a high proportion of serum samples (97%) contained antibodies which recognised recombinant E1-E2/NS1 complexes (Chien *et al.*, 1993). Analysis of partial or complete E2/NS1 sequences from infected individuals indicated that E2/NS1 variants can either coexist simultaneously in an individual or that a particular variant may predominate during different episodes of disease (Weiner *et al.*, 1992). The N-terminus of E2/NS1, encompassing the hypervariable region, was shown to contain multiple linear epitopes (Weiner *et al.*, 1992; Lesniewski *et al.*, 1993) and each hypervariable region contains an epitope that is specific for the homologous HCV isolate. The amino

acid alterations have been shown to occur sequentially in the chronic phase of hepatitis at intervals of several months (Kato *et al.*, 1992a; Kato *et al.*, 1993a; Kurosaki *et al.*, 1993; Taniguchi *et al.*, 1993; Kato *et al.*, 1994), suggesting that mutations in this region are involved in the mechanism of persistent infection as a result of immunological selection. Furthermore, the amino acid changes were associated with an alteration of predicted local secondary structure of the epitope region (Taniguchi *et al.*, 1993) and variants in this region could escape recognition by preexisting antibodies (Weiner *et al.*, 1992; Kato *et al.*, 1994).

Immunisation of chimpanzees with purified recombinant E1-E2/NS1 complexes generated high titres of anti-E1 and -E2/NS1 (Ralston *et al.*, 1993). Furthermore, preliminary data demonstrated E1-E2/NS1 complexes provide protection against HCV infection in chimpanzees (Choo *et al.*, 1994). A humoral immune response was generated to these glycoproteins and following challenge with homologous HCV, five chimpanzees were completely protected while two were infected. However, the disease in unprotected chimpanzees was less severe than that experienced by the unvaccinated chimpanzees (Choo *et al.*, 1994).

1.9.4 Viral RNA detection

During the 'window' period, HCV RNA is the only marker of infection. The low level of circulating virus has led to the use of sensitive techniques for the detection of HCV RNA. Hence, the use of PCR to amplify reverse transcribed cDNA is necessary for the detection of viral RNA in serum to confirm HCV infection (Garson *et al.*, 1990a; Weiner *et al.*, 1990). PCR provides valuable information concerning the viraemic status and potential infectivity of the individual (Garson *et al.*, 1990a). In addition, detection of HCV RNA in serum by PCR may be the only indicator of acute infection. However, the sequence heterogeneity among different HCV isolates has led to the use of primers to target the highly conserved 5' UTR to avoid false negative results (Okamoto *et*

al., 1990b; Bukh *et al.*, 1992a). Recent quantitation of HCV RNA in serum by competitive RT-PCR, based on coamplification of the target RNA with known amounts of synthetic mutated RNA, detected 10^3 to 10^5 genomes/ml (Kaneko *et al.*, 1992), 10^4 to $10^{9.5}$ genomes/ml (Hagiwara *et al.*, 1993), 10^1 to 10^7 genomes/ml (Kato *et al.*, 1993b) and 10^4 to 10^8 genomes/ml (Naito et al., 1994). Furthermore, the level of HCV RNA was lower in asymptomatic blood donors and patients with CPH than in patients with advanced liver disease and CAH or cirrhosis (Hagiwara *et al.*, 1993; Kato *et al.*, 1993b; Naito *et al.*, 1994). This high titre of circulating HCV RNA suggests that conventional Northern- and slot blot-hybridisation may be successful for HCV RNA detection. Although one report detected HCV RNA in infected serum by slot blot-hybridisation using both cDNA and RNA probes from the highly conserved 5' UTR (Hu *et al.*, 1992), this study has not been confirmed by others. Nevertheless, the level of circulating HCV RNA is likely to be lower than that described above, otherwise it is probable that RT-PCR would generally have been replaced by slot blot-hybridisation assays.

1.9.5 Serological events

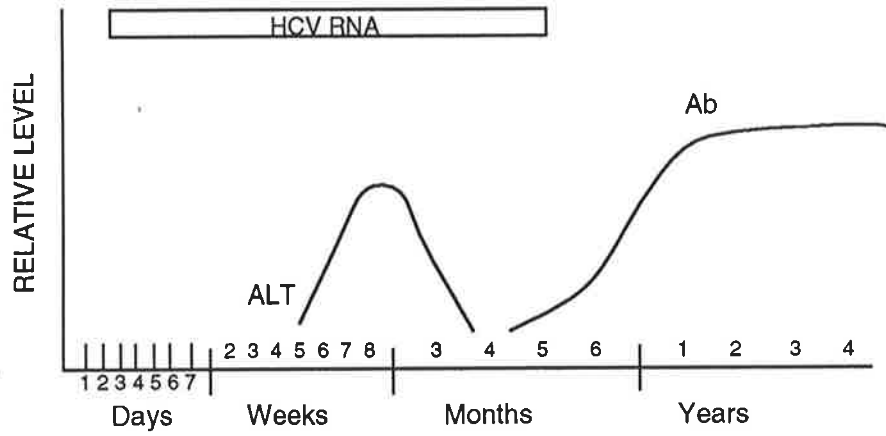
The use of PCR and the second generation ELISA have permitted an examination of the serological events which occur after infection. These serological events have been examined in chimpanzees (Shimizu *et al.*, 1990; Farci *et al.*, 1991; Abe *et al.*, 1992), but similar events have been noted in human infection (Garson *et al.*, 1990b; Farci *et al.*, 1991). Recognised serological profiles are summarised in Figure 1.4. After exposure to the virus, HCV RNA appears within 2 to 3 days in the serum of infected individuals (Farci *et al.*, 1991). The duration of the viraemic phase was found to be variable in acute infection ranging from 10 to 40 weeks followed by a loss of viral RNA. However, greater than 80% of patients progress to chronic infection (Prince *et al.*, 1993) and HCV RNA can persist for greater than 10 years after the initial exposure (Farci *et al.*, 1991; Lok *et al.*, 1992; Prince *et al.*, 1993). A high proportion (88-100%) of anti-HCV positive

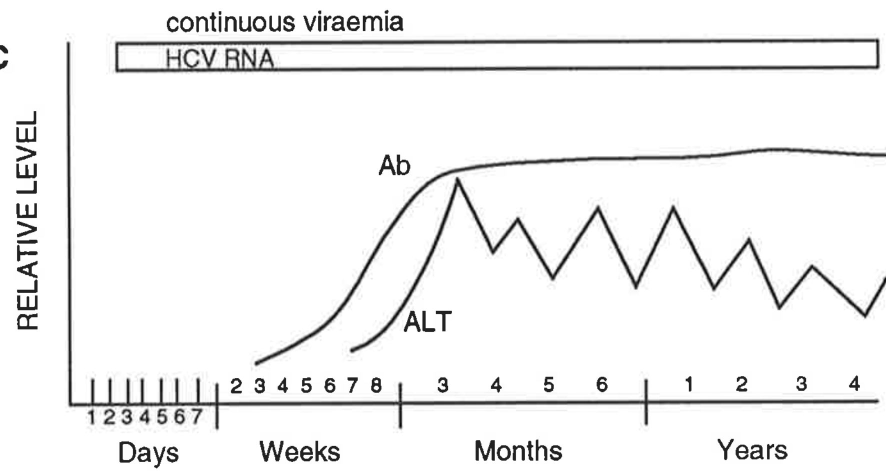
Figure 1.4 Serological profiles associated with HCV infection.

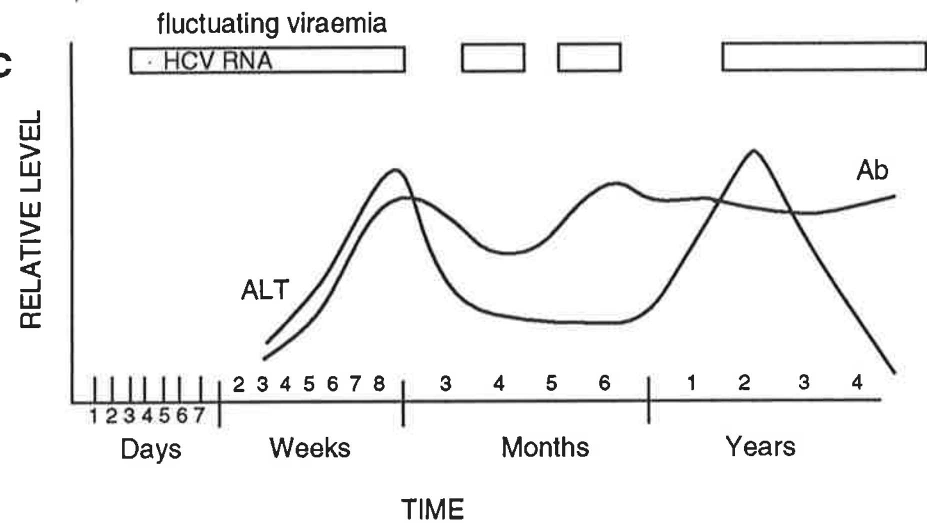
A. Acute infection.

B and C. Serological responses in chronic infection.

Zero on the X axis refers to the day of exposure.


A
ACUTE
HCV RNA
RELATIVE LEVEL
Ab
ALT
2 3 4 5 6 7 8
3 4 5 6
1 2 3 4
1 2 3 4 5 6 7
Days
Weeks
Months
Years


B
CHRONIC
continuous viraemia
HCV RNA
RELATIVE LEVEL
Ab
ALT
2 3 4 5 6 7 8
3 4 5 6
1 2 3 4
1 2 3 4 5 6 7
Days
Weeks
Months
Years


C
CHRONIC
fluctuating viraemia
HCV RNA
RELATIVE LEVEL
Ab
ALT
2 3 4 5 6 7 8
3 4 5 6
1 2 3 4
1 2 3 4 5 6 7
Days
Weeks
Months
Years
TIME

individuals are HCV RNA-positive (Okamoto *et al.*, 1990b; Hagiwara *et al.*, 1992; Lok *et al.*, 1992; Romeo *et al.*, 1993), although the presence of antibody does not always correlate with persistent infection. Two patterns of viraemia, continuous and fluctuating, have been described in chronic carriers. The absence of HCV RNA has been noted in approximately 12% of anti-HCV positive individuals (Hagiwara *et al.*, 1992; Lok *et al.*, 1992). Conversely, the persistence of HCV RNA may not always be associated with a consistent detectable immune response and fluctuating antibody patterns have been observed in chronically-infected patients (Farci *et al.*, 1991; Romeo *et al.*, 1993). However, these patients may represent cases of resolved HCV infection. Several studies failed to observe a strong correlation between elevated levels of ALT in the serum and HCV viraemia (Farci *et al.*, 1991; Abe *et al.*, 1992; Puoti *et al.*, 1992). HCV RNA appeared well before raised ALT levels and was maintained even following the decline in ALT (Garson *et al.*, 1990b; Shimizu *et al.*, 1990). From these studies, it is clear that HCV infection does not follow a predictable pattern and in a number of cases this causes problems in the diagnosis and prognosis of HCV infection.

1.10 TRANSMISSION OF HCV

Epidemiologic and experimental studies indicate that HCV is transmitted by the parenteral route. The high risk groups include intravenous drug users and haemophiliacs. However, transmission by blood or blood products only represents the cause of 60% of HCV infections; only 5-10% of patients with chronic HCV infection have a history of blood transfusion, 40% have a history of intravenous drug use, approximately 5% are health care workers with occupational exposure to blood and 10% may be related to contact with known carriers. Consequently, approximately 40% of chronic carriers have no identifiable risk factors of infection (Alter *et al.*, 1990). Potential modes of nonparenteral transmission are mother to child, sexual and intrafamilial, but none of these are well documented.

Vertical transmission from mother to infant has been reported in mothers coinfected with the human immunodeficiency virus (HIV) and HCV (Giovannini *et al.*, 1990; Thaler *et al.*, 1991; Novati *et al.*, 1992), but it is unclear if transmission of HCV from a non-HIV infected mother to her infant occurs. Recent studies failed to provide evidence for mother to infant transmission of HCV in a group of HCV-positive, HIV-negative women (Reinus *et al.*, 1992; Roudot-Thoraval *et al.*, 1993), suggesting that the absence of coexistant HIV infection drastically reduces the transmission of HCV. In contrast, other studies suggested that vertical transmission of HCV can occur in these circumstances (Chen *et al.*, 1991; Kuroki *et al.*, 1991; Wejstal *et al.*, 1992). Furthermore, three studies provide direct evidence to support HCV transmission from HIV-negative mothers to her infant. Firstly, nucleotide sequence analysis of the C/envelope region revealed transmission of the identical HCV sequence from mother to child through two generations (Inoue *et al.*, 1991, 1992). Secondly, the HCV cDNA sequence, within the C and E1/E2(NS1) genes, from HCV-infected infants was identical to that from the mother (Ohto *et al.*, 1994). Finally, sequence data from the putative E2/NS1 hypervariable region revealed a unique, predominant HCV variant within an infant born to a mother with multiple E2/NS1 variants. Furthermore, the E2/NS1 variant isolated from the infant was related to, but not identical with any of the nine variants identified in the mother at the time of birth (Weiner *et al.*, 1993). Two recent studies suggested the viral titre is the main influence on HCV transmission from mother to child (Lin H-H *et al.*, 1994; Ohto *et al.*, 1994). However, these data contradict that of Hijikata *et al*, (1993c) (see section 1.5) in which the presence of high density HCV (immune complexes) in patients sera was associated with low infectivity, despite the detection of high titre HCV RNA.

Although the exact mode of transmission of HCV in sporadic cases is unclear, transmission of NANBH by saliva has been demonstrated in chimpanzee experiments (Abe *et al.*, 1987) and possible transmission of HCV by a human bite has been reported in one case (Dusheiko *et al.*, 1990). Using PCR, the

presence of HCV RNA in saliva (Takamatsu *et al.*, 1990; Abe and Inchauspe, 1991; Wang *et al.*, 1991; Liou *et al.*, 1992; Wang *et al.*, 1992b; Couzigou *et al.*, 1993; Young *et al.*, 1993) and other human secretions such as urine, seminal fluid and ascites has been documented (Liou *et al.*, 1992). Despite the presence of HCV RNA in saliva, a prospective study revealed that the transmission efficiency was low among spouses (Wang *et al.*, 1992b). This may reflect the low levels of virus found in saliva relative to serum. In contrast, two studies have reported the absence of HCV RNA in human secretions (Hsu *et al.*, 1991; Fried *et al.*, 1992). Hence, further studies are required to determine if these differences may be related to genotype differences, stage of the disease and/or levels of circulating virus.

The detection of HCV RNA in seminal fluid suggests sexual contact as a likely mode of nonparenteral transmission of HCV (Liou *et al.*, 1992). Sexual transmission of HCV has been documented (Alter MJ *et al.*, 1989; Tedder *et al.*, 1991; Akahane *et al.*, 1992; Thomas *et al.*, 1994) and is more efficient with homosexual rather than heterosexual activity (Tedder *et al.*, 1991). Furthermore, antibodies to HCV have been found to be highly prevalent (12%) in prostitutes (Wu *et al.*, 1993). Although the importance of sexual transmission in the epidemiology of HCV infection is still controversial, a number of recent studies using second generation ELISA indicate the absence or very low risk of HCV transmission by sexual contact (van Doornum *et al.*, 1991; Brettler *et al.*, 1992; Osmond *et al.*, 1993). Furthermore, other studies demonstrated low level sexual transmission using both the second generation anti-HCV assays and PCR (Wang *et al.*, 1992b; Bresters *et al.*, 1993; Hallam *et al.*, 1993). In addition, several studies of haemophiliac men, all of whom were anti-HCV positive, and their female partners showed a low risk of sexual transmission (Schulman and Grillner, 1990; Widell *et al.*, 1991; Brettler *et al.*, 1992; Hallam *et al.*, 1993). Further evidence to support the lack of sexual transmission comes from two reports in which HCV RNA was undetected in semen and vaginal secretions of

subjects positive for serum HCV RNA (Hsu *et al.*, 1991; Terada *et al.*, 1992). More extensive investigations need to be undertaken to determine whether sexual transmission actually occurs, although it appears that the risk is reduced compared with that for HIV and HBV.

1.11 HCV PATHOGENESIS

Currently, HCV infection of liver tissue is diagnosed on the basis of characteristic histological appearances. The histopathological features of NANBH have been described previously, but not confirmed following the introduction of diagnostic tests for HCV (Dienes *et al.*, 1982; Bianchi *et al.*, 1987). Several histological features have been observed in liver tissue of HCV chronic carriers; these include portal lymphoid aggregates, bile duct damage and loss, steatosis and moderate degrees of piecemeal and lobular necrosis (Bach *et al.*, 1992; Scheuer *et al.*, 1992). Hepatic fibrosis and regenerating nodules, characteristic of cirrhosis, develop in approximately 50% of HCV-positive patients (Bach *et al.*, 1992; Scheuer *et al.*, 1992). Although these features are not unique to HCV infection, they provide a useful diagnostic clue. Furthermore, it is unclear which of these histological changes represent a direct effect of HCV infection and which are a reflection of host response.

Ideally, RNA and protein detection within the liver would provide direct evidence for HCV infection. The relatively low HCV RNA copy number found in serum from HCV-positive individuals (see section 1.9.4), has led to the use of RT-PCR in initial studies to detect HCV RNA in liver tissue. By analogy with flavivirus replication (section 1.6), it seems likely that negative strand virus RNA will serve as a template for the synthesis of positive sense RNA. A number of studies describe the detection of positive, as well as negative, HCV RNA within infected liver tissue by RT-PCR (Fong *et al.*, 1991; Shibata *et al.*, 1991; Takehara *et al.*, 1992; Horiike *et al.*, 1993; Muller *et al.*, 1993a; Sherker *et al.*, 1993). More

recently, positive sense RNA has been detected (Bouffard *et al.*, 1992; Hsieh *et al.*, 1992; Takehara *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Young *et al.*, 1993; Muratori *et al.*, 1994), along with the negative strand (Bouffard *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Muratori *et al.*, 1994), in peripheral blood mononuclear cells (PBMC), suggesting that these cells are sites of viral replication during the natural course of infection, and possibly represent a reservoir of HCV virions. Moreover, patients without detectable serum HCV RNA had HCV RNA within PBMC (Bouffard *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Young *et al.*, 1993; Muratori *et al.*, 1994). Levels of negative strand HCV RNA in the liver and PBMC were approximately 1 to 100 fold less than that of the positive strand RNA (Fong *et al.*, 1991; Wang *et al.*, 1992a; Horiike *et al.*, 1993). Furthermore, the negative strand RNA has also been identified in the plasma of chronic carriers, suggesting encapsidation during virus assembly or the possibility that negative strands are present within virions in the form of RI (Fong *et al.*, 1991; Zignego *et al.*, 1992; Sherker *et al.*, 1993). The latter finding is in contrast to other reports (Bouffard *et al.*, 1992; Takehara *et al.*, 1992; Wang *et al.*, 1992a; Muller *et al.*, 1993a) and to those related to flaviviruses in which negative strand RNA is not found in mature virions (Westaway, 1987). These conflicting results may be due to the specificity of the conventional RT-PCR assay. Studies from Willems *et al*, (1993) and McGuinness *et al*, (1994) showed that 'positive' and 'negative' RNA could be detected by RT-PCR even if the primers were omitted from the RT reaction. Similarly, Gunji *et al*, (1994) failed to distinguish the two RNA strands during the conventional RT-PCR assay, providing evidence for random priming during the RT step. Chemical modification of RNA samples at the 3' end followed by strand specific RT-PCR made specific detection possible. Positive and negative strands were then detected in liver tissue and PBMC (Gunji *et al.*, 1994). Hence, work published prior to Gunji *et al*, (1994) and/or without the use of recognised strand specific RT-PCR assays may be incorrect [The remainder of this thesis continues to quote the work as published by the authors to save repeated

reference to Willems *et al*, (1993), Gunji *et al*, (1994) and McGuinness *et al*, (1994).] Although the detection of the negative strand in tissue samples is indicative of ongoing viral replication, it provides no information regarding the site of infection and/or replication of HCV.

Knowledge of HCV antigen and RNA expression in infected liver tissue from chronic carriers is accumulating. HCV RNA has been localised by *in situ* hybridisation (ISH) in the hepatocyte cytoplasm by various detection systems (Lamas *et al.*, 1992; Negro *et al.*, 1992; Yamada *et al.*, 1992; Haruna *et al.*, 1993; Nuovo *et al.*, 1993; Nouri Aria *et al.*, 1993; Tanaka *et al.*, 1993; Yamada *et al.*, 1993). HCV RNA has been detected in infiltrating mononuclear cells in infected liver tissue by several groups (Blight *et al.*, 1992; Lamas *et al.*, 1992; Nouri Aria *et al.*, 1993; Yamada *et al.*, 1993). Details of these experiments will be discussed in Chapter 6. The proportion of hepatocytes positive for HCV RNA ranged from 1-5% in human liver tissue (Nouri Aria *et al.*, 1993) to 100% of hepatocytes of infected chimpanzees (Negro *et al.*, 1992) and humans (Nuovo *et al.*, 1993). Furthermore, the negative sense RNA strand has also been detected in hepatocytes by ISH (Lamas *et al.*, 1992; Yamada *et al.*, 1992; Nouri Aria *et al.*, 1993; Yamada *et al.*, 1993).

A number of studies report the detection of HCV antigens in fixed and frozen liver tissue using human antibody (Infantolino *et al.*, 1990; Krawczynski *et al.*, 1992), murine monoclonal antibody (Hiramatsu *et al.*, 1992; Sansonno and Dammacco, 1993; Yamada *et al.*, 1993; Hiramatsu *et al.*, 1994; Yap *et al.*, 1994) or polyclonal rabbit antibody (Infantolino *et al.*, 1990; Blight *et al.*, 1993a, 1993b, 1994; Tsutsumi *et al.*, 1994). Results of similar studies form Chapters 3 and 4 of this thesis. The evidence generated to date from ISH and immunohistochemistry studies are consistent with cytoplasmic replication of HCV.

1.12 HCV AND ITS ASSOCIATION WITH HEPATOCELLULAR CARCINOMA

Previous epidemiological studies have demonstrated a significant association of the HBV carrier state with the development of hepatocellular carcinoma (HCC) (Szmuness, 1978). However, many patients with HCC are serologically negative for hepatitis B surface antigen (HBsAg) particularly in Japan. A high frequency (58-94%) of HBsAg-negative patients with chronic hepatitis were found to be anti-HCV positive in Japan (Kiyosawa *et al.*, 1990; Saito *et al.*, 1990; Nishioka *et al.*, 1991a,b; Watanabe *et al.*, 1991; Hamasaki *et al.*, 1993). Thus, it has been postulated that HCV may play a role in the development of HCC and recent studies indicate a strong association between anti-HCV positivity and the development of HCC worldwide. The prevalence of anti-HCV in HBV-negative patients with HCC was 75% in Spain (Bruix *et al.*, 1989), 65-78% in Italy (Colombo *et al.*, 1989; Simonetti *et al.*, 1989; Sbolli *et al.*, 1990), 53% in the USA (Hasan *et al.*, 1990) and 67% in Taiwan (Tsai *et al.*, 1994). These serological findings have been confirmed by the detection of the HCV RNA genome by RT-PCR in human non-HBV, HCC tumour tissue homogenates (Yoneyama *et al.*, 1990; Gerber *et al.*, 1992; Horiike *et al.*, 1993; Sherker *et al.*, 1993; Sullivan and Gerber, 1994). In addition, negative sense HCV RNA has been detected by RT-PCR in cancerous and non-cancerous liver tissue (Gerber *et al.*, 1992; Horiike *et al.*, 1993; Sherker *et al.*, 1993).

Although long term HCV infection is associated with the development of HCC, it is possible that establishment of HCC is enhanced if the patient is a HBV-carrier. Evidence to support this has come from studies conducted in Italy and Greece where the association between anti-HCV and HCC was substantially higher among subjects positive for HBsAg than among those who were negative for HBsAg (54% vs 19%; Colombo *et al.*, 1989; Kaklamani *et al.*, 1991). Hence, these results indicate that previous estimates of the association between HCV and HCC may be inflated due to the interacting role of HBV in the development of

HCC. Nevertheless, the data suggests a slow, sequential progression from acute HCV through chronic hepatitis and hepatic cirrhosis to HCC and supports a role for HCV in the development of HCC.

1.13 AIMS OF THESIS

The overall aim of this thesis was to examine the markers of HCV replication in infected liver samples. At the commencement of this thesis the mechanism(s) by which HCV causes hepatic disease was ill-defined. Ultrastructural changes (Jackson *et al.*, 1979; Shimizu *et al.*, 1979; Tsiquaye *et al.*, 1980) accompanied by the appearance of a cytoplasmic antigen (described in detail in section 1.2) have been detected in hepatocytes in HCV-infected liver samples (Shimizu *et al.*, 1985, 1986; Maeda *et al.*, 1989), but these represent indirect markers of HCV infection and viral replication. Hence, it became apparent that the detection of HCV proteins and RNA was required to positively identify HCV-infected cells and to investigate mechanisms of pathogenesis. In addition, details of the replication cycle of HCV are still unknown, although by analogy with the Flaviviruses, it is likely that RNA replication occurs through a double-stranded RF (section 1.6).

The specific aims of this thesis were to employ the techniques of immunohistochemistry and ISH to study HCV antigen and RNA expression in liver tissue, respectively. Furthermore, the development of these techniques to detect HCV-specific markers would allow an investigation into the relationship between virus replication, virus antigen expression and liver histology. A second facet of this study was to examine RNA extracts from HCV-infected liver samples for evidence of viral RNA RF.

CHAPTER 2

MATERIALS AND METHODS

2.1 SUBCLONING OF HCV cDNA SEQUENCES

2.1.1 Vectors and plasmids

A 324bp cDNA representing the 5' UTR was cloned and sequenced by Dr. R Trowbridge in the laboratory (Table 2.1). HCV cDNA corresponding to nucleotide positions 223 to 1876, 4652 to 5275 and 8791 to 9258 was a generous gift from Dr. T Miyamura, Tokyo National Institute of Health, Japan (Takeuchi *et al.*, 1990b) and represent the capsid + envelope region, and portions of NS3 and NS5, respectively (Table 2.1). Recombinant plasmids containing regions E1-NS2 (nt 1349 to 2913), NS2-NS3 (nt 2913-4478) and NS4 (nt 4922-6444) were generous gifts from Dr. K Fukai, Osaka University, Japan (Takamizawa *et al.*, 1991) (Table 2.1). Plasmid pBluescript 11KS (Stratagene) was chosen as the vector to subclone the HCV cDNA sequences as it contains multiple unique restriction enzyme sites flanked by T3- and T7-RNA polymerase promoters, allowing subsequent production of strand specific RNA probes (section 2.5.3). The presence of an ampicillin resistance gene within the plasmid permits plasmid selection within the cloning protocol. Probes prepared from this plasmid were used in experiments described in Chapter 6.

2.1.2 Phenol extraction and ethanol precipitation of nucleic acids

Crystallised phenol (ICN) was melted at 65°C then 8-hydroxyquinoline (Aldrich) added to a final concentration of 0.1%. The melted phenol was extracted 3x in 1M Tris-HCl pH 8.0 followed by repeated washes in 0.1M Tris-HCl pH 8.0 until

Table 2.1 Location and size of HCV cDNA cloned into pBluescript.

HCV region	nt position	Size (bp)
5' UTR	1-324	324
C, E1	223-1876	1653
E1, E2/NS1, NS2	1349-2913	1564
NS2, NS3	2913-4478	1565
NS3	4652-5275	623
NS4	4922-6444	1522
NS5	8791-9258	467

the pH of the aqueous phase was ≥7.6. Equilibrated phenol was stored under 0.1M Tris-HCl pH 8.0 at 4°C until required.

DNA and RNA solutions were extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:49:1). After centrifugation at 10000g for 5min at rt, the upper aqueous phase, containing the DNA or RNA, was removed and transferred to a clean sterile tube and the nucleic acid precipitated by the addition of 1/10th volume of 3M NaOAc pH 5.2 and either 2.5 volumes of absolute ethanol or 1.5 volumes of isopropanol at -20°C for 2-18h. The pellet was washed 3x in 70% ethanol, freeze-dried and finally redissolved in the required volume of DDW.

2.1.3 DNA modifying enzymes

(i) Restriction enzymes

1-2μg of recombinant plasmid was incubated with 1-5 units of the appropriate restriction enzyme (RE) (Pharmacia, Boehringer, Amersham) in 10mM Tris-acetate pH 7.5, 10mM magnesium acetate and 50mM potassium acetate. Digestions were performed at 37°C for at least 3h. The samples were then analysed by agarose gel electrophoresis (section 2.1.4) to identify the RE digestion pattern of the plasmid and confirm that the reaction was complete.

(ii) Dephosphorylation

To prevent self ligation, end terminal phosphate groups were removed from RE-digested vector DNA by incubation (37°C, 30min) in a reaction mixture containing 10mM Tris-acetate pH 7.5, 10mM magnesium acetate, 50mM potassium acetate and 0.1 units of alkaline phosphatase (Pharmacia) per μg DNA. At the completion of the reaction, 100μg/ml of proteinase K (Boehringer), 5mM EDTA, 0.5% SDS was added, the preparation was incubated at 56°C for 30min, then the DNA was phenol extracted and ethanol precipitated.

(iii) Blunt ending

3' overhangs, created by RE digestion, were end filled in a reaction containing 0.1mM of each dNTP, 5mM $MgCl_2$, 10mM Tris-acetate pH 7.5, 10mM magnesium acetate, 50mM potassium acetate, 300-500ng of DNA and 1-2 units of T4 DNA polymerase (Promega) per μg DNA at 12°C for 15min. At the completion of the reaction, the enzyme was inactivated by heating at 75°C for 10min prior to phenol extraction and ethanol precipitation.

(iv) Phosphorylation

Terminal 5' phosphates were added to blunt-ended DNA (100-500ng) in a reaction mix containing 10mM Tris-acetate pH 7.5, 10mM magnesium acetate, 50mM potassium acetate, 1mM ATP and 1 unit of polynucleotide kinase (Pharmacia) per μg DNA. The reaction was incubated for 1h at 37°C, the enzyme inactivated at 65°C for 5min and the DNA phenol extracted and ethanol precipitated.

(v) Ligation

Vector and insert DNA were ligated at 12°C for 16h in a mixture containing 1 unit of bacteriophage T4 DNA ligase (Boehringer) per μg DNA, 50mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 10mM DTT, 1mM ATP and 0.5mg/ml BSA (Boehringer). A total of 100-500ng of DNA, using vector: insert molar ratios of 1:1 or 1:3, was added to the reaction and an aliquot of the product examined by agarose gel electrophoresis (section 2.1.4) to determine if ligation was successful. The preparation was then transformed into competent *E. coli* (sections 2.1.6 and 2.1.7).

2.1.4 Agarose gel electrophoresis

DNA was analysed in 1-2% agarose gels. Agarose powder (ICN) in 1xTAE buffer (40mM Tris-HCl pH 8.0, 40mM acetic acid, 1mM EDTA pH 8.0) was heated in a

microwave oven, until the agarose was dissolved. The mixture was then cooled to 50°C, poured into gel trays, and a 1-2mm comb inserted, taking care to exclude air bubbles. The gel was allowed to set (15-20min) at rt then submerged in 1xTAE running buffer, containing 100ng/ml ethidium bromide (Sigma), in an electrophoresis tank. The comb was removed from the gel allowing the wells to fill with buffer.

DNA samples (250ng-1μg) were prepared by the addition of 1/6th volume of 6x loading buffer [final concentrations: 5% glycerol, 0.05% BPB (Sigma) and 0.05% XC (Sigma)] and the final volume of 10-20μl added to the wells of the gel. 100-150 volts was applied to the gel until the BPB dye marker migrated to within 1-3cm of the bottom. DNA was visualised in the gel by UV illumination and photographed on polaroid type 667 film. DNA sizes were determined by comparison with the bands generated from λ DNA (Promega) cut with the RE Pst I (Appendix).

2.1.5 Isolation of DNA fragments from agarose gels

DNA was recovered from 1% low melting point agarose gels by excising the slice of agarose gel, containing the required DNA fragment (determined as described in section 2.1.4), with a sterile scalpel blade. The gel slice was transferred to a punctured sterile Eppendorf microfuge tube plugged with 2-3mm of siliconised glass wool. This tube was placed on top of an open Eppendorf tube in a two-tiered system and centrifuged for 10min at 6000rpm. The eluate, containing the DNA fragment, was collected at the bottom of the second tube. The DNA was further purified by phenol extraction and ethanol precipitation, and the DNA pellet redissolved in 10μl DDW. The concentration of the DNA fragment was determined by gel electrophoresis by comparison with known concentrations of Pst I-digested λ DNA.

2.1.6 Preparation of competent cells

A single colony from a 2YT agar plate (Appendix), containing an overnight culture of rec A^- *E. coli* DH5-α (supplied by Prof. P Manning, University of Adelaide, Adelaide), was transferred to 30ml of 2YT broth (Appendix) and incubated with shaking at 37°C for approximately 4h (until OD_{600} = 0.3-0.4). The culture was then centrifuged (2500g, 10min, 4°C) and the pellet resuspended in 10ml of cold 100mM $CaCl_2$ in DDW. The cells were incubated on ice for 20min, repelleted under the same conditions and resuspended in 2ml of cold 100mM $CaCl_2$. For optimal transformation efficiency, competent cells were left at 4°C for 16h before use.

2.1.7 Transformation of competent cells

5-500ng of recombinant plasmid DNA, in a volume of <20μl, was mixed gently with 200μl of competent DH5-α cells and incubated on ice for 30min. The cells were then heat shocked for 2min at 42°C, returned to ice for 2min, 1ml of 2YT broth (without antibiotics) added and incubated for a further 60min at 37°C with constant shaking. The cells were spread on 2YT agar plates containing 50μg/ml ampicillin (Sigma) and incubated inverted at 37°C for 24h or until antibiotic-resistant colonies developed.

2.1.8 Mini preparation of plasmid DNA

Individual bacterial colonies were grown overnight in 3ml of 2YT broth, containing 50μg/ml ampicillin, at 37°C with agitation. 1.5ml of the confluent culture was then pelleted at 13500rpm for 1min at rt in an Eppendorf tube. The supernatant was decanted, the cell pellet resuspended in 350μl of lysis buffer [0.1M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA and 5% Triton X-100], 25μl of 10mg/ml lysozyme (Boehringer) added and boiled for 40sec. Cell debris and chromosomal DNA was

removed by centrifugation at 13500rpm for 10min and the plasmid DNA was ethanol precipitated. The mixture was centrifuged and the pellet washed 3x in 70% ethanol. The plasmid DNA was dried, redissolved in 25μl DDW containing 0.5mg/ml preboiled RNase A (Boehringer), the identity confirmed by RE analysis and stored at -20°C.

2.1.9 Large scale plasmid purification from *E.coli*

The method used was described by Holmes and Quigley (1981). Individual transformed DH5-α colonies were grown overnight at 37°C with shaking in 20ml of 2YT containing 50μg/ml ampicillin. The 20ml culture was added to two bottles containing 250ml of superbroth (Appendix) and 50μg/ml ampicillin, and incubated with shaking at 37°C for 5h (until OD_{600} = 0.9-1.0). Chloramphenicol (Parke Davis) was then added to a final concentration of 160μg/ml and the incubation continued overnight.

The cells were pelleted by centrifugation (7000g, 15min, 4°C) and resuspended in 100ml STET (5% Triton X-100, 50mM EDTA, 50mM Tris-HCl pH 8.0, 50mM sucrose), transferred to a 250ml flask, then 50mg lysozyme added. This mixture was heated in a boiling water bath for 5min with constant swirling. The solution of lysed cells was transferred to a 250ml polypropylene centrifuge tube and held on ice for 15min. Following centrifugation (20000g, 15min, 4°C), the supernatant was transferred to a clean 250ml polypropylene centrifuge tube, an equal volume of cold (-70°C) isopropanol added, mixed thoroughly and the precipitate collected immediately by centrifugation at 20000g for 30min at -5°C. The pellet was redissolved in 17ml TE8 by swirling for 5-10min at rt, then transferred to a 50ml polypropylene centrifuge tube, containing 17.9g CsCl dissolved in 0.7ml of 10mg/ml ethidium bromide in DDW. The mixture was then centrifuged at 16500g for 20min at 20°C, the upper denatured protein layer removed, and the remainder of the solution collected, loaded into two Beckman 13.5ml heat seal tubes and

balanced with a 1.05g/ml CsCl solution. The preparation was centrifuged in a 70.1 Ti rotor in an ultracentrifuge (175000g, 24h, 20°C) to band the plasmid DNA.

The CsCl gradient was examined under UV light and the band corresponding to the supercoiled plasmid DNA (the lowest band on the gradient) was collected through the side of the tube using a 19 gauge needle attached to a 5ml syringe. Ethidium bromide was removed by 3-4 extractions with water-saturated butanol and the DNA then ethanol precipitated. The DNA was then redissolved in 1ml TE8, transferred to a 10ml polypropylene tube and reprecipitated. The DNA pellet was washed 3x in 70% ethanol, redissolved in 0.5ml TE8, the process then repeated and the DNA finally redissolved in 0.5ml TE8. The DNA concentration was determined by OD at 260nm (where 1 OD_{260} unit = 50μg/ml), diluted to the working concentration and stored at -20°C.

2.2 IMMUNOHISTOCHEMISTRY

2.2.1 Tissue preparation

Liver biopsy samples, collected with a Tru-cut needle, were embedded in OCT compound (Miles Diagnostic Division), snap frozen in liquid nitrogen, and stored at -70°C until use. In addition, a portion of the liver samples was fixed in 10% buffered formalin for 12-18h at rt, then processed routinely, and embedded in paraffin wax. 5μm frozen sections were mounted on 3-aminopropyltriethoxysilane (APES)-coated glass slides (Appendix), and air-dried for 30-60min. The sections were then fixed by immersion in either: (i) acetone or chloroform for 5min at 4°C followed by air drying for a further 30min; or (ii) carbon tetrachloride or Methyl Carnoys (methanol:acetic acid = 3:1) for 10min at 4°C followed by air drying; or (iii) 1% formalin in PBS at 4°C for 5min. All sections were rinsed 2x 5min in PBS prior to immunostaining.

2.2.2 Primary and secondary antibodies for antigen detection

Antibodies were titrated on HCV-positive and -negative liver tissue to determine the dilution that produced the optimal signal to background ratio.

(i) HCV protein detection

Primary: Rabbit polyclonal antibodies (a gift from Dr. R Lesniewski, Abbott Laboratories, Chicago, USA) were raised to synthetic peptides deduced from the nucleotide sequence of an American HCV isolate (Choo *et al.*, 1991); C (aa 1-75), E2/NS1 (aa 380-436), NS3 (aa 1192-1240), NS4 (aa 1694-1735) and NS5 (2302-2352) (Table 2.2). All antibodies were strongly reactive with their specific peptide antigen by enzyme immunoassay (EIA) (Table 2.2), and prior to use they were adsorbed against NHL homogenate (sections 2.2.3 and 2.2.4) and diluted 1:20 in 5% BSA.

Secondary: Fluorescein isothiocyanate (FITC)-conjugated sheep-anti-rabbit IgG (Wellcome) or Rhodamine-conjugated goat-anti-rabbit IgG (Boehringer), both adsorbed against NHL and diluted 1:40 in 5% BSA.

(ii) HBcAg detection

Primary: Polyclonal rabbit-anti-HBcAg (Dako) adsorbed against NHL and diluted 1:100 in PBS.

Secondary: As above.

(iii) CD marker detection

Primary: Mouse monoclonal anti-CD4, -CD8, -CD20 (Dako) diluted in PBS (1:15) and monoclonal anti-CD14 (a gift from Dr. I Beckman, Flinders Medical Centre, Adelaide).

Secondary: FITC-conjugated sheep-anti-mouse IgG (Silenus) adsorbed against NHL and diluted 1:40 in PBS.

Table 2.2 Location of the peptides relative to the HCV polyprotein.

Peptide location (aa)	Antigen target	EIA titre
1-75	C	1:800 K
380-436	E2/NS1	1:400 K
1192-1240	NS3	1:400 K
1694-1735	NS4	1:2 M
2302-2352	NS5	1:1 M

aa = amino acid
EIA = enzyme immunoassay
K = 10^3
M = 10^6

(iv) Double-stranded RNA detection

Primary: Rabbit polyclonal antibody to dsRNA (a gift from Dr. R Francki, University of Adelaide, Adelaide) adsorbed against NHL and diluted 1:40 in PBS.

Secondary: As described in sections (i) and (ii) above.

2.2.3 Preparation of NHL homogenate

An autopsy liver from a patient serologically negative for all markers of HCV, HBV and HDV, that was stored frozen at -70°C, was thawed and minced through a sieve. The homogenised liver was collected (10000g, 5min, 4°C) and washed 3x in sterile ice-cold PBS, then resuspended in sterile PBS containing 0.2% sodium azide and stored at -20°C until use.

2.2.4 Adsorption of antibodies against NHL homogenate

All antibodies, except those to the CD markers, were adsorbed against a NHL homogenate to eliminate cross reactions with normal liver constituents. The NHL homogenate was thawed, washed in ice-cold PBS containing 0.08% sodium azide and pelleted at 10000g for 5min at 4°C. An equal volume of diluted antibody solution was mixed well with the homogenate and incubated at 37°C for 1h, followed by 4°C overnight. The mixture was then centrifuged at 10000g at 4°C for 20min, and the supernatant, containing the antibody, was filter sterilised through a 0.2µm membrane prior to titration on liver sections to determine the optimum working strength. The adsorbed antibody was aliquoted and stored at -20°C.

2.2.5 Indirect immunofluorescence

Conjugated antibodies were centrifuged in a microfuge (13500rpm, 10min, rt) before use to remove FITC and rhodamine aggregates. The sections were first

incubated for 30min with a combination of NHS and NSS (each diluted 1:5 in 5% BSA) at 37°C in a humidity chamber. Excess serum was removed and the sections were then incubated with 30-50µl of the primary antibody for 1h at 37°C, followed by 4°C overnight. The sections were then washed 2x 5min in PBS at rt, and then 30-50µl of the adsorbed species-specific conjugated secondary antibody applied for a further 60min under the same conditions. The slides were washed as above, the sections mounted in 90% glycerol saline containing 50mM Tris-HCl pH 8.8 and viewed under a UV microscope. The sections were photographed using Kodak black and white 'Tri-X' film.

2.2.6 Immunoperoxidase staining

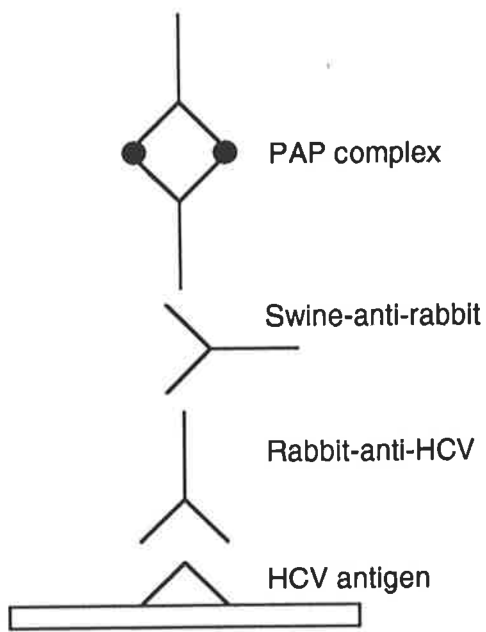
Sections of formalin-fixed, paraffin wax-embedded tissues were dewaxed in xylene for 2x 10min and rehydrated through descending ethanol concentrations to PBS. Endogenous peroxidase activity was destroyed by washing the sections for 15min at rt in 3% H_2O_2 in PBS, followed by washing for 2x 5min in PBS.

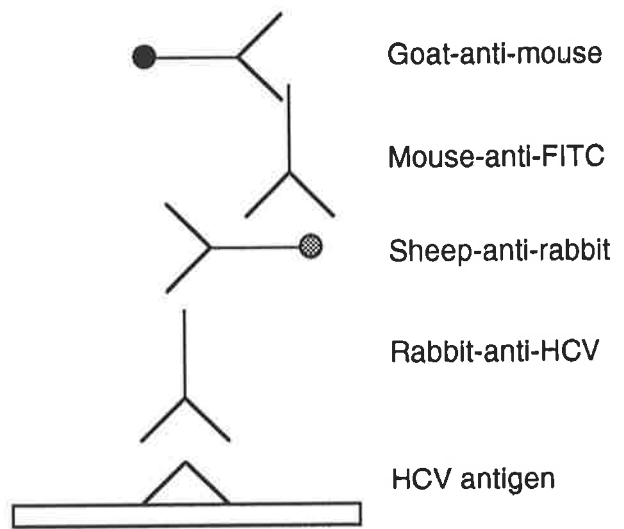
(i) 3 layer method

Dewaxed sections were incubated in normal swine serum (diluted 1:5 in PBS) at 37°C for 30min and then in the rabbit primary antibody at 37°C for 1h, followed by 4°C overnight. The sections were washed for 2x 5min in PBS at rt, incubated for 30min at rt in swine-anti-rabbit (dilution 1:50; Dako), followed by peroxidase-anti-peroxidase (PAP; dilution 1:200; Dako) for 1h at rt. Between antibody steps, the sections were washed for 2x 5min in PBS. The complex was visualised by the addition of 0.5mg/ml diaminobenzidine (DAB; Boehringer) in 100mM Tris-HCl pH 7.6, 0.03% H_2O_2 at rt for 6-10min, the sections counterstained with haematoxylin (Appendix), washed 2x 5min in PBS, dehydrated through ascending concentrations of ethanol, washed 2x 10min in xylene and finally mounted in DPX (Gurr). A schematic diagram of the reaction is shown in Figure 2.1.

Figure 2.1 A schematic representation of the steps involved in the 3 layer (A) and 4 layer (B) immunoperoxidase staining methods.

Shaded circle represents the FITC-conjugate and the horse radish peroxidase is depicted by the solid circle.


PAP complex
Swine-anti-rabbit
Rabbit-anti-HCV
HCV antigen
A


Goat-anti-mouse
Mouse-anti-FITC
Sheep-anti-rabbit
Rabbit-anti-HCV
HCV antigen
B

(ii) 4 layer method

Dewaxed sections were blocked with a combination of NHS and NSS (each diluted 1:5 in 5% BSA), and then incubated with the rabbit primary antibody for 1h at 37°C, followed by 4°C overnight. This was followed in turn by FITC-conjugated sheep-anti-rabbit IgG, mouse anti-FITC (dilution 1:120; Boehringer) and horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG (dilution 1:200; Dako) (Figure 2.1) and each incubated for 1h. The immunostaining procedure was performed at 37°C in a humidity chamber and the sections were rinsed in PBS for 2x 5min between each antibody layer. The complex was visualised and counterstained as described for the 3 layer method above. The sections were photographed using colour Kodak 'Ektachrome' 64 film.

2.3 PREPARATION OF VIRAL RNA

2.3.1 Extraction of RNA

The RNA extraction method was modified from that described by Chomczynski and Sacchi (1987). RNA was extracted from tissue and serum as follows:

(i) Liver tissue

Approximately 0.5mg of human liver tissue was homogenised in a Dounce homogeniser in 8ml guanidine thiocyanate buffer (GIT: 4M guanidine thiocyanate (IBI), 25mM Na_3citrate pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol), and incubated on ice for 5min. After homogenisation, 0.8ml of 3M NaOAc pH 5.2, 8ml of phenol, 1.6ml of chloroform:isoamylalcohol (49:1) were added sequentially, with thorough mixing after the addition of each reagent, and incubated on ice for 10min. The mixture was centrifuged (10000g, 5min, rt), the aqueous phase removed and the RNA then ethanol precipitated. The sample was redissolved in 500μl of RNase-free DDW.

(ii) Serum

RNA was extracted from 200µl of human serum as described above without homogenisation, except that the solution volumes were reduced by 13-fold. The final RNA pellet was dissolved in 11µl of RNase-free DDW.

2.3.2 RNase A digestion

The sensitivity of HCV RNA to RNase A was determined by the addition of RNase A to 0 (control) or 50µg/ml in DDW or high salt (0.75M NaCl). After incubation for 5min at 37°C or 100°C, RNA samples were phenol extracted and ethanol precipitated.

2.3.3 Lithium chloride fractionation

Total liver RNA was fractionated in 2M LiCl by a modification of the method published by Spector and Baltimore (1975). Liver RNA was mixed with total cellular RNA, extracted from a human hepatoma-derived cell line (HepG2) and 10% sarcosyl, 300mM EDTA pH 7.4 and 10M LiCl to yield final concentrations of 1mg/ml, 1%, 10mM and 2M, respectively. After 16h at -20°C, the RNA was centrifuged at 15000g for 30min at 4°C. The LiCl supernatant (which contains dsRNA) was collected and ethanol precipitated. The LiCl pellet, containing single-stranded and partially dsRNA, was dissolved in DDW and also ethanol precipitated.

2.3.4 Chemical modification of RNA samples

Chemical modification of RNA samples at the 3' end was primarily performed by periodate oxidation followed by reduction with $NaBH_4$. Following denaturation of RNA samples at 95°C for 5min, 200µl of 50mM NaOAc pH 5.2 and 50µl of 20mM $NaIO_4$ were added and incubated at 30°C for 12h. The reaction was stopped by

the addition of 60μl of 10% ethylene glycol and the RNA was ethanol precipitated. The RNA was redissolved in 300μl of RNase-free DDW and incubated with 100μl of 0.1M $NaBH_4$, dissolved in 0.05M NaOH, on ice for 1h. The reaction was stopped by the addition of 20μl of ice-cold acetic acid, followed by ethanol precipitation.

2.4 AMPLIFICATION OF HCV RNA BY PCR

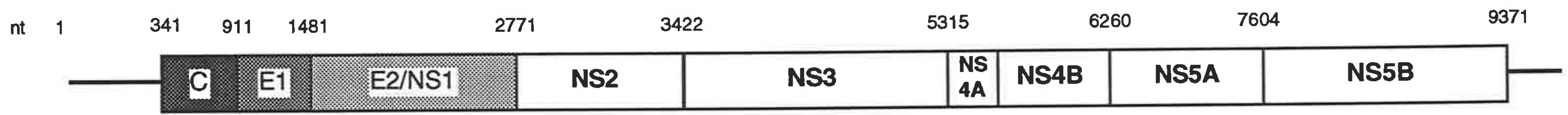
2.4.1 Synthesis of HCV cDNA

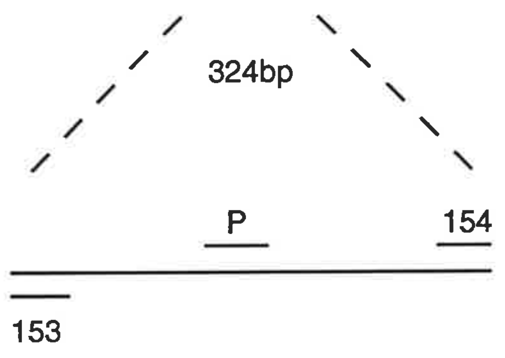
HCV-specific cDNA was generated by RNase H-negative Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (RTase; BRL) using either a sense or antisense primer. The sense and antisense primers annealed to position 1-20 and 304-324 in the 5' UTR, respectively (Okamoto *et al.*, 1990c; Figure 2.2). After denaturation of RNA samples at 94°C for 5min, cDNA was synthesised in 20μl containing 11μl of RNA solution [from serum or liver (section 2.3)], 50mM Tris-HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 0.5mM of each dNTP, 10pmol of either sense or antisense primer and 50 units of MMLV RTase and overlaid with mineral oil (Sigma). After reverse transcription (37°C for 40min), the reaction was heated to 100°C for 30min to destroy RTase activity, chilled on ice, and then incubated with 10μg of RNase A at 37°C for 20min, 95°C for 30min, followed by 37°C for 15min to degrade residual RNA. The tubes were then placed on ice until the PCR was performed. The RNase digestion step prior to PCR was only performed in experiments related to strand specificity described in Chapter 7.

2.4.2 Amplification of reverse transcribed cDNA

The cDNA generated by RT was amplified by PCR in a final volume of 50μl containing 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 10pmol of each

Figure 2.2 Position of oligonucleotide primers used in RT-PCR.





154 (-) (324-304) 5' GGTGCACGGTCTACGAGACC 3'

153 (+) (1-20) 5' GGCGACACTCCACCATAGAT 3'

Probe(P) (133-159) 5' CAATTCCGGTGTACTCACGGTTCCGC 3'

antisense or sense primer and 1 unit of Taq polymerase (Perkin-Elmer). Amplification was performed by initially denaturing the samples at 94°C for 3min followed by 35 cycles of denaturation for 50sec at 94°C, annealing for 30sec at 55°C and extension for 40sec at 72°C. 10µl aliquots were analysed by gel electrophoresis (section 2.1.4) and a positive reaction noted by a 324bp product. The specificity was confirmed by Southern blot hybridisation (section 2.6) using a ^{32}P-end-labelled oligonucleotide (nt 133-159; Okamoto *et al.*, 1990c; Figure 2.2) internal to the PCR product.

2.5 PREPARATION OF RADIOLABELLED PROBES

2.5.1 Efficiency of radiolabelling reactions

The efficiency of all radiolabelling reactions was determined by measuring the incorporation of radiolabelled nucleotides into nucleic acid by TCA precipitation. This was carried out by spotting 1µl samples of the reaction mix onto two pieces of Whatman 542 filter paper. One filter was washed for 10min in ice-cold 10% TCA in DDW, then dehydrated by successive washes in 100% ethanol, 50% ethanol/ether and 100% ether. The second filter was allowed to air dry for 10min. Both filters were counted in a toluene-based liquid scintillant (Appendix) in a scintillation counter. The percentage incorporation of ^{32}P, ^{125}I or ^{33}P was determined by the ratio of counts in the TCA washed filter compared with the air-dried filter.

2.5.2 Random prime labelling of excised plasmid DNA

The random prime labelling method was adapted from Feinberg and Vogelstein (1983). 25ng of excised, gel purified DNA (section 2.1.5) was labelled in a 25µl reaction mixture containing 50mM Tris-HCl pH 8.0, 5mM $MgCl_2$, 200mM Hepes pH 6.6, 10mM 2-mercaptoethanol, 0.02mM of dGTP, dATP and dTTP, 25µCi of

α-^{32}P-dCTP (3000Ci/mmol; Bresatec), 160μg/ml of random hexanucleotides (Pharmacia) and 2 units of Klenow (Boehringer). The reaction was incubated at rt for 2-18h, then stopped by the addition of 1μl of 0.5M EDTA pH 8.0. DDW was added to 100μl and the TCA-precipitable counts monitored. The probe was ethanol precipitated to remove unincorporated nucleotides and washed 3x in 70% ethanol, freeze-dried and finally redissolved in 20μl DDW and stored at -20°C.

2.5.3 RNA probes and transcripts

^{32}P-, ^{125}I- and FITC-labelled RNA probes were prepared from HCV cDNA sequences inserted into the cloning vector pBluescript (section 2.1). The plasmid was linearised by digestion with a unique RE and the RNA transcribed using T3- or T7-RNA polymerase.

The reaction mixture, in a final volume of 20μl, comprised:

1x transcription buffer (40mM Tris-HCl pH 7.5, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl)

10mM DTT

1 unit/μl of RNasin (Promega)

0.5mM of each ATP, GTP and CTP or UTP (to use with ^{32}P-, FITC- or ^{125}I-labelled probes, respectively)

12μM of UTP or CTP

50μCi of α-[^{32}P]-UTP (3000Ci/mmol; Bresatec) or 50μCi of dried α-[^{125}I]-CTP (2000Ci/mmol; Amersham) or 250μM of FITC-12-UTP (Boehringer)

1μg of linearised template

30 units of T3- or T7-RNA polymerase (Promega)

The reaction mix was incubated at 37°C for 75min, 1 unit of RQ1 DNase (Promega) per μg DNA added and incubated for a further 20min. The reaction

was stopped by the addition of 1μl of 0.5M EDTA pH 8.0 and DDW added to 100μl. Prior to ethanol precipitation, the TCA-precipitable counts were monitored. Finally, the probes were redissolved in 50μl DDW at 65°C for 10min. For FITC-labelled RNA probes the specific activity was monitored by the incorporation of 5μCi of α-^{32}P-UTP. Synthesis of cold RNA transcripts was performed in a reaction volume of 100μl essentially as described above, except that the radioactive nucleotide was replaced with 0.5mM of UTP, and 2-3μg of linearised template and 40 units of the T3- or T7-RNA polymerase were used. RNA probes and transcripts prepared in this manner were analysed for length and specificity as described in sections 2.5.6 and 2.6, respectively.

2.5.4 End-labelling of synthetic oligonucleotides

150ng of the oligonucleotide was labelled at the 5' end in a 10μl reaction mixture comprising 10mM Tris-acetate pH 7.5, 10mM magnesium acetate, 50mM potassium acetate, 50μCi of γ-^{32}P-ATP (3000Ci/mmol; Bresatec), 10 units of polynucleotide kinase, and incubated at 37°C for 1h. At the completion of the reaction, the volume was adjusted to 100μl with DDW, TCA-precipitable counts measured and the probe ethanol precipitated.

2.5.5 Preparation of ^{32}P-labelled molecular weight markers

^{32}P-labelled Pst-I digested λ DNA markers were prepared in a 10μl reaction mix containing 10mM Tris-acetate pH 7.5, 10mM magnesium acetate, 50mM potassium acetate, 1mM of each dATP, dGTP and dTTP, 10μCi aqueous of α-^{32}P-dCTP, 125ng of λ/Pst I digested DNA and 2 units of Klenow. The reaction was incubated at 37°C for 15min, prior to denaturing agarose gel electrophoresis (section 2.5.6).

2.5.6 Size determination of ^{125}I-, ^{32}P- and FITC-labelled probes

The length of ^{125}I- and ^{32}P-labelled RNA probes was determined by denaturing agarose gel electrophoresis; agarose (1%) was dissolved in DDW by heating, cooled to 50°C, then 10xMOPS buffer (Appendix) and formaldehyde (37% solution; BDH) added to final concentrations of 1x and 2%, respectively. Samples, containing 4×10^4 cpm/μl of labelled RNA probes, were denatured by boiling for 5min in 9μl loading buffer [50% ultrapure deionised formamide (FSB), 1xMOPS buffer, 6.5% formaldehyde, 5% glycerol, 0.1% BPB], snap cooled and loaded onto the gel. Samples were electrophoresed at 80V for 2-3h in 1xMOPS buffer. Following electrophoresis, the gel was dried at 80°C under vacuum for 2h, wrapped in plastic film, exposed to X-ray film (X-Omat RP or AR, Kodak Australasia) at -70°C or rt in cassettes with intensifying screens, and developed in an X-ray processor after the required exposure time. The length of FITC-labelled RNA probes was determined by non-denaturing agarose gel electrophoresis as described in section 2.1.4.

2.6 NUCLEIC ACID HYBRIDISATION

2.6.1 Preparation of hybridisation membranes

(i) Southern blot hybridisation

After agarose gel electrophoresis, the gel was treated in 0.5M NaOH, 1.5M NaCl for 2x 20min with shaking, rinsed in DDW and then neutralised in several 20min changes of 0.5M Tris-HCl pH 7.4, 1.5M NaCl, 0.001M EDTA. The DNA was then transferred for 16h by capillary transfer with 20xSSC (1xSSC: 0.15M NaCl, 0.015M Na_3citrate pH 7.0) to a nylon membrane (Hybond N^+; Amersham) presoaked in 20xSSC. The membrane was then baked at 80°C for 2h.

(ii) RNA slot blot hybridisation

The nitrocellulose membrane (Hybond C-extra; Amersham) was presoaked in 2xSSC before assembly in the slot blot manifold (BRL). *In vitro* transcribed RNA (prepared as described in section 2.5.3) was denatured in a solution of 6xSSC, 7% formaldehyde for 15min at 65°C then quenched on ice. Serial dilutions of the RNA (10ng-10pg), in a final concentration of 6xSSC, were added to the wells and a vacuum applied. The wells were washed with 6xSSC, the vacuum released, the membrane rinsed in 2xSSC and finally baked under vacuum at 80°C for 2h.

(iii) Northern blot hybridisation

(a) Sample preparation

The Northern blot method was performed as described by Thomas (1980). 15μg of total liver RNA (extracted in section 2.3.1(i)) was denatured by incubation in 1M glyoxal, 50% DMSO, 1mM EDTA, 10mM sodium phosphate buffer pH 7.0 and 0.1% SDS at 50°C for 1h. The reaction mix was snap cooled and 2.5μl of dye mix, containing 0.1% BPB, 25% glycerol, 0.1% SDS and 1mM EDTA, was added.

(b) Electrophoresis and transfer

Samples were electrophoresed on horizontal 1% agarose gels in 10mM sodium phosphate buffer pH 7.0 at 25mA for 6-7h with constant buffer recirculation to maintain the pH at 7.0. Following electrophoresis, the gel was treated with 0.05M NaOH for 20min (to increase the transfer efficiency of larger RNA species), rinsed in DDW and soaked for 45min in 20xSSC. The nitrocellulose membrane was soaked in 2xSSC, then in 20xSSC and the transfer performed as described above (section 2.6.1(i)). Prior to hybridisation, the membrane was immersed in 500ml of 10mM Tris-HCl pH 8.0 at 100°C and incubated with shaking until the temperature was equilibrated to rt. This step removed the glyoxal from the membrane and allowed efficient hybridisation.

2.6.2 Hybridisation

(i) RNA and DNA probes

Prehybridisation and hybridisation of the nylon and nitrocellulose filters were performed in polythene bags for 6h and 16h respectively at 50°C in 50% deionised formamide, 0.2% each of PVP, BSA and Ficoll, 1% SDS, 10% dextran sulphate, 0.1% sodium pyrophosphate, 1M NaCl, 50mM Tris-HCl pH 7.6 and 100μg/ml denatured salmon sperm DNA (Boehringer). Following prehybridisation, ^{32}P-labelled probes (5-10x 10^6 cpm/ml) were boiled for 5min, snap cooled and added to the filters in the polythene bags. After hybridisation, the filter was washed 4x 5min in 2xSSC at rt, 2x 30min in 0.1xSSC/0.1% SDS at 60-75°C and finally 2x 5min in 0.1xSSC at rt. The filters were wrapped in plastic film and exposed to X-ray film as described in section 2.5.6.

(ii) Oligonucleotide probes

Prehybridisation and hybridisation using end-labelled oligonucleotide probes (1-5x 10^6 cpm/ml) were performed at 37°C for 4h and 16h respectively in a solution containing 6xSSC, 10% dextran sulphate, 50mM $NaPO_4$ pH 6.8, 0.2% each of PVP, BSA and Ficoll and 100μg/ml denatured salmon sperm DNA. After hybridisation, the membranes were washed 3x 5min in 6xSSC at rt, 2x 20min in 2xSSC/0.1% SDS at 55°C and 2x 5min in 2xSSC at rt. The nylon membranes were dried and exposed to X-ray film as described in section 2.5.6.

2.7 *IN SITU* HYBRIDISATION

2.7.1 Frozen liver sections and cells

For the detection of HCV RNA, frozen liver sections and cell smears (prepared as described in sections 2.7.3 and 2.7.4) were air-dried on APES-coated slides for at least 30min at rt, then fixed in 4% paraformaldehyde (TAAB laboratories) in

PBS containing 5mM $MgCl_2$ for 5min at rt. The slides were then washed 2x 5min in PBS, preceding and following a 10min acetylation step at rt in 0.25% acetic anhydride (BDH), 0.1M triethanolamine (BDH) pH 8.0. Finally, the sections and cells were dehydrated through ascending concentrations of ethanol and air-dried. Prior to acetylation, the cell smears were treated in 0.2% Triton X-100 in PBS for 5min to permit probe access during hybridisation.

2.7.2 Formalin-fixed, wax-embedded liver sections

Formalin-fixed, wax-embedded liver sections on APES-coated slides were dewaxed in xylene for 2x 10min, rehydrated through descending ethanol concentrations to PBS and then postfixed in 4% paraformaldehyde as described in section 2.7.1. The slides were then washed for 2x 5min in PBS before and after a 15min treatment in 50μg/ml proteinase K in 20mM Tris-HCl pH 7.4, 2mM $CaCl_2$ and 10mM EDTA. The sections were refixed for 5min in 4% paraformaldehyde, washed 2x 5min in PBS then acetylated and dehydrated as described in section 2.7.1.

2.7.3 Preparation of mononuclear cells from human peripheral blood

PBMC were prepared from heparinised blood using Lymphoprep (Nycomed). 9ml of Lymphoprep (specific gravity 1.077) was warmed to rt in a 50ml sterile centrifuge tube, 20ml of heparinised blood, diluted 1:2 in PBS, was overlaid and centrifuged at 500g for 20min at rt. The leukocyte layer was collected from the interface between the pellet (containing red blood cells and neutrophils) and the plasma. The PBMC were washed 2x in PBS, then resuspended in RPMI 1640 (Flow) supplemented with 10% non-inactivated foetal bovine serum (Cytosystems), 100units/ml penicillin and 40μg/ml gentamycin. The yield was approximately $2x10^6$ cells from 10ml of blood. At this stage, the B lymphocytes were separated from the T lymphocytes using a nylon wool column; the total cells

in 2.5ml were layered on the column which was equilibrated previously in RPMI 1640 at 37°C for 1h and incubated for 1h at 37°C in 5% CO_2 to allow the B cells to adhere to the nylon wool. T cells were eluted from the column with 10-12ml of RPMI 1640, collected, washed once in PBS and resuspended in 1ml RPMI 1640. The T lymphocyte-enriched and total PBMC populations were cultured overnight in 24 well plates (Costar) at 37°C in humidified 5% CO_2.

2.7.4 PBMC stimulation

B lymphocytes (contained in the total PBMC) were stimulated with 10μg/ml *E.coli* K12-derived LPS (supplied by Gary Penney, University of Adelaide, Adelaide) for 24h at 37°C in humidified 5% CO_2. T lymphocytes (contained in the total PBMC and T cell-enriched populations) were stimulated with 1μg/ml Con A (Pharmacia) for 24h. As negative controls, cells were cultured in medium alone. After stimulation, the lymphocytes were pelleted and resuspended in 200μl of RPMI 1640, then 20μl of the suspension smeared ($2x10^5$ cells/ml) onto APES-coated slides. The cells were air-dried and then fixed in 4% paraformaldehyde as described in section 2.7.1.

2.7.5 *In situ* hybridisation

The final hybridisation mixture contained 2xSSC, 50mM Tris-HCl pH 7.6, 10mM NaH_2PO_4, 10mM Na_2HPO_4, 10% dextran sulphate, 50% ultrapure deionised formamide, 0.02% of each Ficoll and PVP, 500μg/ml denatured salmon sperm DNA, 500μg/ml tRNA (Boehringer), 1.25mg/ml nuclease-free BSA (BRL), 20mM DTT and 1unit/μl of RNasin. The hybridisation mixture was assembled by boiling 2-5μl of the ^{32}P- and ^{125}I-labelled probe ($1x10^5$ cpm/section) or the FITC-labelled RNA probe (50-400ng/section) with 67μl of 1.5x FDST [1.5x FDST: 75% formamide, 15% dextran sulphate, 750μg/ml denatured salmon sperm DNA and 750μg/ml tRNA] for 5min. The mixture was quenched immediately on ice before

the addition of 1/5th volume of 5x hybridisation mix (10xSSC, 250mM Tris-HCl pH 7.6, 50mM NaH_2PO_4, 50mM Na_2HPO_4, 0.1% each of Ficoll and PVP, 2.5µl 800mM DTT, 2.5µl RNasin (40units/µl), 2.5µl nuclease-free BSA (50mg/ml) and finally DDW to 100µl. ^{125}I probe mixtures also contained 2mM KI.

2.5µl of the above probe mixture, was spotted onto the pretreated cells or sections and overlaid with a sterile 13mm diameter siliconised glass coverslip (Appendix), taking care to exclude air bubbles. Prior to the addition of the probe mix, the coverslip and slide were blown gently by a stream of air to remove any dust. The slides and coverslips were either immersed in paraffin oil or the coverslips sealed with rubber cement to prevent dehydration and incubated for 16-40h at 42°C for DNA probes or 50°C for RNA probes.

After hybridisation, the slides that were incubated under oil were removed and placed in slide racks to drain, washed for 2x 10min in chloroform at rt to remove all traces of oil, and then air-dried to evaporate all residual traces of chloroform. The rubber cement was gently peeled off from around the coverslips prior to post hybridisation washing.

2.7.6 Washing

The slides were transferred to a large volume of 2xSSC and washed at rt until the coverslips detached. The slides were then transferred to fresh 2xSSC for 2x 60min with stirring. Experiments performed using ^{125}I-labelled probes were washed as above, but the first 2xSSC wash contained 100µM KI to further reduce the background (Gowans *et al.*, 1989). A high stringency wash was carried out in 0.1xSSC for 2x 10min at 65°C and 75°C for DNA and RNA probes respectively, and then followed by 2x 60min washes in 0.1xSSC at rt. All washes were carried out with stirring. For radiolabelled probes, the samples were then dehydrated through ascending ethanol concentrations, air-dried and bound probe

detected by autoradiography, described below. For FITC-labelled probes, after the final post hybridisation wash, the sections were mounted in 90% glycerol saline containing 25mM Tris-HCl pH 8.8 and bound probes were detected by examination with a UV microscope.

2.7.7 Detection of bound radiolabelled probes

(i) Autoradiography

In the darkroom, an equal volume of K2 emulsion (Ilford) was mixed with 2% glycerol in DDW and melted at 45°C for 30min. The melted emulsion was mixed carefully to avoid the formation of air bubbles and poured into a chamber, into which the slides were dipped vertically. The slides were drained for a few seconds, the back of the slide wiped clear of emulsion and the resultant thin layer of emulsion was gelled immediately by placing the slide on an ice-cold flat metal plate for 10min. The slides were transferred to a level bench in complete darkness overnight at rt to ensure thorough drying of the emulsion. Finally, the slides were placed into light tight boxes containing silica gel and stored at 4°C for the duration of the exposure time.

(ii) Development and staining

After exposure, the slides were warmed to rt, then developed in D19 developer (Kodak) for 4min and fixed for 6min in Hypam rapid fixer (Ilford), diluted 1:4 in DDW. At this stage, the slides were removed from the darkroom and the fixer slowly replaced with cold running tap water and washed for 15min. The cells were counterstained in haematoxylin for 30sec, rinsed in 0.1xSSC, washed for 15min in 2xSSC, dehydrated through ascending concentrations of ethanol, and stained in eosin (Appendix) for 1min. The slides were washed in ethanol 2x 2min followed by 2x 10min washes in xylene, then mounted in DPX. The slides were viewed by light microscopy and photographed using black and white Kodak 'Pan-Tech' film.

CHAPTER 3

DETECTION OF HCV PROTEINS IN FROZEN LIVER TISSUE

3.1 INTRODUCTION

HCV causes persistent infection which often develops into severe liver disease and although the histological features have been described recently (Bàch *et al.*, 1992; Scheuer *et al.*, 1992), the mechanisms of pathogenesis are still unclear. Indirect markers of HCV infection, such as ultrastructural changes within the cytoplasm and the detection of an antigen associated with the microtubular aggregates, have been described (see section 1.2). However, the detection of the S and NS proteins of HCV are required to determine the cellular tropism of HCV in natural infection and may help to explain the pathogenesis of hepatocyte injury in chronic HCV infection. Consequently, this chapter describes the detection of a number of HCV-specific antigens in frozen liver sections from anti-HCV positive patients.

3.2 EXPERIMENTAL DESIGN

Liver biopsy specimens were collected from 12 anti-HCV positive patients from the Royal Adelaide Hospital, Adelaide. Patient 1 was a recipient of a bone marrow transplant from an anti-HCV positive donor and patients 2-12 were blood donors who were recreational intravenous drug users in the 1970s. Uninfected liver samples were also taken by biopsy or collected at autopsy within 6 to 24h after death. In addition, serum was collected from all patients and tested for HCV RNA by RT-PCR (section 2.4). Liver samples were either: (i) fixed in 10% buffered formalin, processed routinely and embedded in paraffin wax for

histological analysis; or (ii) embedded in OCT compound, snap frozen and stored at -70°C (section 2.2.1).

5μm cryostat sections of liver biopsy samples from patients 1-8 were air-dried and fixed in acetone (section 2.2.1) prior to immunostaining (section 2.2.5) to detect HCV antigens, C, E2/NS1, NS3, NS4 and NS5 (section 2.2.2). Subsequent experiments studied liver samples from patients 9-12 to compare the effect of fixation on the detection of HCV antigens. Frozen sections were fixed by immersion in either acetone or chloroform for 5min at 4°C, or carbon tetrachloride or Methyl Carnoys for 10min at 4°C and again air-dried (section 2.2.1). Alternatively, the sections were fixed in 1% or 10% buffered formalin at 4°C for 1, 2, 5 or 10min. All sections were rinsed in PBS prior to immunostaining (section 2.2.5).

Finally, simultaneous detection of HCV antigens and lymphocytic CD markers was performed on unfixed tissue sections from patients 11 and 12 to determine the HCV-infected cell population. The sections were first stained for the CD marker, using monoclonal antibodies to CD4, CD8, CD14 and CD20 (section 2.2.2), that were subsequently detected by FITC-conjugated sheep-anti-mouse IgG. Thereafter, the sections were stained with anti-E2/NS1 or -NS3 followed by rhodamine-conjugated goat-anti-rabbit IgG. In some experiments, sections were first stained for the HCV antigen followed by the CD marker.

3.3 RESULTS

3.3.1 PCR analysis of serum samples

HCV RNA in patients sera was detected by RT-PCR using primers from the 5' UTR (section 2.4; Figure 2.2). The products were analysed by agarose gel electrophoresis (section 2.1.4) followed by Southern blot hybridisation using a

^{32}P-end-labelled internal oligonucleotide (sections 2.5 and 2.6; Figure 2.2). All 12 patients were shown to be HCV RNA positive (Figure 3.1), whereas HCV RNA was not detected in an anti-HCV negative serum sample (Figure 3.1).

3.3.2 Histological analysis

Liver samples from patients 1-12 were examined by routine histology. The liver histology of sample 1 was indicative of acute phase hepatitis. Portal tracts were expanded and contained a mixed inflammatory infiltrate with moderate density. The histological appearance of samples 2-6 and 11 was consistent with low-grade CAH with mild lobular inflammation. The basic liver architecture in these samples was preserved and a mild to severe steatosis was present. Portal tracts were expanded by a mononuclear inflammatory cell infiltrate, consisting principally of lymphocytes. Samples 3, 5, 6 and 11 displayed hepatic fibrosis with focal piecemeal necrosis. A CPH pattern was observed in samples 7-10 with a dense lymphocytic infiltrate within the portal tracts. No evidence of steatosis was observed in these samples. The liver architecture in sample 12 was distorted and showed expansion of the portal tracts with mild lymphocytic infiltrates. Moderate steatosis was also observed. The appearance of this sample was consistent with mild CPH. This part of the study was performed by Dr. R Rowland, Institute of Medical and Veterinary Science, Adelaide.

3.3.3 Detection of HCV antigens in liver tissue

Acetone-fixed frozen sections from samples 1-8 were stained for the C, E2/NS1, NS3, NS4 and NS5 antigens of HCV, as described in section 2.2.2. The location of the targets of the anti-peptides is shown in Figure 3.2. Clear differences were noted in the ability of the different antibodies to detect HCV-specific antigens in the biopsy samples (Table 3.1). E2/NS1 and NS3 were detected as discrete foci (punctate staining) within the cytoplasm of scattered widespread hepatocytes

Figure 3.1 Southern blot hybridisation analysis of the 324bp RT-PCR products synthesised from HCV RNA extracted from human serum.

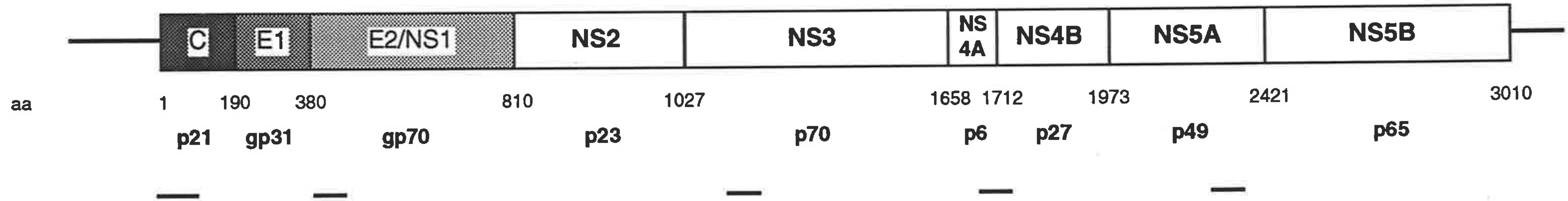
Lane 1-12 Products amplified from serum of patients 1-12, respectively.

Lane 13 Normal human serum analysed in the same manner as the samples in lanes 1-12.

1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3.2 The location of the targets of the anti-peptides on the HCV polyprotein.

(-) represents the peptide position



Structural
Non-Structural
C
E1
E2/NS1
NS2
NS3
NS 4A
NS4B
NS5A
NS5B
aa
1
190
380
810
1027
1658
1712
1973
2421
3010
p21
gp31
gp70
p23
p70
p6
p27
p49
p65

Table 3.1 Detection of HCV antigens in liver biopsy samples by immunofluorescence.

		Antigen				
Patient	Liver Histology	C	E2/NS1	NS3	NS4	NS5
1	ACUTE	-	+++	++++	+	+
2	CAH	-	+++	++++	+/-	+
3	CAH	-	+++	++++	+	+/-
4	CAH	-	+++	++++	+/-	+
5	CAH	-	+++	++++	+/-	++
6	CAH	-	+++	++++	+/-	+
7	CPH	-	+++	++++	+	+
8	CPH	-	+++	++++	+	+

The immunofluorescence intensity was quantitated + to ++++.
+ corresponds to a fluorescence signal just detectable, while ++++ represents an intense fluorescent staining.
CAH = chronic active hepatitis
CPH = chronic persistent hepatitis

(50-80%) in all 8 cases (Figure 3.3A). A weaker signal was observed for NS4 (Figure 3.3B) and NS5, and the C antigen was undetected (Table 3.1). Despite the different histological diagnoses, the distribution of HCV antigen-positive cells was similar in all the liver samples from the chronic carriers. However, the sample from the acute phase patient displayed a higher proportion of infected hepatocytes. The positive hepatocytes were randomly distributed within lobules and the nuclei were uniformly negative. Lymphoid aggregates, a characteristic feature of HCV infection, were also positive for the four HCV antigens detected in the hepatocytes (Figure 3.3C). The C antigen remained undetected even when an additional anti-C antibody (raised against aa 24 to 39; a gift from Dr. D Rowlands, Wellcome Research Laboratories, UK) was used, despite the fact that this antibody detected the C protein in rVV-infected cells (Dr. D Rowlands, personal communication).

3.3.4 Specificity of the indirect immunofluorescence assay for HCV antigen detection

The specificity of all four antibodies for the detection of HCV-specific antigens was established by the absence of staining in hepatocytes and lymphoid cells when the following controls were performed.

(i) Substitution of anti-HCV with pre-immune rabbit serum.

(ii) Immunostaining of liver tissue from uninfected or HBV-infected individuals (Figure 3.3D).

(iii) Immunostaining of lymphocytes in tonsil tissue from a HCV-negative individual (Figure 3.3E).

(iv) Preadsorption of the E2/NS1, NS4 and NS5 antisera with the specific synthetic peptide prior to immunostaining. This was consistent with a parallel loss of reactivity in EIA. In contrast, the immunofluorescence and EIA signals were retained following a mock adsorption of these antisera against the peptide diluent. For this final specificity control, the E2/NS1, NS4 and NS5 antisera were

Figure 3.3 The detection and distribution of HCV-specific antigens in frozen sections of liver samples by indirect immunofluorescence using antibodies raised against synthetic peptides.

A. Chronic phase biopsy sample (Case 3, Table 3.1). E2/NS1 antigen-positive hepatocytes. Note the prominent fluorescent foci or granules within the cytoplasm.

B. NS4 antigen-positive hepatocytes in chronic phase tissue (Case 3, Table 3.1).

C. Chronic phase patient (Case 4, Table 3.1). NS3 antigen-positive cells in inflammatory areas.

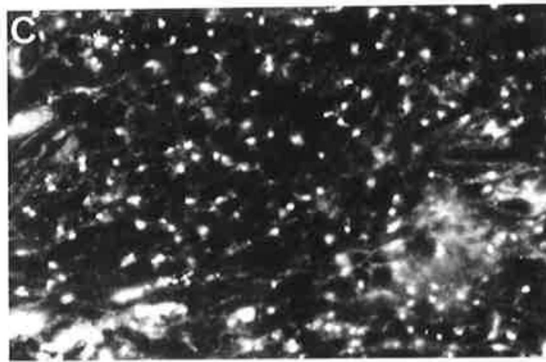
D. Uninfected liver tissue stained with anti-NS3.

E. Tonsil tissue from a HCV-negative individual stained with anti-E2/NS1.

Original magnification x400.

A

B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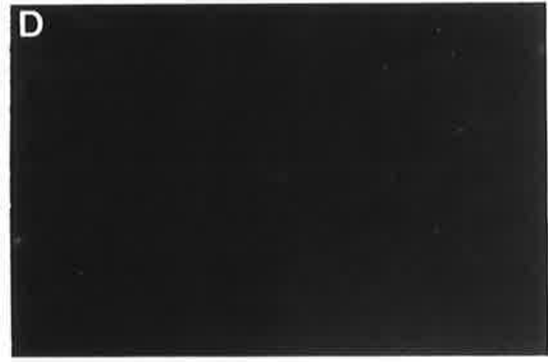
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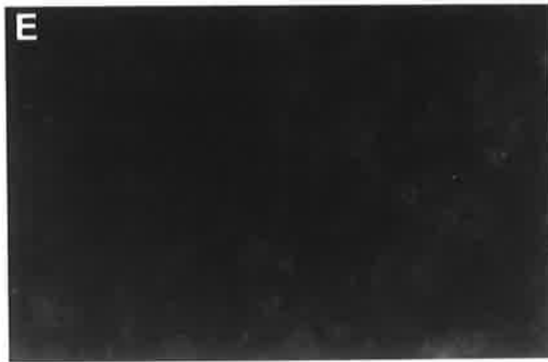

E

adsorbed against their respective synthetic peptide by Abbott Laboratories. The NS3 peptide was unavailable for adsorption of the specific antiserum.

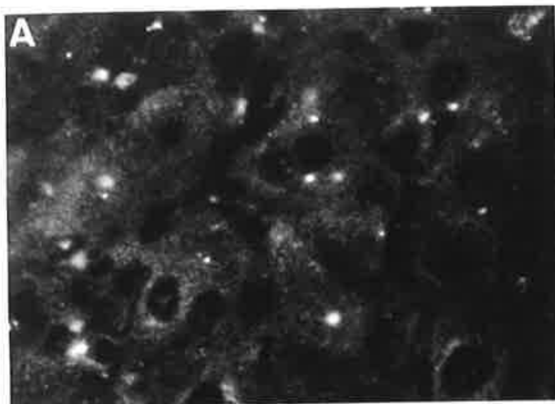
3.3.5 Fixation of sections for HCV antigen detection

The detection of viral antigens is often most successful in unfixed tissues, but to preserve the histological appearance, a fixation step is often introduced. The detection of HCV antigens described above (section 3.3.3) used acetone-fixed frozen sections. Acetone was chosen because it had been used successfully for viral antigen detection in liver tissues in previous studies in the laboratory. However, as the immunofluorescence staining intensity of NS4 and NS5 was weak, and the C antigen was undetected, it was decided to examine the role of different fixatives on the detection of the HCV antigens. Hence, the expression and distribution of HCV antigens in unfixed- and fixed-frozen sections of liver biopsy samples from patients 9-12 was examined.

Immunofluorescence to detect the C, E2/NS1, NS3, NS4 and NS5 antigens was performed, and the effect of the various fixation protocols on the staining intensity and distribution of positive cells was examined by comparison with that in unfixed sections. The unfixed sections showed a poor histological appearance, but the intensity of the staining pattern (as described above) was enhanced with all antibodies (Figure 3.4A), including anti-NS4 and anti-NS5, except anti-C. In contrast, although fixation of the sections improved the histological appearance, the HCV antigen staining intensity varied with different fixatives. Sections which were fixed in acetone (Figure 3.4B), chloroform or carbon tetrachloride, showed a distinct reduction in the staining intensity and in the proportion of positive hepatocytes (Table 3.2). Furthermore, the background staining intensity was increased. Methyl Carnoys, on the other hand, completely destroyed the antigenicity (Figure 3.4C), whereas 1% formalin for 5min retained the highest level of HCV antigenicity (Figure 3.4D,E; Table 3.2). Although intense staining

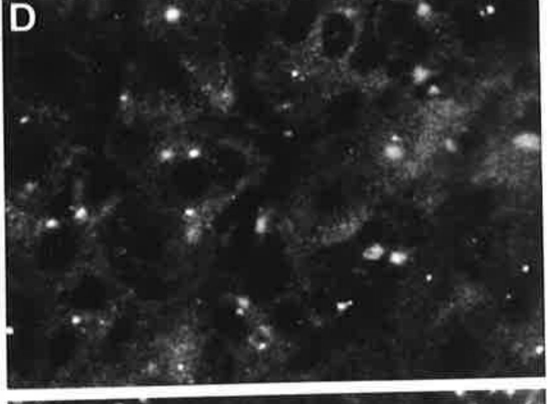
Figure 3.4 A comparison of the staining intensity and distribution of HCV-antigen positive cells in unfixed- and fixed-cryostat sections after indirect immunofluorescence.

Detection of NS3 in hepatocytes in unfixed sections (A), compared with acetone (B), Methyl Carnoys (C) and 1% formalin (D) fixed sections and E2/NS1 in lymphoid aggregates in 1% formalin (E) and acetone (F) fixed sections.
Original magnification x400.


A

B

C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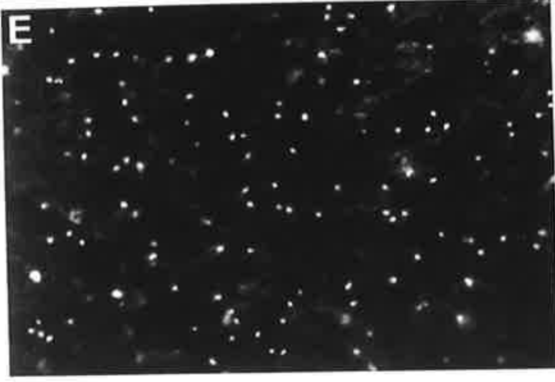
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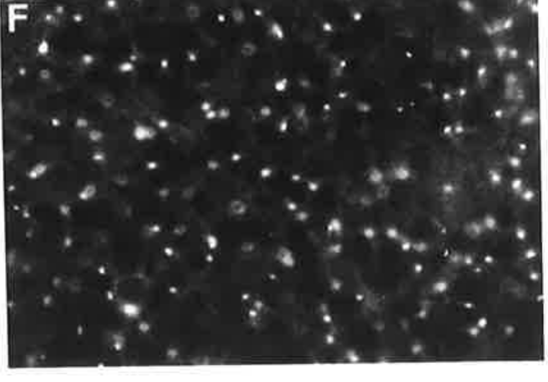

F

Table 3.2 A comparison of fixatives for the detection of HCV antigens in cryostat sections.

	Signal intensity	
Fixative	Hepatocytes	Lymphocytes
Unfixed	++++	++++
1% formalin	++++	++++
Chloroform	++	++++
Acetone	++	++++
Carbon tetrachloride	++	++++
Methyl Carnoys (methanol: acetic acid, 3:1)	-	-

The signal intensity was scored from + to ++++; + represents a weak fluorescent signal and ++++ represents a strong fluorescent signal.

patterns were observed in hepatocytes and lymphocytes following fixation in 10% formalin, the background fluorescence was very high making accurate interpretation difficult. In complete contrast, it was noticeable that HCV antigens, localised to cells in lymphoid aggregates, were completely unaffected by fixation in acetone (Figure 3.4F), chloroform or carbon tetrachloride, all of which reduced the antigenicity in hepatocytes (Table 3.2). Furthermore, the anti-C antibodies failed to detect the C antigen irrespective of the fixative used.

3.3.6 Detection of endogenous IgG in liver tissue

The inability to detect the C antigen in infected liver tissue may be due to IgG deposition masking antigenic determinants. Hence, liver sections from samples 1-8 were stained for endogenous IgG by direct immunofluorescence using a FITC-conjugated sheep-anti-human IgG (Wellcome) to determine if IgG was bound to infected cells. High levels of IgG deposits were identified in hepatocytes in liver samples 2-4, 6 and 7 (Figure 3.5A,B), whereas lower levels were detected in sample 1. No samples from patients 5 and 8 remained, and as a result immunostaining was not performed on these samples. In addition, uninfected liver tissue showed low level staining (Figure 3.5C), and no staining was observed when HCV-infected liver sections were stained with FITC-conjugated sheep-anti-mouse IgG or FITC-conjugated sheep-anti-rabbit IgG. Hence, high levels of IgG deposits were detected in hepatocytes in the liver samples previously stained for HCV antigens, E2/NS1, NS3, NS4 and NS5. It could be postulated that circulating anti-C saturates C antigenic sites in the liver, preventing C detection by immunofluorescence (described in more detail in section 3.4).

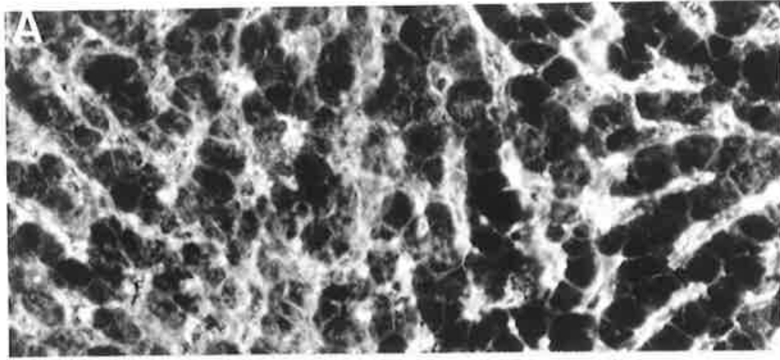
Figure 3.5 Staining for endogenous IgG in frozen sections of liver samples by direct immunofluorescence.

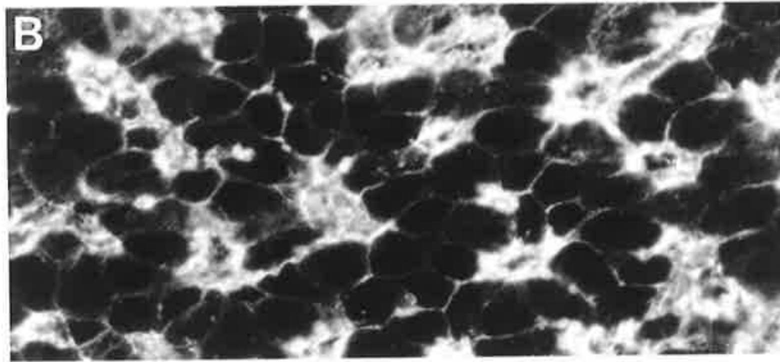
A. Low power view of IgG in hepatocytes (Case 2, Table 3.1).

B. IgG staining in hepatocytes (Case 4, Table 3.1).

C. HCV-negative liver sample.

Original magnification x200 (A) and x400 (B,C).


A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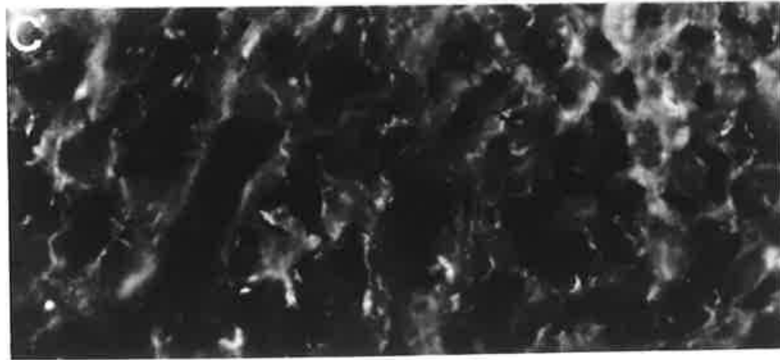

C

3.3.7 Identification of the lymphocytic population infected with HCV

The above data showed that HCV antigens were expressed not only in hepatocytes, but also in cells contained in lymphoid aggregates. Consequently, the identity of these cells was examined by dual staining experiments in liver samples 11 and 12 which were considered to be representative. The E2/NS1- and NS3-antibodies were chosen for this study because the above experiments showed that these antibodies produced a superior signal (Table 3.1). The CD markers CD4, CD8, CD14 and CD20 were chosen because these represent cell surface markers on helper T cells, cytotoxic/suppressor T cells, monocytes and B cells respectively, that have been shown to be predominant in the inflammatory infiltrates in HBV- (Montano *et al.*, 1983) and HCV- (Mosnier *et al.*, 1993) infected liver.

Following dual immunostaining (CD markers followed by HCV antigen detection or *vice versa*), lymphoid aggregates were chosen randomly and care was taken to avoid scoring the same aggregate more than once. Cells were selected by the presence of CD4, CD8 or CD20 markers in different sections and then examined for the expression of either E2/NS1 or NS3 antigens. The results were remarkably consistent and showed that approximately 40% of the lymphoid cells were E2/NS1-positive (Table 3.3) and 40-50% were NS3-positive (Table 3.4). Similarly, when HCV antigen-positive cells were selected and examined for the expression of CD4, CD8 or CD20, the results were equally consistent. However, only 2% of E2/NS1 antigen-positive cells were CD4-positive, 50% were CD8-positive and 62% were CD20-positive (Table 3.5; Figure 3.6), while 4% of NS3-positive cells were CD4-positive, 47% CD8-positive and 63% CD20-positive (Table 3.5; Figure 3.6). A correlation with CD14-positive cells was attempted, but this was not possible due to the lack of CD14-positive cells in the liver samples. Antiglobulin interspecies cross-reactivity in these dual immunostaining experiments was excluded by the absence of staining when the rabbit or mouse

Table 3.3 HCV-E2/NS1 antigen positivity in cells selected on the basis of CD4, CD8 or CD20 markers.

	Total cells	
CD marker	E2/NS1-positive	E2/NS1-negative
CD4	21 (40%)	32 (60%)
CD8	20 (43%)	27 (57%)
CD20	21 (41%)	30 (59%)

Table 3.4 HCV-NS3 antigen positivity in cells selected on the basis of CD4, CD8 or CD20 markers.

	Total cells	
CD marker	NS3-positive	NS3-negative
CD4	21 (41%)	30 (59%)
CD8	23 (44%)	29 (56%)
CD20	57 (50%)	57 (50%)

Table 3.5 Identification of CD markers of cells selected on the basis of HCV-E2/NS1 and -NS3 positivity.

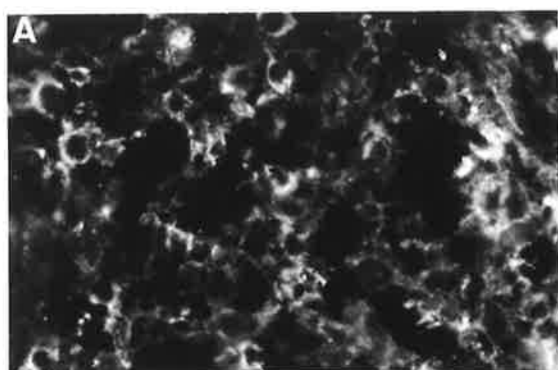
		E2/NS1	NS3
CD4	Positive	1 (2%)	2 (4%)
	Negative	50 (98%)	46 (96%)
CD8	Positive	26 (50%)	24 (47%)
	Negative	26 (50%)	27 (53%)
CD20	Positive	31 (62%)	35 (63%)
	Negative	19 (38%)	21 (37%)

Figure 3.6 Identification of the lymphocytic cell population infected with HCV in unfixed frozen tissue by dual immunostaining.

Detection of CD8 (A) and E2/NS1 (B) antigens within lymphoid aggregates photographed from the same field of view.

Lymphoid aggregates stained for CD20 (C) and NS3 (D) antigens photographed from the same field of view.

Original magnification x400.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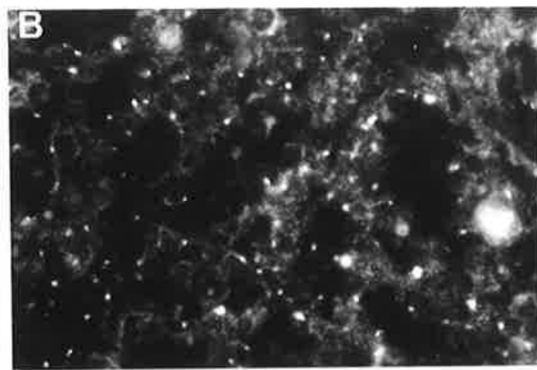
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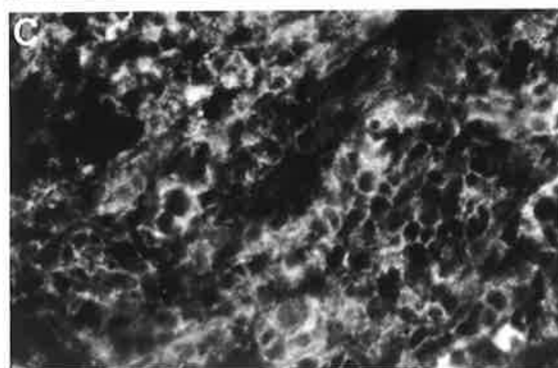
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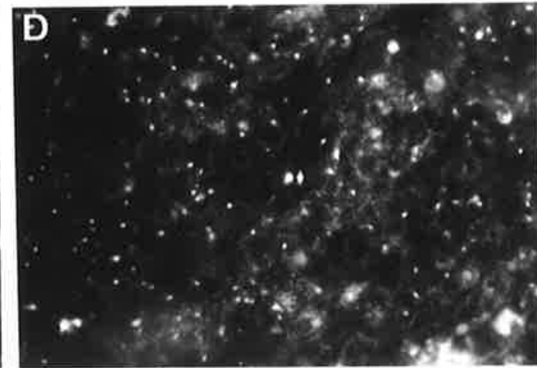

D

antibodies were substituted with pre-immune serum or unrelated antibodies. In addition, identical staining patterns were observed regardless of the order of antigen detection in these experiments (ie. CD marker followed by HCV antigen or *vice versa*). The above figures do not simply reflect the distribution of cells expressing these specific CD markers within the aggregates, since CD4-, CD8- and CD20-positive cells were present in approximately equal numbers. The distribution of CD4-, CD8- and CD20-positive cells within lymphoid aggregates is consistent with a previous report (Mosnier *et al.*, 1993). However, CD4-positive cells were reported to be more numerous than CD8-positive cells (Mosnier *et al.*, 1993). In conclusion, the results of the dual staining experiments showed that about 40% of CD4-, CD8- and CD20-positive cells, present in lymphoid aggregates, were HCV-infected, while the bulk of HCV-infected lymphocytes were either CD8- or CD20-positive and only a small proportion were CD4-positive.

3.4 CONCLUSIONS AND DISCUSSION

Data presented in this chapter demonstrated that fixation of cryostat sections in 1% formalin was optimal for the detection of HCV-specific antigens and for improved histological appearance of the tissue. In contrast, Methyl Carnoys completely abolished HCV antigen staining, and the staining intensity and the number of HCV antigen-positive hepatocytes was reduced considerably when either acetone, chloroform or carbon tetrachloride was used as the fixative. However, the staining pattern in CD4-, CD8- and CD20-positive lymphocytes was unaffected by the latter 3 fixatives. Hence, it is possible that HCV antigens are expressed or presented in a different manner in the two cell types, emphasising the importance of appropriate tissue fixation for HCV protein detection prior to immunostaining.

All four antigens which were detected in frozen liver tissue sections (E2/NS1, NS3, NS4, NS5) were present in cells which were widely distributed and showed a similar intra- and inter-cellular distribution pattern, and it is likely that the same population of cells were positive. Dual immunostaining for HCV-E2/NS1 and -NS3 was carried out in an attempt to show colocalisation of the S and NS proteins. However, this proved unsuccessful because both antibodies were raised in rabbits and did not permit the two antigens to be distinguished by dual immunostaining. The punctate or granular staining pattern seen in the cytoplasm of infected hepatocytes and in CD4-, CD8- and CD20-positive lymphocytes may represent virus replicative complexes. This pattern of staining is consistent with other studies in which punctate staining was observed in hepatocytes in frozen chimpanzee- and human-liver sections using HCV-positive human serum (Krawczynski *et al.*, 1992), murine monoclonal antibodies (Sansonno and Dammacco, 1993; Yap *et al.*, 1994) or polyclonal rabbit antibodies (Tsutsumi *et al.*, 1994). Immunoelectron microscopic examination showed that the NS5 antigen was localised along the endoplasmic reticulum (Tsutsumi *et al.*, 1994). Furthermore, immunofluorescence studies of mammalian cells transfected with full-length and selected domains of HCV cDNA showed the staining pattern of C, E1, E2/NS1, NS2 and NS5 proteins to be intense, punctate and perinuclear, consistent with endoplasmic reticulum localisation (Selby *et al.*, 1993).

The detection of a NS protein viz. NS3 in CD4-, CD8- and CD20-positive lymphocytes suggests that these cells are likely to be supporting HCV replication and lead to the conclusion that HCV is not only hepatotropic, but also lymphotropic. Thus, the data in this chapter are consistent with reports of HCV replication in PBMC (Bouffard *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Gunji *et al.*, 1994; Muratori *et al.*, 1994), circulating B lymphocytes (Zignego *et al.*, 1992; Muller *et al.*, 1993a) and circulating T lymphocytes (Zignego *et al.*, 1992). These studies report the detection, by RT-PCR, of positive and negative sense HCV RNA. Nevertheless, HCV RNA was

undetected in CD4- and CD8-positive T lymphocytes purified from PBMC of infected individuals (Muller *et al.*, 1993a). This latter result may reflect the stage of disease, as the timing of infection of PBMC during the chronic carrier stage is unknown, as is the duration of infection of these cell types. However, HCV replication has been demonstrated in T lymphocyte- (Shimizu *et al.*, 1992, 1993) and B lymphocyte- (Bertolini *et al.*, 1993) cell lines inoculated with HCV-positive human serum, in which negative strand HCV RNA, and the C and NS4 antigens were detected. Hence, the results in this chapter provide additional data which may also prove useful in the choice of cells for *in vitro* culture experiments.

The detection of NS4 and NS5 was relatively poor and may be due to: (i) the quality of the antisera, particularly related to the ability of an anti-peptide to detect the complete protein which may be folded in a different manner to that of the peptide; or (ii) antigenic variability of HCV; or (iii) a low level of antigen expression. Support for the latter hypothesis comes from *in vitro* translation studies in which the NS proteins were poorly expressed in rVV-infected cells expressing the entire HCV polyprotein; in contrast, high expression of the NS proteins was achieved by infection with rVV designed to express the NS proteins only (Grakoui *et al.*, 1993c). It is possible that a similar problem is encountered in natural infection. Furthermore, although the C gene has been shown to be highly conserved, it was not possible to detect the C protein in naturally-infected liver biopsy samples using two different antibodies. This result is consistent with one study which failed to detect the C protein in frozen liver tissue unless the tissue was pre-treated with acid, suggesting that the antigenic determinants may be masked by antibody deposition (Sansonno and Dammacco, 1993). In support of endogenous IgG in infected liver tissue, this chapter detected IgG deposits in liver samples from selected patients. However, if the C antigen was masked by IgG, then why doesn't antibody to NS3 block NS3 antigen detection in liver sections, given that anti-NS3 circulates in infected individuals. Furthermore, three

studies detected the C antigen in only 11 of 48 (Hiramatsu *et al.*, 1992), 6 of 20 (Yamada *et al.*, 1993) and 21 of 28 (Yap *et al.*, 1994) liver samples examined.

There may be several explanations for the lack of C antigen detection: (i) the antibody raised to a peptide may fail to recognise conformational epitopes in infected cells; or alternatively the C protein may be: (ii) unstable; or (iii) rapidly enveloped and secreted from infected cells. Thus if the latter explanation is true, it is possible that C expression is rate limiting for virus replication, although other positive strand viruses which replicate to high levels must face a similar problem. This may be an important regulatory mechanism and explain the low level of viraemia in infected individuals. Similarly, limited expression of NS4 and NS5, as discussed above, may also be rate limiting, although this may be less likely.

CHAPTER 4

IMMUNOHISTOCHEMICAL DETECTION OF HCV-NS4 AND ITS RELATION TO HISTOPATHOLOGY

4.1 INTRODUCTION

Several histological features have been associated with HCV infection of the liver. Common findings include portal lymphoid aggregates, bile duct damage and loss, steatosis and hepatocyte necrosis. The most prominent single feature in HCV-infected liver tissue is the lymphoid aggregate (Bach *et al.*, 1992; Scheuer *et al.*, 1992). Although these histological changes are suggestive of hepatitis C liver disease, they often do not appear in a single specimen and the same histological features are seen in HBV infection and autoimmune liver disease. Furthermore, it is unclear which of these changes may represent a direct effect of HCV or which reflect the host response.

The detection of HCV-specific antigens in liver tissue would confirm HCV infection and also allow a correlation between the histological features and antigen expression. Few studies have been performed to determine the relationship between HCV-infected hepatocytes and the patterns of chronic liver injury. Hence, the aim of this chapter was to investigate methods to detect HCV antigens in routinely processed formalin-fixed, paraffin wax-embedded liver sections from anti-HCV positive individuals, and to relate viral antigen detection to histopathological features.

4.2 EXPERIMENTAL DESIGN

Liver biopsy specimens were collected from 10 anti-HCV positive patients different from those studied in Chapter 3, fixed and embedded in paraffin wax as described in section 2.2.1. Paraffin sections rather than frozen sections were

used to permit a study of the HCV antigen-histopathological correlation in chronic HCV infection. The majority of the patients were individuals who were found to be anti-HCV positive after blood donation. On interview they were found to be recreational intravenous drug users in the 1970's. In addition, serum from the majority of patients was collected prior to the date of biopsy and tested for HCV RNA (section 2.4) and ALT levels. Samples of liver tissue from 3 HBV-carriers and 3 uninfected individuals were used as negative controls.

4.3 RESULTS

4.3.1 Preliminary study of HBcAg by 3 and 4 layer assays

Initially, a comparison of the sensitivity of 3 and 4 layer immunoperoxidase assays (section 2.2.6; Figure 2.1) for the detection of HBcAg was undertaken on formalin-fixed liver tissue from HBV-carriers. This study was undertaken due to; (i) readily available autopsy tissue samples: (ii) the well characterised commercially-available rabbit anti-HBcAg (section 2.2.2); and (iii) the well defined staining pattern in infected liver tissue. Using the 3 layer method (section 2.2.6(i)), HBcAg was only detected within the nuclei of infected hepatocytes in the formalin-fixed liver tissue samples studied. The sensitivity of detection was assessed by the percentage of hepatocytes positive for nuclear HBcAg and by the intensity of the nuclear staining.

A 4 layer horseradish peroxidase assay (section 2.2.6(ii)) was developed in an attempt to increase the sensitivity of detection. The assay was optimised for the detection of HBcAg by titration of all antibody layers on the same HBV-positive liver tissues and compared with the above 3 layer method. A clear difference was noted between the 3 and 4 layer methods; in the 4 layer method the nuclear HBcAg signal was more intense and appeared to be present in a higher

proportion of hepatocytes. Hence, in subsequent experiments the 4 layer assay was used to detect HCV antigens in HCV-infected liver samples.

4.3.2 Histological diagnosis

A histological analysis of the liver biopsy samples from the anti-HCV positive individuals was made under code by a system which scores activity in chronic hepatitis, as described by Scheuer (1991). Three categories, lobular activity, portal/periportal activity and fibrosis, were graded from 0 to 4. Portal tract inflammation was a feature in all specimens, and the infiltrate consisted mainly of lymphocytes with occasional scattered macrophages; 6 of 10 samples contained lymphoid aggregates within the portal tracts (Table 4.1). The histological diagnosis of each sample was determined to be CAH, ranging from mild to severe, with fibrosis and some with cirrhosis (Table 4.1). All samples showed evidence of mild to moderate steatosis.

4.3.3 PCR analysis of serum samples

Serum samples were available from 8 of the 10 patients. These were examined for HCV RNA by RT-PCR using primers from the 5' UTR (section 2.4). The products were analysed by agarose gel electrophoresis (section 2.1.4) (Figure 4.1) and the specificity confirmed by Southern blot hybridisation using a ^{32}P-end-labelled internal oligonucleotide (section 2.5.4) (data not shown); all 8 patients were viraemic (Table 4.1; Figure 4.1).

4.3.4 HCV antigen detection

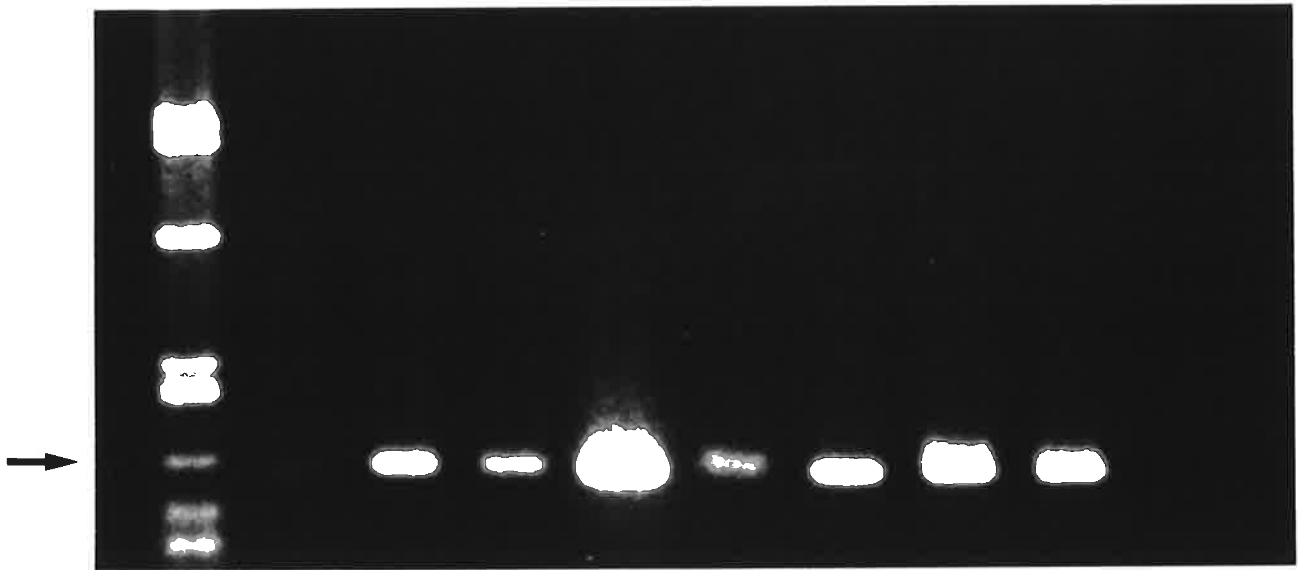
Initially, attempts were made to detect E2/NS1, NS3, NS4 and NS5 in formalin-fixed liver tissue using the same antisera described in Chapter 3. However, only anti-NS4 produced a positive result, and no convincing staining was observed with antibodies to E2/NS1, NS3 and NS5, despite the fact that these antigens were detected in frozen sections (Chapter 3).

Table 4.1 Histological diagnosis of liver biopsy specimens from 10 chronic hepatitis C patients.

Patient	Age (yr)/ sex	Serum			Liver Histology				
		ALT/(IU/L)	Anti-HCV	HCV-RNA	Diagnosis	Fibrosis*	Lobular* activity	Portal/periportal* activity	Lymphoid aggregates
A	28M	116	+	+	severe CAH (cirrhosis)	4	1	4	+
B	58M	NT	+	NT	moderate CAH	3	1	3	-
C	54M	NT	+	NT	moderate CAH (cirrhosis)	4	2	3	+
D	36M	NT	+	+	moderate CAH (cirrhosis)	4	1	3	-
E	35F	91	+	+	severe CAH (cirrhosis)	4	4	4	+
F	34M	68	+	+	mild CAH	1	1	2	-
G	33M	227	+	+	severe CAH	3	2	4	+
H	30M	225	+	+	moderate CAH	2	1	3	+
I	40M	34	+	+	mild CAH	1	1	2	-
J	45M	99	+	+	moderate CAH	2	1	3	+

CAH = chronic active hepatitis ALT = alanine aminotransferase NT = not tested * According to Scheuer, 1991.

Figure 4.1 Ethidium bromide stained agarose gel showing HCV-specific RT-PCR products generated from HCV RNA isolated from serum samples from patients A, D-J (lanes 2-9) and an anti-HCV negative patient (lane 10).
The position of the 324bp product is shown by an arrow and is consistent with the λ DNA marker digested with Pst I (lane 1).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10

In addition, attempts were made to enhance the signal by inclusion of a trypsin digestion step (0.1% trypsin, 8 min, 37°C) prior to immunostaining (Swoveland and Johnson, 1979; Van Noorden, 1986). This treatment had no positive effect and actually decreased the staining intensity in the liver samples tested (Figure 4.2). Consequently, all liver sections were stained for NS4 by the 4 layer immunoperoxidase assay (section 2.2.6(ii)) omitting the trypsin digestion step. Following staining, the sections were examined independently under code by two histopathologists, Dr. R Rowland, Institute of Medical and Veterinary Science, Adelaide and Assoc. Prof. P Hall, Flinders Medical Centre, Adelaide.

4.3.5 Correlation between NS4 expression and histopathology

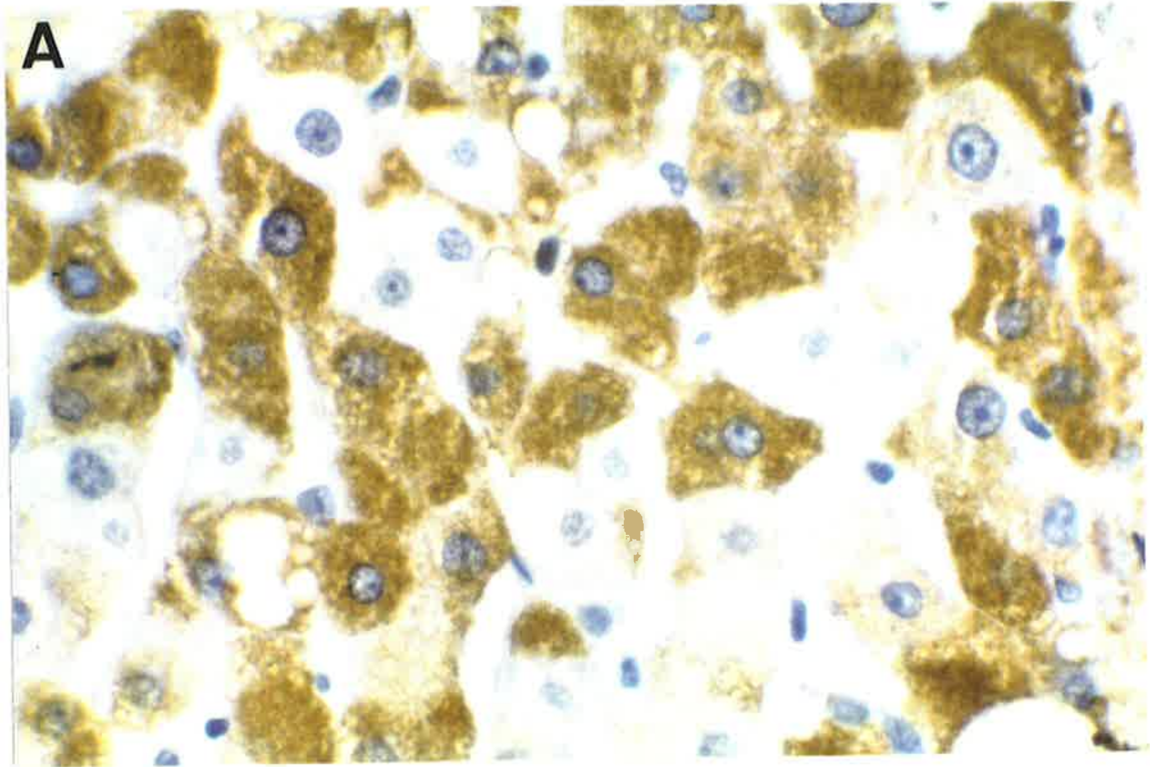
NS4 was detected within the cytoplasm of 60% to 90% of hepatocytes in biopsy samples from all 10 patients with persistent HCV infection. NS4 was localised to the hepatocyte cytoplasm. The staining pattern was crisp and finely granular, and was never found within nuclei nor in cells contained in the inflammatory infiltrate (Figure 4.3A). Although HCV antigens were detected in lymphocytes in the cryostat sections (Chapter 3), NS4 was undetected in these cell types in the sections from the formalin-fixed tissue samples. The staining intensity within the hepatocyte cytoplasm was variable, and although a high proportion of the hepatocytes were weakly stained, intensely stained hepatocytes were observed as either individual isolated hepatocytes (Figure 4.3B) and/or in small groups (Figure 4.3C). No consistent cytological abnormality was noted within infected cells, regardless of the concentration of antigen localised within the cytoplasm. No strong association was noted between antigen-positive hepatocytes, steatosis and other pathological features, such as focal hepatocyte necrosis within the lobules, regenerating nodules, piecemeal necrosis or lymphoid aggregates, within the HCV-infected liver. However, one sample (case E) contained intensely stained hepatocytes situated in the periportal region close to sites of piecemeal necrosis, but this was not consistent. HCV antigen-positive cells were not

Figure 4.2 Effect of trypsin digestion on the detection of NS4 in formalin-fixed liver tissue.

A. Sections with no trypsin digestion.

B. Sections digested with trypsin prior to immunostaining.

Original magnification x400.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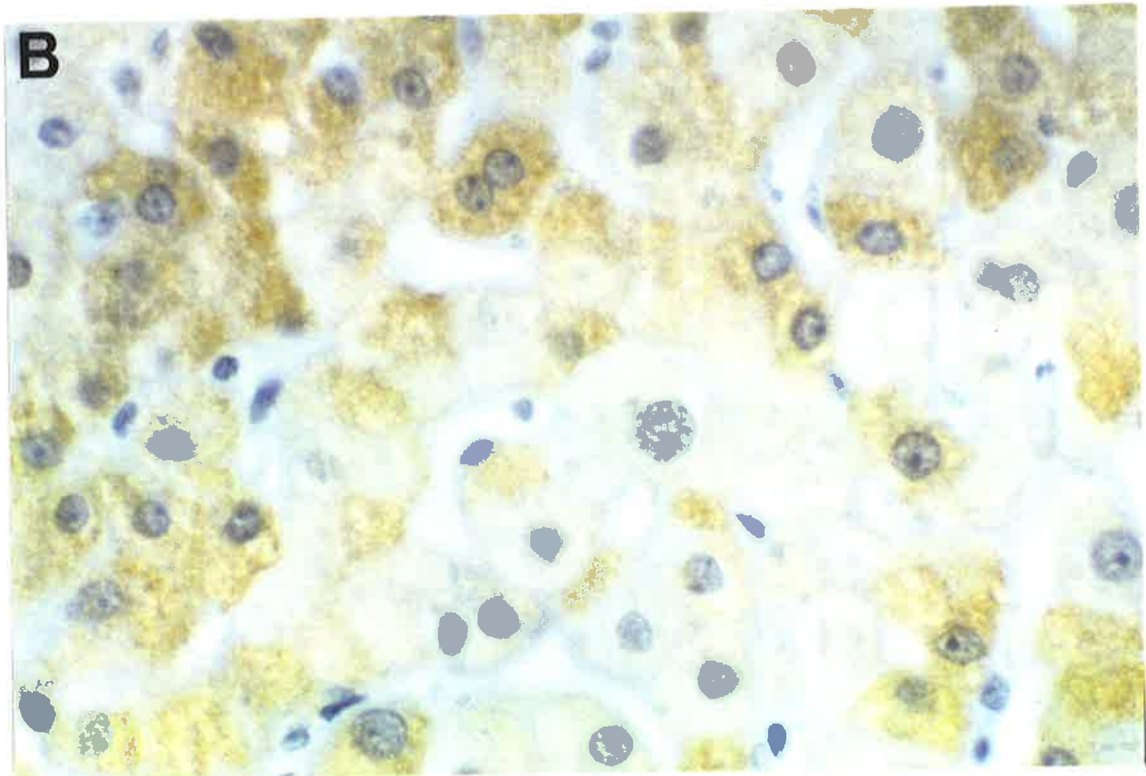

B

Figure 4.3 Immunohistochemical detection of HCV antigen NS4 within formalin-fixed, paraffin wax-embedded liver sections.

A. Low power view of NS4 in hepatocytes, but not within adjacent mononuclear cells (Case H, Table 4.1).

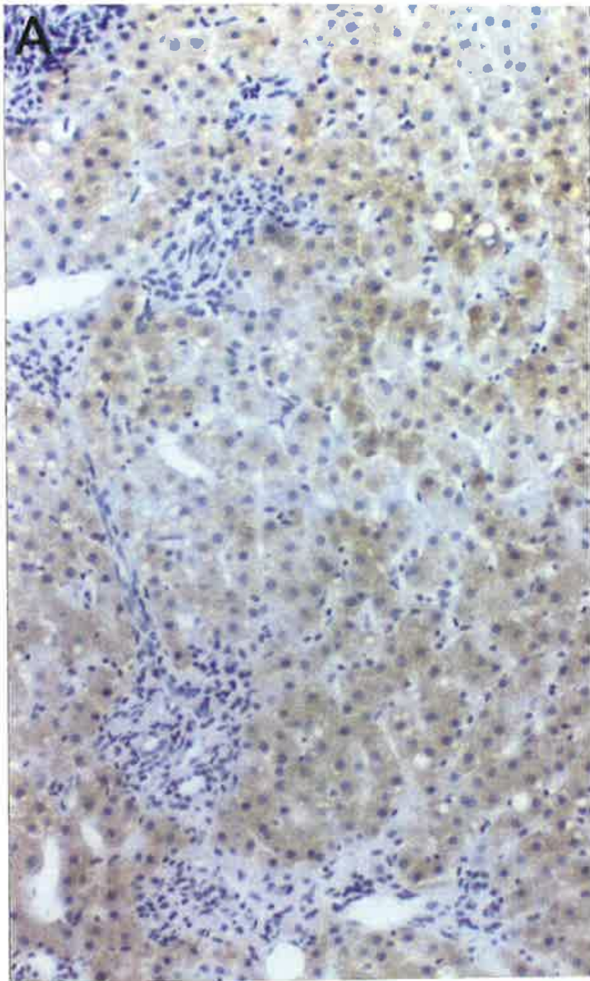
B. Intense staining within the cytoplasm of isolated hepatocytes (Case I, Table 4.1).

C. Localisation of NS4 within clusters of hepatocytes (Case F, Table 4.1).

D. HCV-negative liver sample.

E. The resultant staining pattern following preadsorption of the antiserum with the NS4 synthetic peptide (Case G, Table 4.1) or with the peptide diluent (Case G, Table 4.1) (F).

Haematoxylin counterstain, original magnification x100 (A), x400 (B, C, D, E, F).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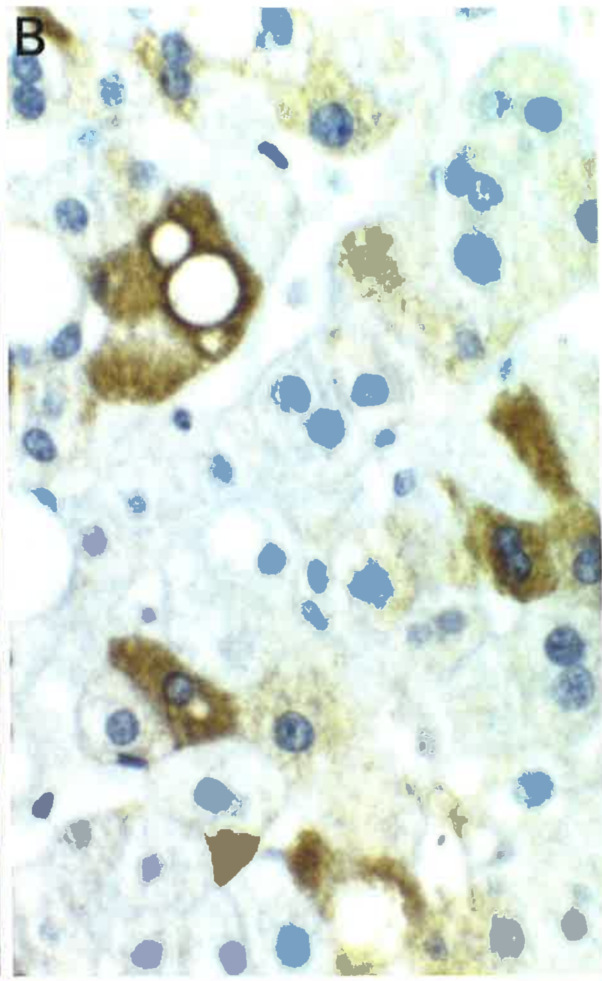
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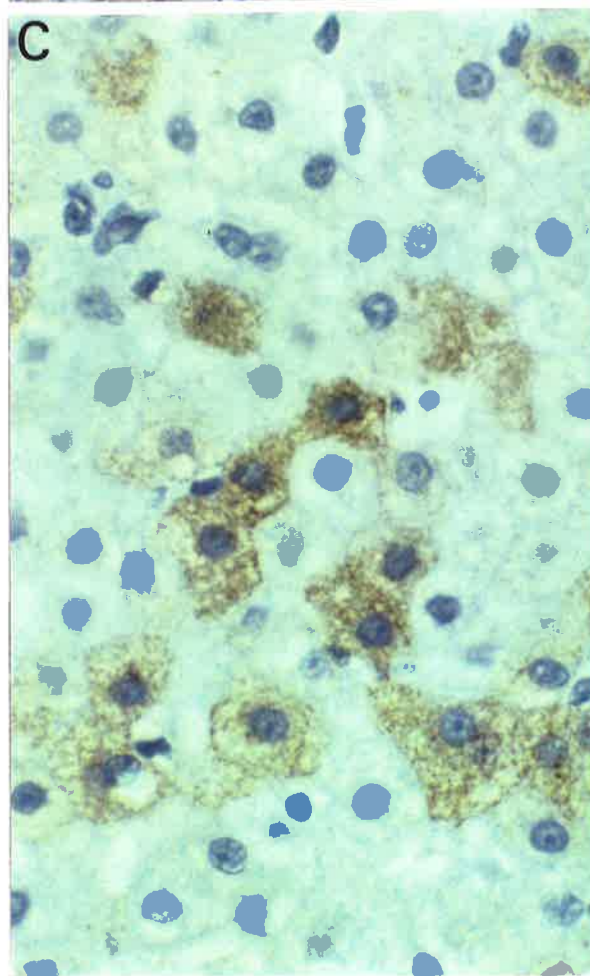
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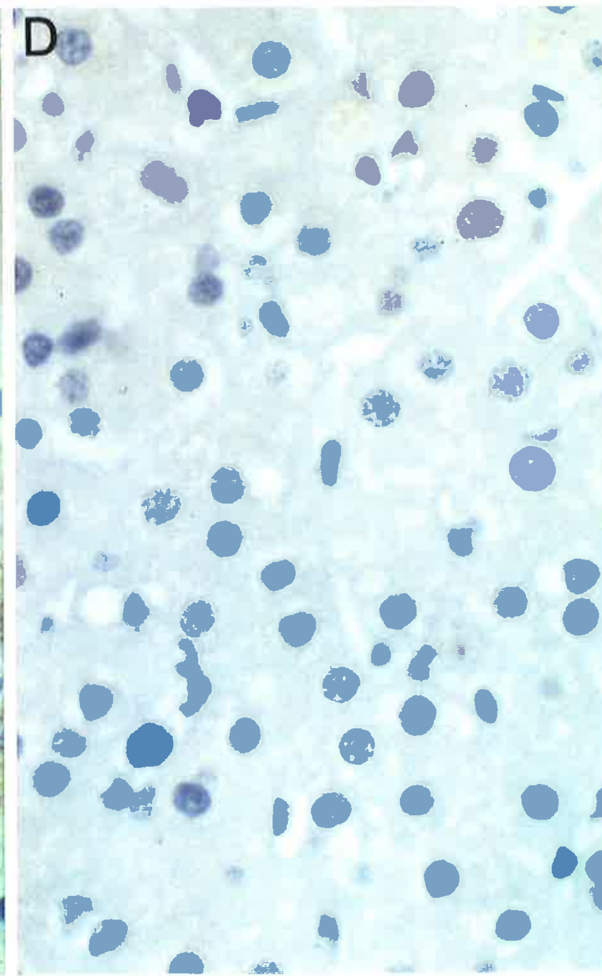
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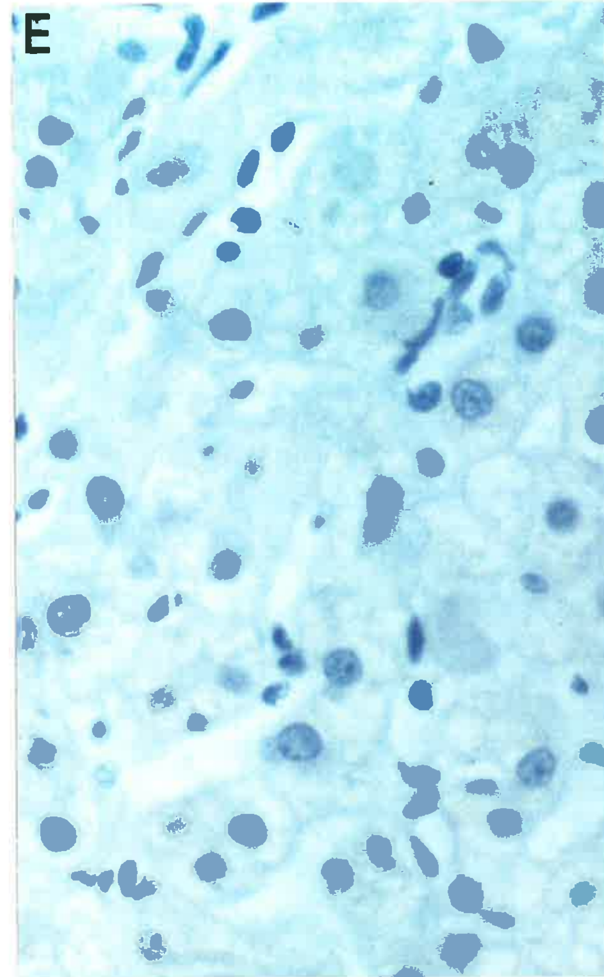
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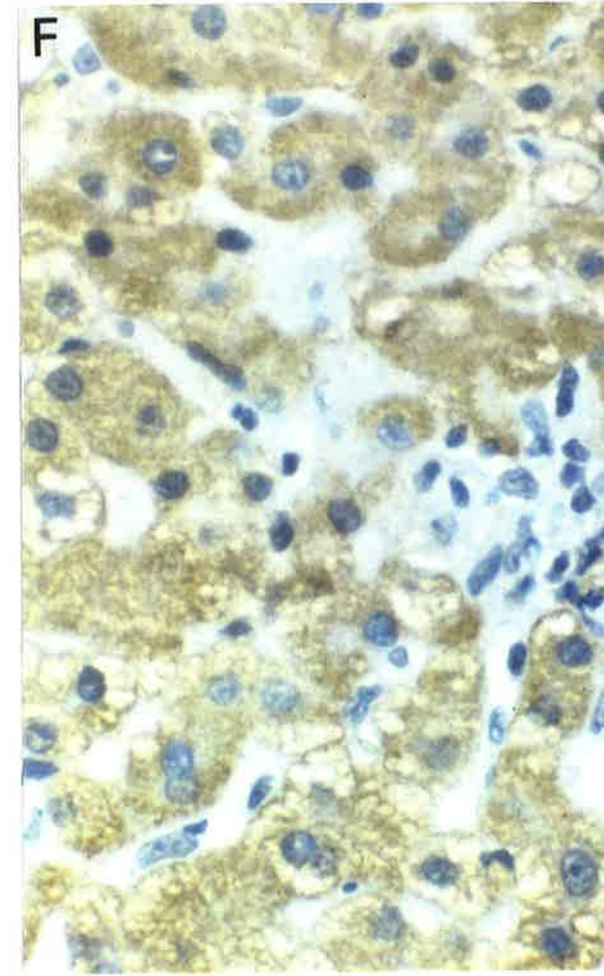

F

restricted to areas with active hepatocyte necrosis. Generally, the cirrhotic samples only contained weakly stained hepatocytes, whereas non-cirrhotic samples with portal fibrosis showed both weakly staining hepatocytes and focal areas of intense staining within the hepatocytes.

4.3.6 Specificity of the reaction for NS4 detection

No reaction was observed in samples from uninfected or HBV-infected individuals (Figure 4.3D) or in HCV-positive liver tissue after immunostaining with the pre-immune rabbit serum. Preadsorption of the NS4 antiserum with the NS4 synthetic peptide prior to immunostaining, consistent with a parallel loss of reactivity in EIA, abolished the signal (Figure 4.3E). In contrast, the peroxidase and EIA signals were retained following a mock adsorption of the antiserum against the peptide diluent (Figure 4.3F). Furthermore, single substitution of any of the four antibody layers with the diluent abolished the signal.

4.4 CONCLUSIONS AND DISCUSSION

This chapter clearly demonstrates the expression of NS4 within the cytoplasm, but not in the nucleus, of a high proportion of hepatocytes in liver biopsy samples from patients with chronic hepatitis C. NS4 detection in formalin-fixed liver sections is consistent with the data in Chapter 3, in which four HCV antigens (E2/NS1, NS3, NS4 and NS5) were detected in frozen sections, and with other studies which used human antibody (Infantolino *et al.*, 1990; Krawczynski *et al.*, 1992), murine monoclonal antibody (Hiramatsu *et al.*, 1992; Sansonno and Dammacco, 1993; Yamada *et al.*, 1993; Yap *et al.*, 1994) or polyclonal antibody (Infantolino *et al.*, 1990; Tsutsumi *et al.*, 1994). Although three studies (Hiramatsu *et al.*, 1992; Krawczynski *et al.*, 1992; Sansonno and Dammacco, 1993) report a lower percentage of infected cells in chronic carriers, this may reflect the sensitivity of detection, and in this chapter a 4 layer immunoperoxidase assay

was developed to increase the sensitivity. In support of these observations, HCV RNA was detected by ISH in 100% of hepatocytes of infected chimpanzees (Negro *et al.*, 1992) and more recently in a majority of hepatocytes in formalin-fixed human liver tissue by RT-PCR/ISH (Nuovo *et al.*, 1993).

Hepatocytes containing higher levels of NS4 were distributed randomly among the bulk of hepatocytes, which contained low levels of antigen. A proportion of the hepatocytes were negative. The detection of viral proteins in a high proportion of hepatocytes was unexpected since viraemia in infected individuals is low (Weiner *et al.*, 1990; Fong *et al.*, 1991). However, the staining was considered to be specific for the following reasons: (i) negative hepatocytes were contained within areas of positive staining (Figure 4.3B,C); (ii) preadsorption of the antiserum against the synthetic peptide abolished staining; (iii) no reaction was observed with the pre-immune serum; (iv) HBV-infected and uninfected liver samples showed no reaction; (v) there was a variation in the intensity of the NS4 staining pattern as well as the proportion of infected hepatocytes in different samples; and (vi) the staining pattern within some hepatocytes was restricted to a portion of the cytoplasm. Consequently, these controls confirm the specificity of the reaction for HCV-NS4.

There was no association of HCV antigen with regions of active hepatocyte necrosis or areas of lymphoid aggregates, although the cirrhotic livers did not contain the randomly distributed hepatocytes with higher levels of NS4. The detection of the C100 antigen with murine monoclonal antibodies in frozen liver tissue from chronic carriers, in which positive hepatocytes showed no direct relationship with necrotic areas and inflammatory cell infiltration (Sansonno and Dammacco, 1993), is consistent with these observations. Since expression of NS4 is a likely marker of HCV replication, these results could be interpreted to suggest that HCV is non-cytopathic, consistent with recent suggestions (Mosnier *et al.*, 1993; Nouri Aria *et al.*, 1993; Nuovo *et al.*, 1993; Tanaka *et al.*, 1993).

However, the apparent lack of cytotoxicity may be due to the low level of virus replication (Weiner *et al.*, 1990; Fong *et al.*, 1991). The putative non-cytopathic nature of HCV is consistent with the data from this chapter in which samples F and I, which contained the highest levels of NS4, displayed mild liver disease and no lymphoid aggregates. Furthermore, although RT-PCR is not absolutely quantitative, it was noted that those patients (samples F, H, I and J) with the highest levels of NS4 antigen within the liver had a higher level of viraemia (Figure 4.1). Thus, higher levels of antigen expression were directly related to (relatively) higher levels of viraemia. However, this is in contrast to three studies in which serum RNA levels, measured by quantitative RT-PCR, tended to increase directly with an increase in the severity of histopathological changes in the liver (Hagiwara *et al.*, 1993; Kato *et al.*, 1993b; Naito *et al.*, 1994).

CHAPTER 5

DETECTION OF THE NS4 PROTEIN IN HEPATOCELLULAR CARCINOMA AND ADJOINING NONTUMOROUS LIVER TISSUE

5.1 INTRODUCTION

Progression of acute HCV through chronic hepatitis and hepatic cirrhosis is a major risk factor for the development of HCC. An association between anti-HCV positivity and the development of HCC has been suggested (Saito *et al.*, 1990; Blum, 1994) and there is a worldwide increased incidence of HCC in patients with HCV-positive cirrhosis. However, the pathogenetic mechanism(s) for the development of HCV-associated HCC is unknown, but it may be related to a non-specific mechanism linked to the presence of cirrhosis (Tabor and Kobayashi, 1992). Recently, positive and negative sense HCV RNA was demonstrated in HCC tissue from chronic HCV carriers (Gerber *et al.*, 1992; Horiike *et al.*, 1993; Sherker *et al.*, 1993), but to date HCV protein detection in HCC tissue has not been documented. Hence, the aim of this chapter was to examine the expression of the NS4 protein in formalin-fixed liver samples from HBsAg-negative patients with HCC, and thus to study the association of HCV protein expression with HCC. Anti-NS4 was chosen for this study because the experiments described in Chapter 4 showed that this antibody produced a superior signal in formalin-fixed liver tissue.

5.2 EXPERIMENTAL DESIGN

Liver samples from five HBsAg-negative patients with histologically proven HCC from the Royal Adelaide Hospital, Adelaide were studied. The liver specimens were collected at autopsy (sample D) or by biopsy (samples A, B, C and E), prior

to the development of serological assays for anti-HCV, fixed in formalin and embedded in paraffin wax as described in section 2.2.1. The liver samples were known to be HBcAg- and HBsAg-negative, and thus the aetiology of the liver injury and progression to HCC were unknown. Serum samples from these individuals were not available and the sections were stained for NS4 using the 4 layer detection assay developed in Chapter 4. Samples of liver tissue from 3 HBV-carriers and 1 uninfected individual were used as negative controls. Following staining, the sections were examined independently under code by two histopathologists, Dr. R Rowland, Institute of Medical and Veterinary Science, Adelaide and Assoc. Prof. P Hall, Flinders Medical Centre, Adelaide.

5.3 RESULTS

5.3.1 Histological diagnosis

Liver samples from the five patients were examined by routine histological diagnosis. The histological appearance of all samples was consistent with chronic hepatitis. The basic hepatic architecture in liver samples C and E was disrupted and showed expansion of the portal tracts with chronic inflammatory cells. Cirrhosis was evident in samples B, C, D and E, and mild to moderate steatosis in samples A, B, and D. HCC was evident in all samples and the tumour showed predominantly a trabecular growth pattern of tumour cells with large nuclei and moderate to large eosinophilic cytoplasm.

5.3.2 NS4 detection

NS4 was detected within the cytoplasm of a high proportion of hepatocytes in patient samples B, C, D and E (Figure 5.1A), but not in sample A. The staining intensity within the cytoplasm of nontumorous hepatocytes was variable and negative hepatocytes were found interspersed with positive hepatocytes in a

Figure 5.1 Immunohistochemical detection of HCV antigen NS4 in HCC and adjoining nontumorous liver tissue.

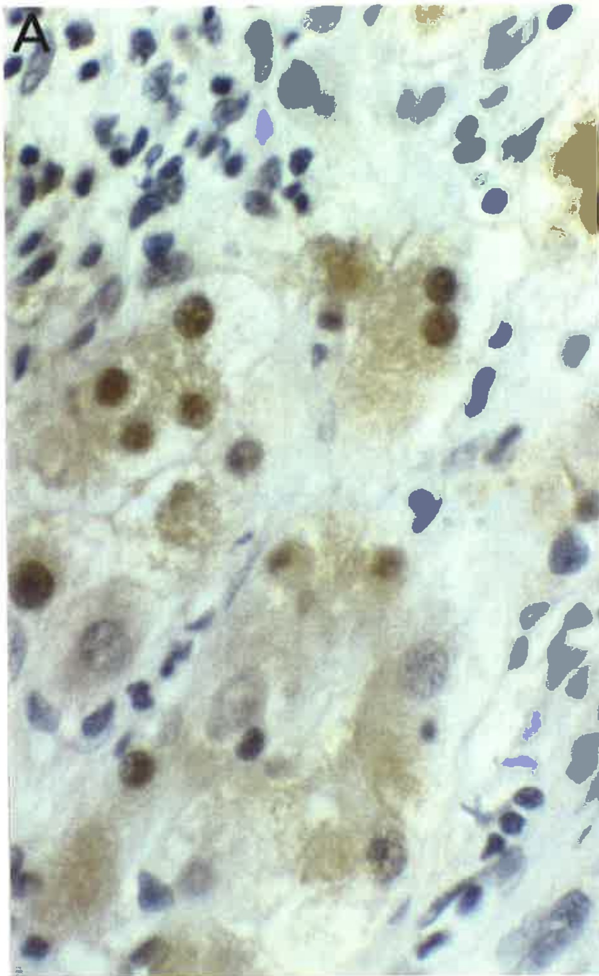
A. Cytoplasmic and nuclear NS4 within nontumorous hepatocytes (Case C).

B. NS4 staining within intranuclear inclusions (Case E).

C. Cytoplasmic NS4 within malignant cells (Case B).

D. HCV-negative liver sample.

Original magnification x400.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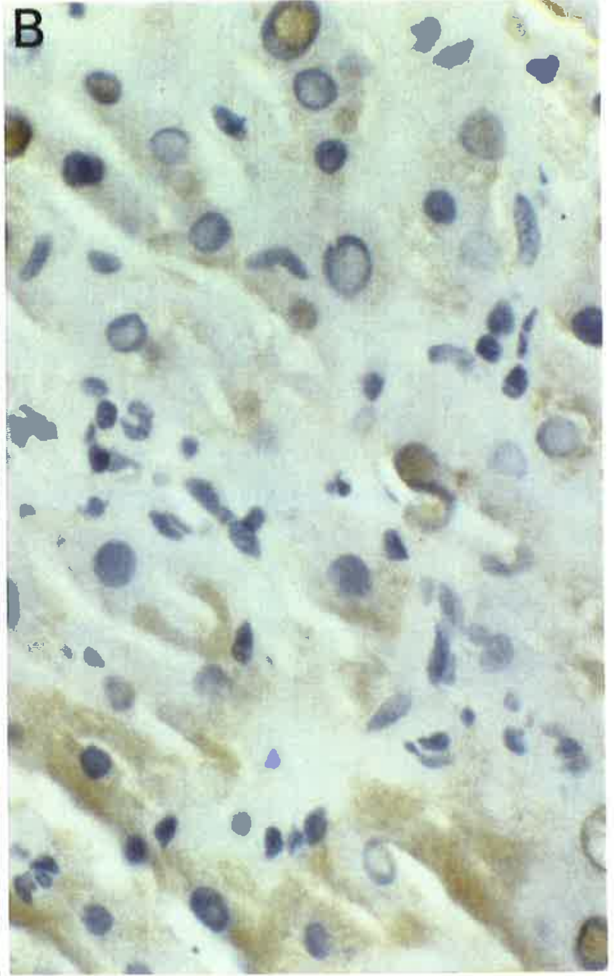
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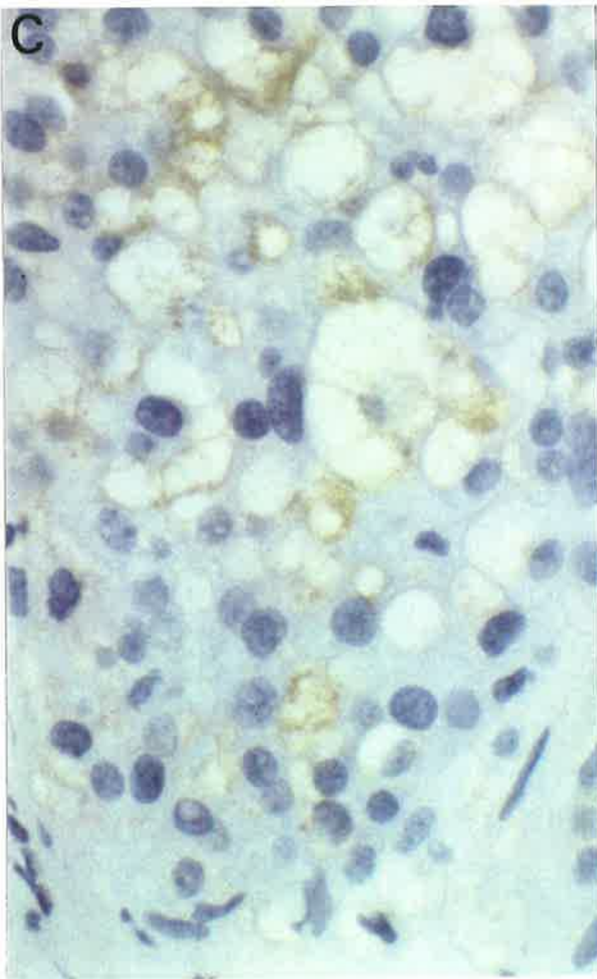
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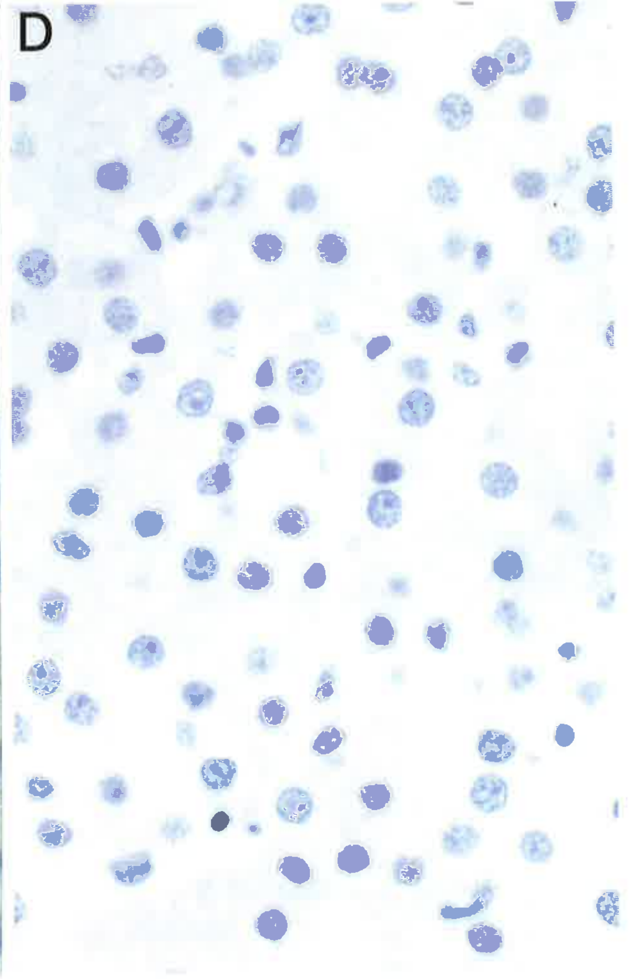

D

similar manner to that described in Chapter 4. High background staining was observed in the tumorous and nontumorous liver tissue from sample D. This was probably due to the autopsy nature of the sample, and as a result interpretation of a positive signal was difficult. However, definite positive nontumorous hepatocytes were observed in sample D that were absent in the negative controls (see section 5.3.3). In addition to cytoplasmic NS4 in sample C, intense staining was also observed in the nuclei of approximately 5% of these nontumorous hepatocytes. The hepatocytes with nuclear NS4 appeared to be mainly localised at the edge of the nodule (Figure 5.1A). Hepatocytes with NS4-positive nuclei always contained cytoplasmic NS4 and staining within the nuclei was stronger than that seen in the cytoplasm (Figure 5.1A). Furthermore, pseudo-intranuclear staining of NS4 within nontumorous hepatocytes was observed in sample E (Figure 5.1B). This was considered to be due to inclusion of cytoplasmic material into the nucleus commonly referred to as pseudo inclusions or intranuclear inclusions. The tumorous cells in samples A and D were negative, whereas in samples B, C and E approximately 40-60% contained a low level of NS4 antigen in the cytoplasm (Figure 5.1C). Finally, infiltrating lymphocytes were negative for NS4 in all samples studied (Figure 5.1A) as observed in Chapter 4.

5.3.3 Specificity of staining for NS4

NS4 staining was absent in samples from uninfected or HBV-infected individuals (Figure 5.1D) or in HCV-positive liver tissue after immunostaining with the pre-immune rabbit serum. Furthermore, preadsorption of the NS4 antiserum with the NS4 synthetic peptide prior to immunostaining, consistent with a parallel loss of reactivity in EIA, abolished the signal. In contrast, the peroxidase and EIA signals were retained following a mock adsorption of the antiserum against the peptide diluent.

5.4 CONCLUSIONS AND DISCUSSION

In this chapter, HCV antigen NS4 was detected in the hepatocyte cytoplasm in nontumorous liver tissue as well as in the cytoplasm of malignant hepatocytes. The staining intensity of NS4 in tumorous cells was lower than that seen in the nontumorous hepatocytes. In one case, nuclear staining was evident in nontumorous hepatocytes and in another sample NS4 was localised in intranuclear inclusions. The importance of nuclear NS4 remains unclear and consequently requires further investigation.

Liver cirrhosis is a precancerous state frequently leading to HCC regardless of the aetiology. Although cirrhosis develops in the liver of HBV carriers, HBV has been shown to integrate into cellular DNA during chronic infection (Shafritz *et al.*, 1981), suggesting a direct role for HBV in the initiation of hepatocyte transformation and progression to HCC. To date, the mechanism by which HCC occurs in HCV-infected individuals is unknown. However, HCV may merely be an aetiological agent for liver cirrhosis and the progression of cirrhosis to HCC may be a separate phenomena. Work presented in this chapter is consistent with this hypothesis since all liver samples with HCC were cirrhotic; however the sample number was small.

Nevertheless, the detection of a NS HCV protein in malignant hepatocytes, coupled with the previous observation of positive and negative sense HCV RNA in tumorous liver tissue by RT-PCR (Gerber *et al.*, 1992; Horiike *et al.*, 1993; Sherker *et al.*, 1993), suggest that HCV replication may be ongoing in these cells. This is in contrast to HBV-associated HCC in which HBV replication and gene expression are absent in poorly differentiated tumour cells (Nagaya *et al.*, 1987). Two studies found that HCC can occur in anti-HCV positive patients with inactive or mild chronic liver disease (Kiyosawa *et al.*, 1990; Watanabe *et al.*, 1994), suggesting that HCV may be an oncogenic virus. To date, the existence of an

oncogene or reverse trancriptase activity in the HCV genome has not been identified. The significance of HCV in the oncogenic process is still unclear, as NS4 was only detected in 40-60% of tumour cells and it is unknown if these were monoclonal.

Hence, this chapter represents a preliminary study that highlights the need to study an adequate number of biopsy samples from individuals with HCV-associated HCC. This will allow further investigation into the possible significance of HCV antigens in the tumour and a comparison of the levels of antigen within the tumorous and nontumorous cell populations.

CHAPTER 6

DETECTION OF HCV RNA BY *IN SITU* HYBRIDISATION

6.1 INTRODUCTION

ISH was originally developed in 1969 to detect specific nucleic acid sequences in cytological preparations (John *et al.*, 1969; Pardue and Gall, 1969), and has subsequently been adapted to permit detection of either DNA or RNA sequences in cell smears and tissue sections. ISH has been used to identify the chromosomal location of particular DNA sequences or to assess the level of expression of these genes by mRNA detection. In particular, mRNA is a common target for ISH reactions in studies of gene expression and cell differentiation. Furthermore, ISH can now be used to detect integrated viral DNA sequences, RNA transcripts or viral nucleic acid replicative intermediates in virus-infected cells and tissue sections. Finally, ISH is especially valuable where the histological identity and relationship of positive cells within the tissue are sought.

Prior to the development of an assay to detect HCV RNA by ISH, the viral genome was detected in extracts of liver samples from chronically-infected individuals by RT-PCR (Fong *et al.*, 1991; Shieh *et al.*, 1991; Shibata *et al.*, 1991; Takehara *et al.*, 1992). However, unless the RT-PCR assay is designed to identify negative strand RNA, present as a replicative intermediate, positive results may simply reflect serum contamination rather than active virus replication. Furthermore, PCR provides no information relating to the distribution and cell type which either supports HCV replication or is persistently infected. The low level of HCV RNA in the liver and serum suggests that the proportion of infected cells, or the viral load in infected cells, or both, are low. Hence, the aim of the work in this chapter was to develop a sensitive ISH assay to localise HCV RNA in infected liver tissue.

6.2 TECHNICAL CONSIDERATIONS FOR ISH

The sensitivity of ISH depends on: (i) the effect of tissue preparation on the retention and accessibility of target sequences; (ii) the effect of probe construction and hybridisation conditions on hybridisation efficiency; and (iii) the choice of the indicator molecule for probe labelling.

(i) Optimal fixation and tissue preparation should preserve the maximum level of cellular target sequences while maintaining optimal morphological details and allowing probe access. Successful fixation procedures were developed previously in the laboratory and consequently this aspect was not examined in detail.

(ii) Probe constructions vary and the available options include: (a) synthetic oligonucleotides which have a reduced sensitivity because they represent a low percentage of the total target sequence; (b) double-stranded cDNA labelled by random priming or nick translation to high specific activity (eg. 0.5-1x 10^9 dpm/μg). In the presence of dextran sulphate, cDNA probes show an ability for network formation over specific target sequences with an accompanying increase in sensitivity (Gerhard *et al.*, 1981); and (c) RNA transcripts generated by transcription from plasmids containing RNA polymerase promoters, as either sense or antisense RNA with high specific activity (eg. 3x 10^8 dpm/μg).

(iii) A number of indicator molecules are available for probe labelling and these include isotopic (eg. ^{32}P-, ^{125}I-, ^{3}H-, ^{35}S-, ^{33}P-dNTPs and ^{32}P-, ^{125}I-, ^{33}P-rNTPs) or non-isotopic molecules (eg. biotin-, digoxygenin (DIG)-, FITC-dNTPs and rNTPs). Generally, radioactive ISH reactions provide: (a) higher sensitivity; (b) the ability to measure probe specific activity; and (c) the ability to quantitate the signal by autoradiography.

However, in recent years there has been a move towards the use of non-isotopic methods, mainly due to the expense and half life of radioisotopes as well as the safety aspects. Biotin-, DIG- and fluorochrome-labelled probes provide a rapid means to detect virus nucleic acids at high resolution without many of the problems associated with the use of radioisotopes. FITC-labelled DNA probes have been used for the detection of DNA in chromosome smears, but to date no one has reported viral nucleic acid detection by these probes.

6.3 EXPERIMENTAL DESIGN

Initially, ^{32}P-labelled cDNA probes, prepared by the random prime method (section 2.5.2), were evaluated for the detection of HCV RNA in smears of PBMC and bone marrow cells by ISH (section 2.7). PBMC were chosen because RT-PCR detection of positive sense HCV RNA in PBMC had been described (Takehara *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a), and as a result thought to represent a good positive control. PBMC were isolated as described in section 2.7.3 from an anti-HCV, HCV RNA-, HIV-positive individual and the bone marrow cells isolated from an anti-HCV, HCV RNA-positive, HIV-negative donor. Briefly, lymphocytes were purified into T cell-enriched and mixed populations (T and B cells), then stimulated with either Con A (T cell stimulus) or LPS (B cell stimulus) for 24h (section 2.7.4). cDNA (section 2.5.2) and RNA (section 2.5.3) probe cocktails were then compared for the detection of HCV RNA in autopsy liver tissue by ISH (section 2.7). Radiolabelling was chosen initially over non-isotopic methods because of the increased efficiency of detection and because of previous experience with radiolabelled probes in the laboratory. ^{125}I was chosen to label RNA transcripts as this radioisotope provides a higher resolution of autoradiographic grains.

Initially, FITC-labelled RNA probes were assessed for the detection of viral RNA in infected liver tissue and the results compared with those of the radioisotopic

technique. HDV RNA has been detected previously in liver tissue by an ^{125}I-labelled RNA probe representing the complete HDV genome (Gowans *et al.*, 1988), and the intracellular copy number of HDV RNA estimated to be 0.5x 10^6 genomes per cell. Consequently, FITC-labelled RNA probes were used to detect HDV RNA in HDV-infected liver tissue to evaluate non-isotopic ISH.

FITC-labelled RNA probes were then used in the second part of this chapter to study positive strand HCV RNA localisation in both frozen and formalin-fixed liver biopsy tissue from anti-HCV positive individuals. Furthermore, as HCV may replicate via RF (section 1.6), the inclusion of a denaturation step prior to hybridisation was thought to be crucial to disassociate positive and negative RNA strands. Thus, following application of the probe to the tissue section, the slide was heated to 95°C for 5min and immediately cooled to 50°C prior to hybridisation (section 2.7.5).

6.4 RESULTS

6.4.1 Preface

Although radiolabelled probes provide higher sensitivity and allow quantitation of the autoradiographic signal, they are expensive and problems are encountered with safety and disposal. Hence, non-isotopic probes were investigated. However, DIG-labelled RNA probes may not be sensitive enough for detection of viral nucleic acids, and consequently FITC-labelled RNA probes were examined. Furthermore, FITC was thought to be a useful marker due to the experience in the laboratory with FITC-conjugated antibodies.

(i) Direct visualisation

Initially, HDV RNA was detected in formalin-fixed liver samples by a FITC-labelled RNA probe representing the complete HDV genome. A titration of the

FITC-labelled probe concentration from 10ng to 100ng per section was undertaken and at concentrations of ≥25ng, genomic HDV RNA was detected. The results were striking: genomic sense HDV RNA detection showed a distribution of HDV RNA-positive cells similar to that observed previously with ^{125}I-labelled probes (Gowans *et al.*, 1988), but in addition showed intranuclear distribution patterns which were not detected previously (Figure 6.1A,B). Very few nuclei showed homogeneous distribution patterns but even in those that did, the nucleoli were spared. In a majority of nuclei, a central region of varying size was negative indicative of marginated HDV RNA. These results clearly demonstrate the advantages of FITC-labelled probes. Thus, although FITC-labelled probes detected genomic sense HDV RNA, the copy number within infected cells is very high (see above section 6.3), and quantitation of non-isotopic detection systems is difficult.

The detection of antigenomic HDV RNA was then investigated since the ratio of HDV RNA genomic:antigenomic species has been reported to be approximately 20 to 30:1 (Gowans *et al.*, 1988). Greater than 50ng of FITC-labelled probe per section was required to detect antigenomic RNA. However, although the intranuclear distribution pattern was similar to that of genomic RNA, the intensity of the signal was reduced (Figure 6.1C). Thus, if we assume that the level of antigenomic HDV RNA was around 25,000 copies per cell, then 42x 10^6 bases of HDV RNA was detected within infected cells. This represents approximately 4468 copies of the HCV genome. Consequently, although the sensitivity of the system was not determined exactly, FITC appeared to be an appropriate non-radioactive isotope for the detection of HCV RNA.

(ii) Amplification of the FITC RNA signal

The direct detection of target RNA by FITC-labelled probes is dependent on the hybridisation efficiency and probe access to target sequences. Hence, amplification (analogous to autoradiography to detect bound radiolabelled

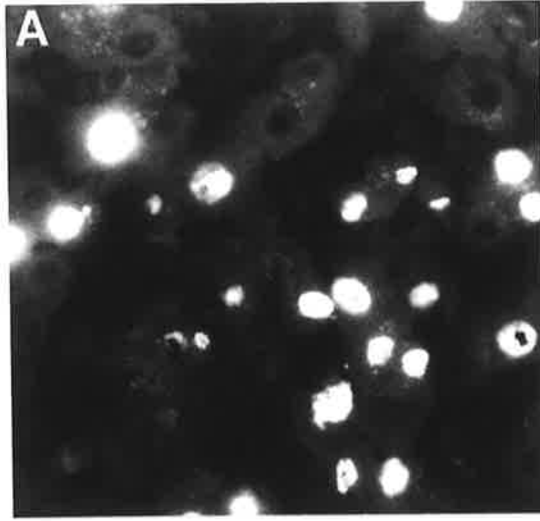
Figure 6.1 The detection of genomic and antigenomic sense HDV RNA in a HDV-infected liver sample using FITC-labelled RNA probes.

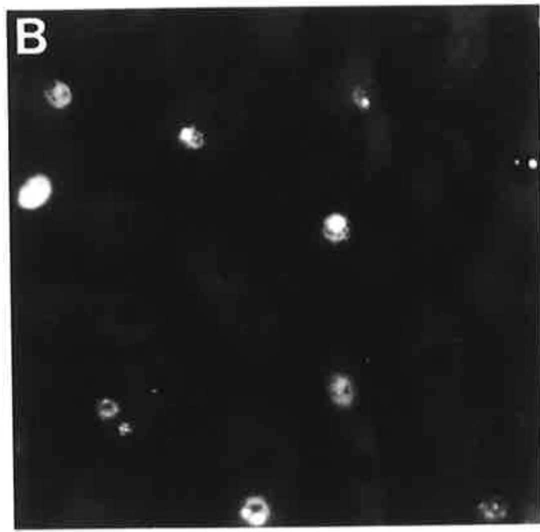
A. Intense staining for genomic RNA in the majority of infected hepatocyte nuclei.

B. Various nuclear localisation patterns of genomic RNA.

C. Detection of antigenomic RNA in infected hepatocytes.

Original magnification x400.


A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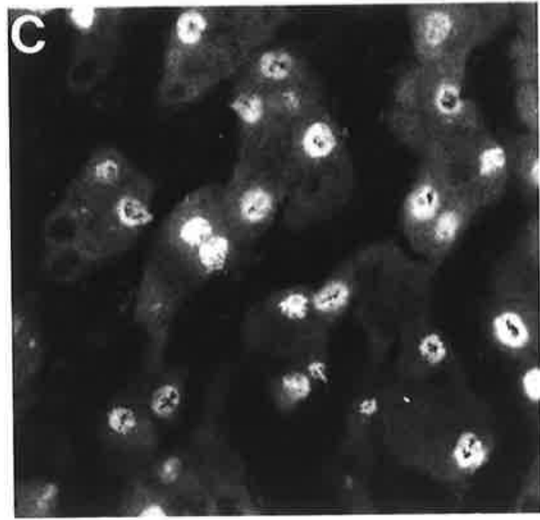
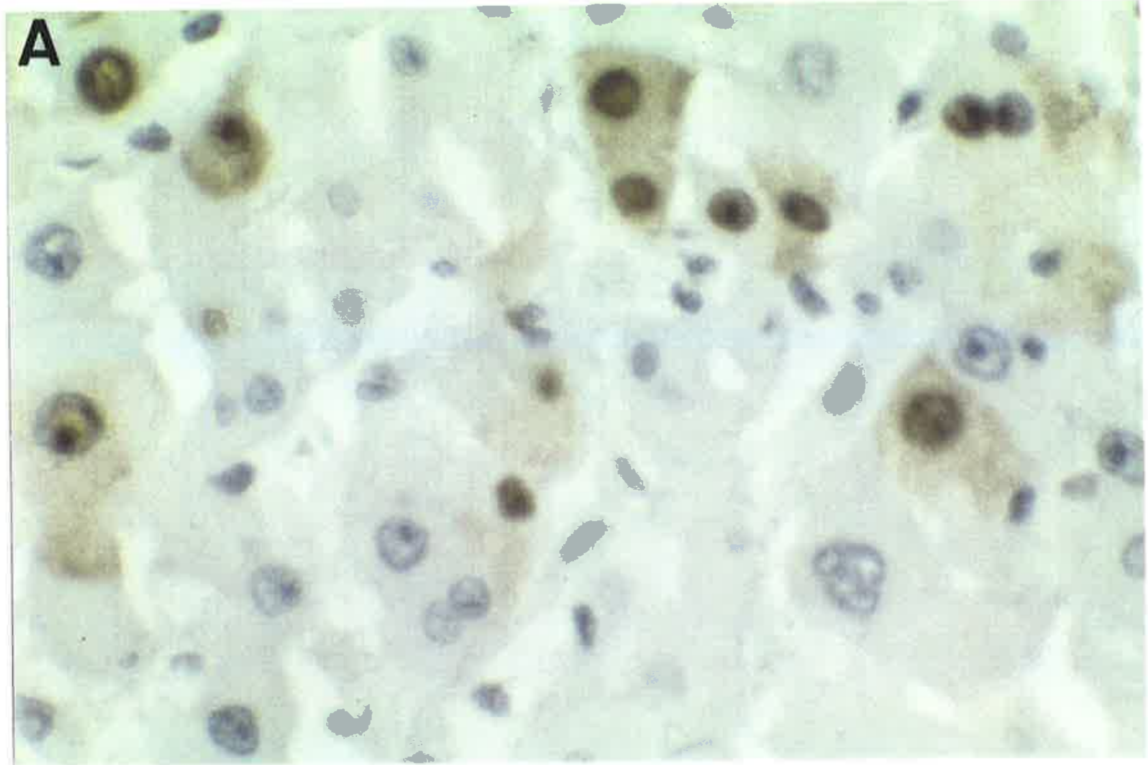

C

probes) may be necessary to detect the specific hybridisation signal. Furthermore, an additional advantage of amplification is that it allows conversion of a transient detection system into a permanent stain with good histological detail. Amplification of the FITC signal was achieved with mouse anti-FITC followed by HRP-conjugated goat-anti-mouse IgG as described in section 2.2.6(ii). Amplification of the genomic HDV RNA signal was assessed by the percentage of hepatocytes with nuclear and cytoplasmic staining. No amplification of the genomic HDV RNA could be distinguished as the proportion of positive nuclei remained the same as that generated by direct detection. However, a cytoplasmic signal was observed within infected hepatocytes that was not detectable by direct FITC-labelled probes (Figure 6.2A). Furthermore, an intense nuclear and weak cytoplasmic staining was observed following amplification of the antigenomic HDV RNA signal (Figure 6.2B). Hence, the FITC signal was amplified resulting in an increase in the sensitivity of detection.

6.4.2 Preliminary study of HCV RNA in peripheral blood and bone marrow cells

A number of studies have demonstrated the presence of the positive and negative strands of HCV RNA within PBMC by RT-PCR (Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Gunji *et al.*, 1994). Hence, a preliminary study was conducted to detect HCV RNA in mononuclear cells isolated from the peripheral blood of infected and uninfected individuals by ISH. A cocktail of cDNA probes representing approximately 35% of the HCV genome (Figure 6.3) was employed to increase the representation of the genome during hybridisation. When these studies were undertaken cDNA representing the complete HCV genome was unavailable. However, 4 cDNA fragments (genotype 1b sequences), 5' UTR (supplied by Dr. R Trowbridge; section 2.1.1), C-E1, NS3 and NS5 (a gift from Dr. T Miyamura; section 2.1.1) (Figure 6.3) were available to perform this work. ISH of the resultant cell smears, probed with either 200ng/ml or 50ng/ml of ^{32}P-

Figure 6.2 Amplification of the FITC hybridisation signal of genomic (A) and antigenomic (B) sense HDV RNA in a HDV-infected liver sample.
Original magnification x400.



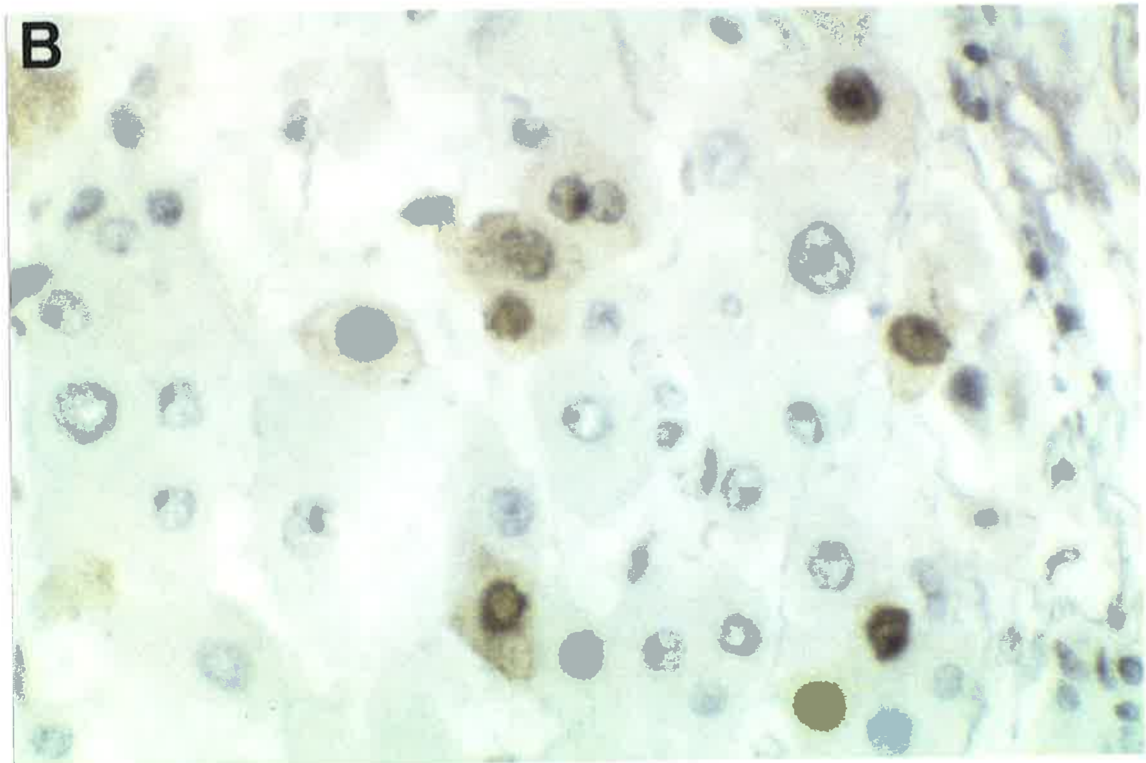


Figure 6.3 Location of HCV cDNA representing 35% of the genome.

Structural
Non-Structural
nt
1
341
911
1481
2771
3422
5315
6260
7604
9371
5'
C
E1
E2/NS1
NS2
NS3
NS
4A
NS4B
NS5A
NS5B
3'
5'UTR
NS3
NS5
C-E1

labelled random primed probes, was performed and the density of autoradiographic grains graded visually from + (~1 in 4900 cells positive) to ++++ (~1 in 800 cells positive). The maximum hybridisation signal was observed when cell smears were probed with 200ng/ml of the ^{32}P-labelled cDNA. The purity of these cell populations was not assessed. However, cell counts revealed that 1 in 3700 lymphocytes in the T lymphocyte-enriched fraction contained detectable levels of HCV RNA. Following Con A stimulation, HCV RNA was detected in 1 in 800 T cell-enriched lymphocytes (Table 6.1; Figure 6.4A), and since Con A stimulates T cells, it is likely that the T lymphocyte population were infected with HCV. Furthermore, LPS, a B cell stimulus, did not significantly increase the HCV RNA signal in the mixed (T and B lymphocytes) and T cell-enriched populations. However, ISH was not performed on B cell-enriched smears due to the difficulties of purifying the B lymphocyte fraction. HCV RNA was undetected in lymphocytes from an anti-HCV negative individual (Table 6.1; Figure 6.4B). In addition, HCV RNA was also detected in 1 in 3000 bone marrow cells purified from a potential anti-HCV positive donor (Figure 6.4C). However, no further studies were performed to determine the exact cell population infected with HCV due to the lack of PBMC samples, and since the experiments were focussed to permit the detection of HCV RNA in infected liver tissue. These results are discussed further in section 6.5.

6.4.3 Examination of liver autopsy samples for HCV RNA

(i) Histological diagnosis

Liver samples were collected from 2 anti-HCV positive patients who died as a result of myocardial infarct. The histological diagnoses were: Case 1 displayed active irregular cirrhosis, and piecemeal necrosis and bile duct proliferation was evident. The fibrous trabeculae separating the nodules contained lymphocytes, including aggregates, macrophages, some lipid-containing droplets and a few plasma cells. Case 2 was diagnosed as CPH. The portal tracts were expanded and fibrotic, and contained mild to moderate numbers of lymphocytes, some

Table 6.1 Detection of HCV RNA in lymphocyte cultures by *in situ* hybridisation.

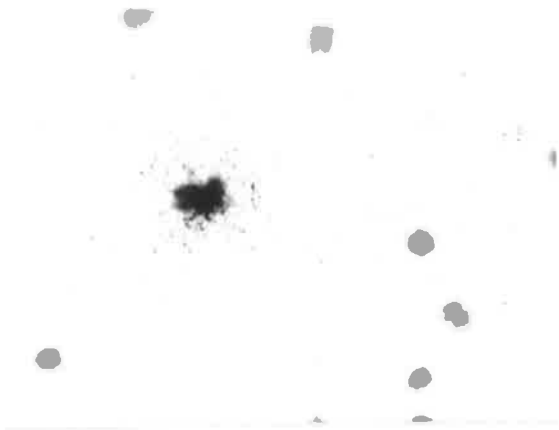
Patient	Mixed lymphocytes Stimulated with			T lymphocytes Stimulated with		
	None	Con A	LPS	None	Con A	LPS
HCV-positive	+	+++	++	++	++++	++
HCV-negative	-	-	-	-	-	-

Level of autoradiographic grains quantitated from + to ++++; + represents approximately 1 in 4900 cells positive and ++++ represents approximately 1 in 800 cells positive.
None = no stimulus
Con A = concanavalin A stimulus
LPS = lipopolysaccharide stimulus

Figure 6.4 Detection of HCV RNA by ^{32}P-labelled cDNA probes in peripheral blood lymphocytes isolated from a HCV-positive carrier (A) and a HCV-negative patient (B). HCV RNA in bone marrow cells from an anti-HCV positive individual (C).

Original magnification x400.

A



macrophages and a few eosinophils. The limiting plates were virtually intact. This part of the study was performed by Dr. R Rowland, Institute of Medical and Veterinary Science, Adelaide.

(ii) Detection of HCV RNA by RT-PCR

RNA was extracted from the 2 autopsy liver samples (section 2.3.1) and analysed for the presence of HCV RNA by RT-PCR (section 2.4). Agarose gel electrophoresis (section 2.1.4) followed by Southern blot hybridisation using a ^{32}P-end-labelled oligonucleotide (section 2.5.4; Figure 2.2), confirmed a 324bp cDNA product which was absent in a liver sample from an anti-HCV negative patient (Figure 6.5).

(iii) Detection of HCV RNA by random primed cDNA probes

Since 200ng/ml of the ^{32}P-labelled cDNA probe cocktail (35%) appeared to be the most sensitive for the detection of HCV RNA in PBMC, this concentration of probe was used to perform ISH on frozen sections from the 2 autopsy livers. Despite the different histological diagnoses, the distribution and abundance of HCV RNA-positive cells was similar in liver samples from both patients. The samples contained very few positive cells, determined as 3 to 4 cells per section on average, although some sections contained no positive cells. HCV RNA-positive cells were identified as small round cells possibly lymphocytes or macrophages (Figure 6.6A,B). No positive cells were noted in the uninfected liver tissue (Figure 6.6C), nor in the anti-HCV positive livers after hybridisation with an unrelated probe; in this case random primed pBluescript DNA was used.

(iv) Detection of HCV RNA by RNA probes

Although the initial experiments described above, using radiolabelled probes synthesised by the random prime method, showed a specific autoradiographic signal, problems were encountered in later experiments due to non-specific random network formation. Consequently, the use of RNA probes was

Figure 6.5 Southern blot hybridisation analysis of the 324bp RT-PCR product synthesised from RNA extracted from liver tissue.

Lane 1 HCV-negative liver sample

Lanes 2 and 3 represent liver tissue from case 1 and case 2 autopsy liver tissue, respectively

Lane 4 Molecular weight standards (Pst I digested λ DNA)

Lane 5 RNA positive control

Lane 6 DDW negative control

1 2 3 4 5 6

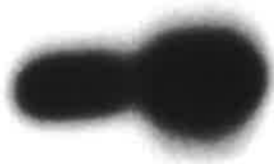
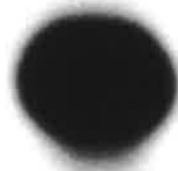
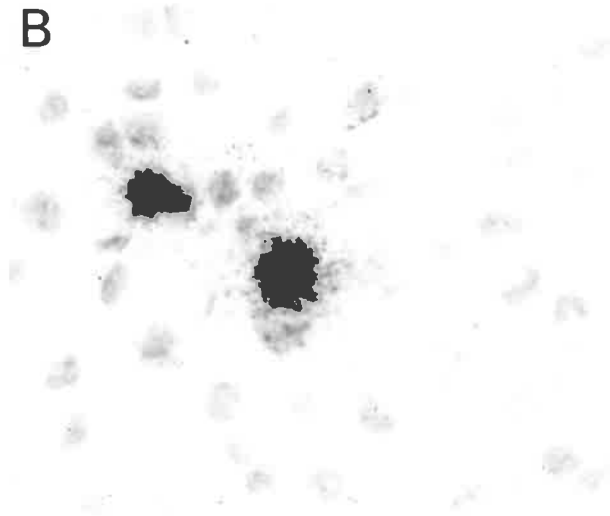



Figure 6.6 Detection of HCV RNA by ^{32}P-labelled cDNA probes representing 35% of the genome in frozen liver tissue from case 1 (A, B).

C. Uninfected liver tissue.

Original magnification x200 (A) and x400 (B, C).

A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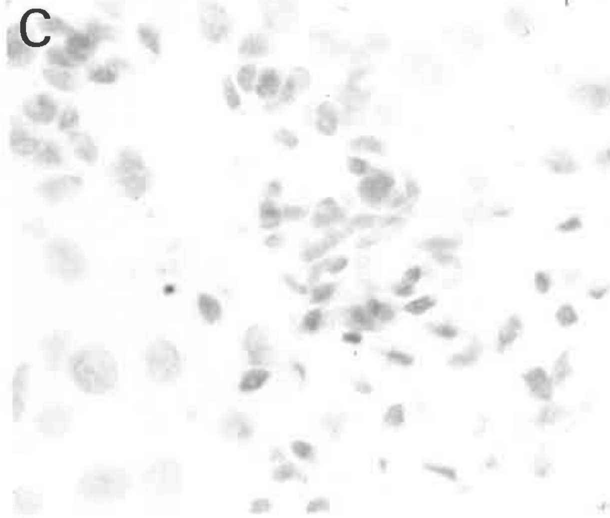

C

investigated due to their high specific activity and their inability to form networks. Furthermore, complementary RNA transcripts can be synthesised separately allowing the detection of either positive or negative sense HCV RNA.

The 4 cDNA fragments representing 35% of the HCV genome (Figure 6.3) were subcloned individually into pBluescript to permit the synthesis of RNA probes. It was hoped that a greater representation of the genome in the probe would result in improved sensitivity. In order to prove this theory, negative strand RNA probes representing 35% of the genome were labelled with ^{125}I and used to detect HCV RNA in the 2 autopsy liver samples. The autoradiographic signal was compared with that generated with a negative strand RNA probe corresponding to the 5' UTR (3.5% of the genome; Figure 6.3). No detectable signal was noted when sections were hybridised with the 5' UTR probe. In contrast, HCV RNA-positive cells were detected following hybridisation with a probe mix representing 35% of the genome. Thus, in later experiments the aim was to increase the representation of the genome in the probe mix.

Within the 2 autopsy liver samples only a few positive cells were identified and these were present as isolated cells found near the edge of the sinusoid, in portal tracts in the fibrotic liver (case 2) or in fibrous trabeculae of the cirrhotic liver (case 1) and occasionally in the parenchyma. The appearance and distribution of the positive cells suggested that they were not hepatocytes. Furthermore, the distribution and appearance of these HCV RNA-positive cells was similar to that observed with ^{32}P-labelled DNA probes (section iii above), suggesting that these represented the same cell types and providing additional confirmation that the autoradiographic signal was specific. Although it was not possible to conclusively identify infected cells, they were most likely to be lymphocytes or macrophages (Figure 6.7A,B). The HCV RNA-positive cells were only identified with probes to detect positive sense RNA; no positive cells were noted after hybridisation with probes to detect negative sense RNA.

Figure 6.7 Detection of positive sense HCV RNA by ISH in liver tissues from 2 anti-HCV positive patients using ^{125}I-labelled RNA probes representing 35% of the HCV genome.

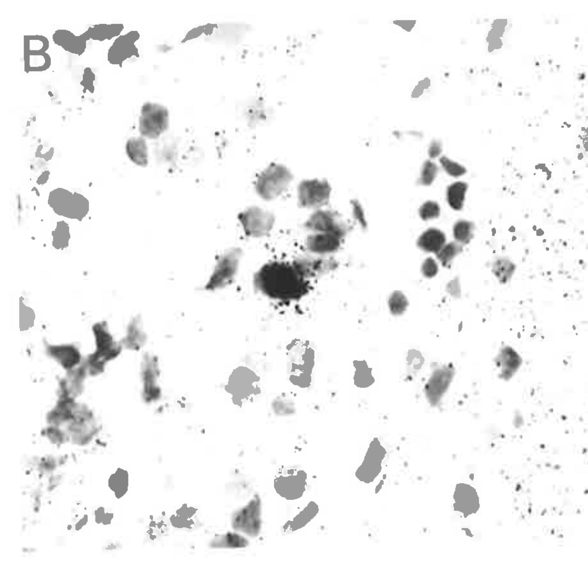
A. Case 1

B. Case 2

C. Anti-HCV negative patient

Original magnification x400.

A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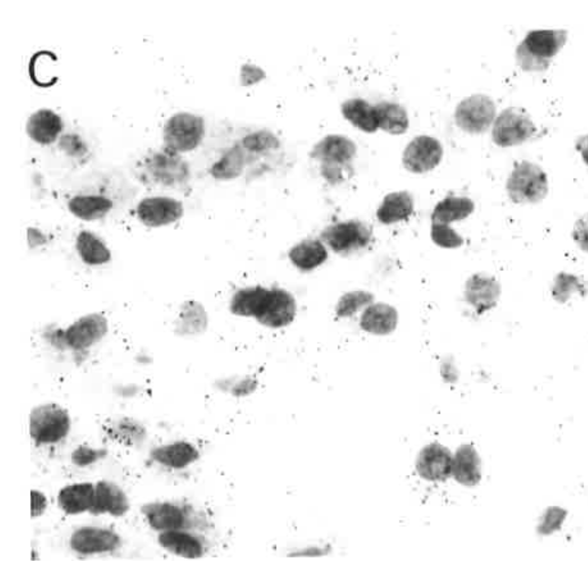

C

(v) Quantitation of the autoradiographic signal

The number of grains overlying 15 positive cells was counted and grains per positive cell found to be 50. Hence, the copy number of HCV RNA within these cells was calculated to be approximately 140 as shown below*.

(vi) Specificity

The specificity of the ISH reaction for HCV RNA detection was validated in a number of ways. Firstly, in sections of frozen liver tissue from uninfected individuals the autoradiographic signal was absent (Figure 6.7C). Secondly, no signal was noted when anti-HCV positive liver tissues were hybridised with an unrelated probe such as HBV, HDV and Duck HBV sequences. Thirdly, a post hybridisation melt curve of ^{125}I-labelled RNA probes bound to frozen sections of the 2 HCV-positive autopsy liver samples was performed. After hybridisation, the sections were washed in 0.1xSSC, 50% formamide at 5°C increments from 55°C to 85°C. Following autoradiography, the grain density over positive cells was graded from + to ++++ and plotted against the stringent wash temperature to construct the melt curve

* 1Da = 1.66×10^{-24}g

1kb ssRNA = 3.4×10^{5}Da

= 5.6×10^{-19}g

Hence, 1 HCV RNA genome = 5.3×10^{-18}g

Specific activity of the probe = 3.5×10^{8}dpm/μg = 3.5×10^{14}dpm/g

Assume the target is 50% saturated with probe

Then 1 HCV genome will produce 5.3×10^{-18}g x 3.5×10^{14}dpm/g x 0.5

= 9.3×10^{-4}dpm

= 4 dp 3 day exposure

Assume autoradiography for ^{125}I is 25% efficient (Gowans *et al.*, 1989)

Then 1 HCV genome will produce 1 grain per 3 days

However, positive cells contained 50 grains and the probe only represents 35% of the genome

Hence, the HCV RNA content was approximately 140 genomes/cell

(Figure 6.8). The Tm for bound RNA probes was found to be 65°C (Figure 6.8). In solution the theoretical Tm in 0.1xSSC, 50% formamide was calculated to be 67°C based on a 58% G+C content of the probe mix containing the 5' UTR, C-E1, NS3 and NS5 sequences (Table 6.2). However, the Tm of RNA:RNA hybrids formed by ISH has been reported to be 5°C lower than those formed in liquid hybridisation (Brahic and Haase, 1978). Hence, the observed value is consistent with these figures. At higher temperatures, the grains remaining over the cells reflected the level of background (Figure 6.8).

6.4.4 Detection of HCV RNA in liver biopsy samples

As work towards this thesis progressed, additional clones of HCV cDNA representing 45% of the genome (a gift from Dr. K Fukai; section 2.1.1) became available that were subsequently subcloned as 3 cDNA fragments into pBluescript (section 2.1) to permit the synthesis of 7 RNA probes representing 80% of the HCV genome (Figure 6.9). The data in section 6.4.3(iv) demonstrated a direct relationship between the genome representation in the probe during hybridisation and the sensitivity of detection. Consequently, for ISH experiments RNA probes representing 80% of the genome were labelled in a cocktail with FITC-UTP. However, for convenience all 7 probes were synthesised in two transcription reactions and the integrity and representation of the RNA probes was initially examined by labelling with ^{32}P only.

(i) Integrity of RNA probes

The linearised templates were pooled into 2 cocktails, ([E-NS2, NS2-3] and [5'UTR, C-E1, NS3, NS4, NS5]), and ^{32}P-labelled RNA probes synthesised. These ^{32}P-labelled RNA probe cocktails were loaded on a denaturing agarose gel (section 2.5.6), separated, and the size of the probes compared with a ^{32}P-end-labelled λ/Pst I standard and with the probes synthesised in individual transcription reactions (Figure 6.10A). The resultant probe cocktails were shown

Figure 6.8 HCV RNA post hybridisation melt curve.

After hybridisation, the sections were washed in 0.1xSSC, 50% formamide at 5°C increments from 55°C to 85°C, and the autoradiographic grain density graded from + to ++++.

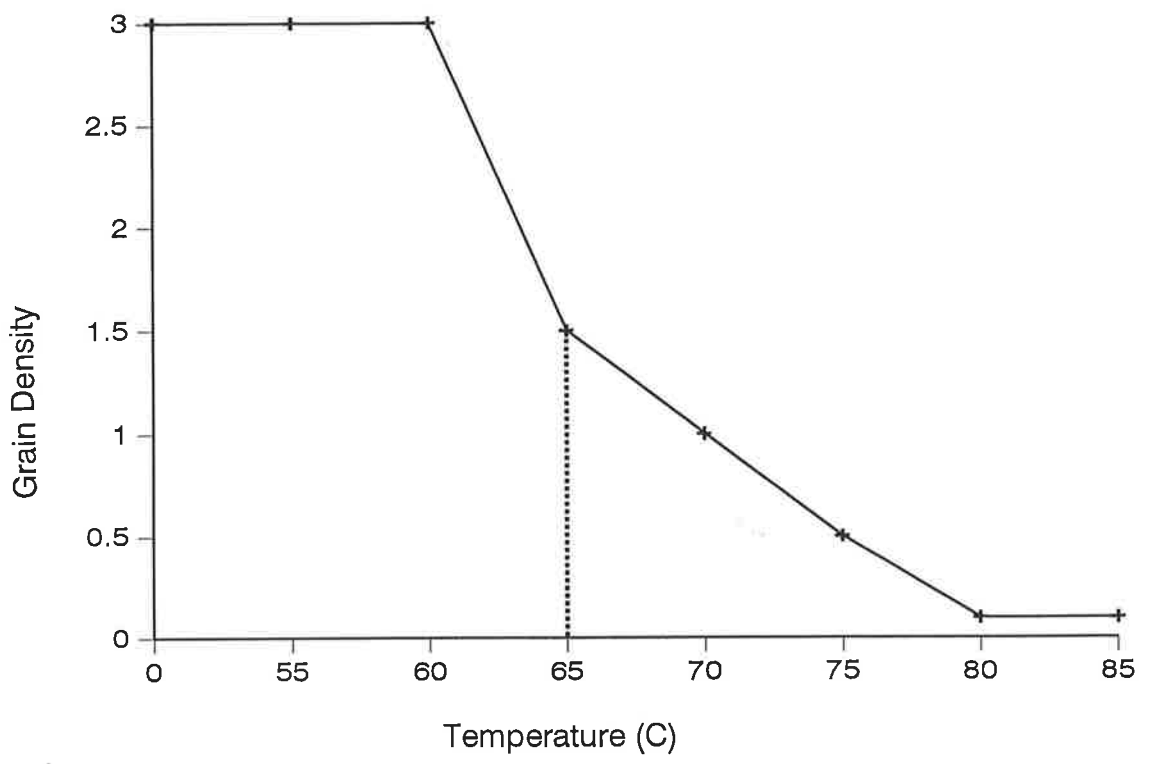

3
2.5
2
1.5
1
0.5
0
Grain Density
0
55
60
65
70
75
80
85
Temperature (C)

Table 6.2 Calculation of the T_m of RNA:RNA hybrids.

Probe	%G+C	T_m (°C) 0.1xSSC	T_m (°C) 0.1xSSC/50% FA
5' UTR	61	87	69
C-E1	60	86	68
E2-NS2	58	85	67
NS2-3	60	86	68
NS3	57	84	66
NS4	59	85	67
NS5	52	80	62

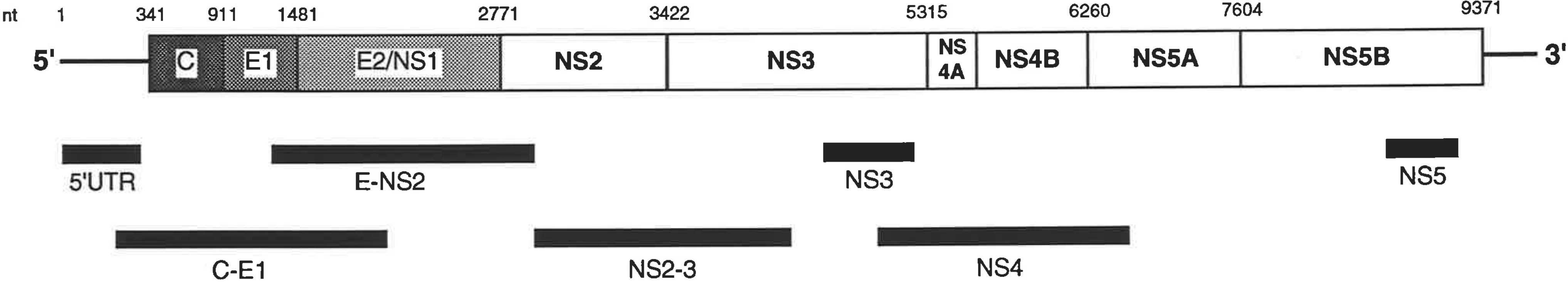
T_m = temperature at which double-stranded RNA hybrids will dissociate in liquid into single-stranded molecules is defined by the equation:

T_m (RNA:RNA) = 18.5 log [Na^+] + 0.584 (%G+C) + 79.8 + 0.0012 $(\%G+C)^2$ - 0.35 (%FA) (Bodkin and Knudson, 1985).

Where [Na^+] = concentration of salt; %G+C = percentage of G+C content; %FA = concentration of formamide.

However, the T_m of RNA:RNA hybrids formed by ISH has been reported to be 5°C lower than those formed in liquid hybridisation (Brahic and Haase, 1978).

Figure 6.9 Location of the HCV RNA probes representing 80% of the genome.



Structural
Non-Structural
nt
1
341
911
1481
2771
3422
5315
6260
7604
9371
5'
C
E1
E2/NS1
NS2
NS3
NS
4A
NS4B
NS5A
NS5B
3'
5'UTR
E-NS2
NS3
NS5
C-E1
NS2-3
NS4

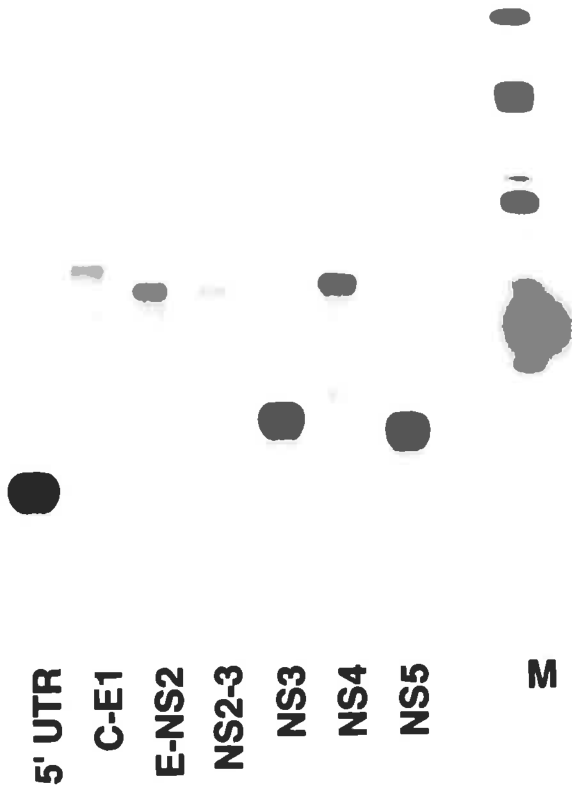
Figure 6.10 Length of ^{32}P-labelled RNA transcripts in denaturing agarose gels prior to use in ISH experiments.

A. RNA probes synthesised from individual DNA templates.

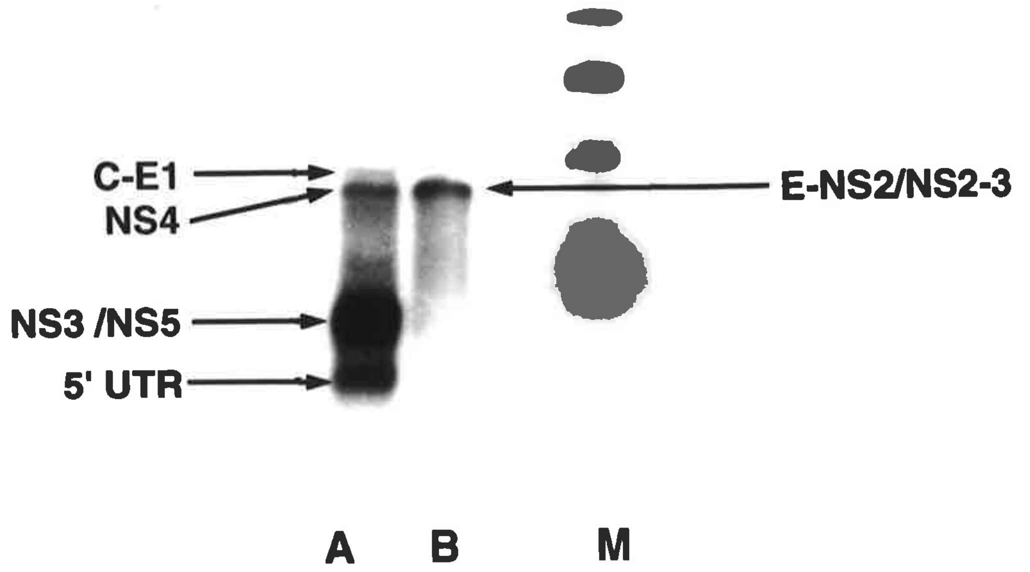
B. RNA probes synthesised from a cocktail of linearised DNA templates. 5' UTR, C-E1, NS3, NS4 and NS5 RNA probe cocktail (A) and E-NS2 and NS2-3 RNA probe cocktail (B).

M λ DNA digested with Pst I

A



B



to contain full-length transcripts with sizes ranging from 324bp to 1650bp as expected (Figure 6.10B).

(ii) Representation of RNA probes

To determine that the 7 probes prepared in the 2 cocktails described above were represented equally, the probe cocktails were hybridised to 1ng and 10pg of cold full-length HCV RNA (transcribed in individual *in vitro* transcription reactions) (section 2.5.3) that was immobilised on nitrocellulose membranes. After hybridisation, the membranes were washed at temperatures increasing in 5°C increments from 75°C to 85°C. At 75°C the radioactive signal from each target was strong (Figure 6.11). Temperatures above 75°C resulted in a reduction of the autoradiographic signal, but HCV RNA was still detected at high stringency (85°C) (Figure 6.11). These results were consistent with the theoretical Tm of 84°C (Table 6.2) and the post hybridisation melt curve constructed in section 6.4.3(vi). However, the stringent washes in the construction of the post hybridisation melt curve (Figure 6.8) were carried out in 50% formamide, reducing the Tm of RNA:RNA hybrids by 18°C (Table 6.2). This reduction in Tm is consistent with data from McConaughy *et al*, (1969) that showed 1% formamide reduces the Tm of RNA:RNA hybrids by 0.36°C. Furthermore, it was clear that the RNA probes transcribed from the 2 cocktails hybridised equally well to each of the immobilised RNA species, and thus the probe was fully representative.

(iii) Histological analysis of liver biopsy tissue

Liver biopsy samples from ten patients were examined by routine histological diagnosis. The liver histology of the patients ranged from mild CPH to moderate CAH. Mild to severe steatosis was observed in the majority of liver tissues. The basic hepatic architecture was preserved in all patients, although portal tracts were expanded by a mixed inflammatory cell infiltrate, and in 6 patients areas of piecemeal necrosis were noted.

Figure 6.11 Slot blot hybridisation to detect HCV RNA targets.

1ng and 10pg of HCV RNA target synthesised *in vitro* was immobilised to nitrocellulose and hybridised with the RNA probe cocktail representing 80% of the HCV genome. Following hybridisation, stringent washes were performed at 75°C, 80°C or 85°C.

5' UTR C-E1 E-NS2 NS2-3 NS3 NS4 NS5

1ng

10pg

75°C

1ng

10pg

80°C

1ng

10pg

85°C

(iv) Detection of HCV RNA in serum by RT-PCR

The presence of HCV RNA in the serum was confirmed by RT-PCR (section 2.4). The products were analysed by agarose gel electrophoresis (section 2.1.4) and the specificity confirmed by Southern blot hybridisation using a ^{32}P-end-labelled oligonucleotide (section 2.5.4; Figure 2.2). All 10 samples were positive for HCV RNA; the results were similar to those shown previously in Figure 3.1 and Figure 4.1.

(v) HCV RNA detection in frozen tissue

Following the detection of HDV RNA within infected liver tissue by FITC-labelled probes (section 6.4.1), the system was evaluated for HCV RNA detection in frozen biopsy tissue samples. The liver samples tested had been shown previously to contain HCV proteins by immunohistochemistry as described in Chapter 3. Consequently, infected cells were thought to be supporting virus replication, and thus were likely to contain HCV RNA.

No convincing signal was observed in the sections after ISH due to a high non-specific FITC signal. There are a number of possibilities to account for this: (a) the temperature of the stringent wash (75°C) was too low and allowed non-specific binding; (b) non-specific hybridisation to frozen liver tissue, since the background seen in formalin-fixed was negligible (see below). Furthermore, when the FITC-labelled HDV RNA probe was used as a negative control on frozen sections, the same background was noted. As a result, formalin-fixed tissues were used in the following experiments to detect HCV RNA.

(vi) HCV RNA detection in formalin-fixed tissue

Formalin-fixed liver tissue from the 10 anti-HCV positive patients was chosen to detect HCV RNA by a FITC-labelled RNA probe cocktail, representing 80% of the HCV genome (Figure 6.9), due to the non-specific FITC signal observed in frozen sections. Initially, a titration of the FITC-labelled RNA probe concentration from

50ng to 400ng per section was performed. Although the background was negligible, no hepatic or lymphocytic FITC signal was observed in all 10 liver samples irrespective of the concentration of the probe cocktail, and inclusion of a denaturation step prior to hybridisation.

As described in section 6.4.1, amplification of the FITC HDV RNA signal resulted in an increased sensitivity of detection. Hence, amplification of the HCV RNA:RNA hybrids, formed during hybridisation was performed. However, no peroxidase signal was observed in the 10 samples following antibody staining for the FITC molecules. Hence, direct detection by FITC-labelled RNA probes and amplification of the RNA:RNA hybrids was unsuccessful for the detection of HCV RNA.

6.5 CONCLUSIONS AND DISCUSSION

This chapter describes the development of an ISH assay to detect HCV RNA in infected tissue sections. Initially, HCV RNA was detected within stimulated and unstimulated PBMC and bone marrow cells using cDNA probes representing 35% of the HCV genome to assess extrahepatic HCV infection and as a positive control. The data suggest that T lymphocytes were the predominant population of PBMC that were infected with HCV, since the number of positive cells was increased following stimulation with Con A, but not LPS. Stimulation may promote an increase in virus replication within individual cells, which in turn may lead to secretion of viral particles able to infect surrounding cells. Considering that the cells were only stimulated for 24h, this hypothesis seems unlikely. Hence, it is more likely that virus replication was increased in stimulated cells resulting in an increase in the level of HCV RNA in infected cells to above the level of sensitivity of detection. This data is consistent with a recent study that detected HCV RNA positive and negative strands in PBMC from HCV-positive patients by ISH (Moldvay *et al.*, 1994). Furthermore, mitogenic stimulation of the

PBMC increased HCV RNA replication as determined by negative strand HCV RNA detection (Moldvay *et al.*, 1994). Finally, this ISH data is consistent with detection of positive and negative HCV RNA in PBMC by RT-PCR (Bouffard *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Gunji *et al.*, 1994; Muratori *et al.*, 1994).

Using radiolabelled DNA and RNA probes, HCV RNA was detected in a small proportion of cells thought to be lymphocytes or macrophages, but not in hepatocytes in the two autopsy liver samples examined. It is unclear if these results are a consequence of the poor sensitivity of the ISH reaction, or due to the autopsy nature of the liver samples. HCV RNA in hepatocytes may be unstable and degraded unless the liver samples are snap frozen immediately after death. Furthermore, since it was not possible to detect negative sense HCV RNA in these cells, it is unclear if HCV replication was actually ongoing in these cells. Positive sense HCV RNA may simply represent phagocytosis. Alternatively, since the positive sense RNA copy number was calculated to be approximately 140, the ISH assay may be too insensitive to detect negative strand RNA which is likely to be 10 to 100-fold less abundant than positive sense RNA (Westaway, 1987; Fong *et al.*, 1991; Wang *et al.*, 1992a; Horiike *et al.*, 1993).

The full range of controls which are desirable for ISH were unable to be performed, due to the paucity of HCV RNA-positive cells in the autopsy liver tissue. However, the control reactions which were performed, viz. unrelated probes, uninfected tissue samples and the construction of a post hybridisation melt curve suggested that the reaction was specific. Furthermore, the fact that only the antisense probes generated a signal is additional evidence for the specificity of the reaction.

Despite attempts to increase the sensitivity of ISH detection, such as 80% representation of the HCV genome during hybridisation, FITC-labelled RNA

probes were unable to detect HCV RNA in infected liver tissue. Although FITC ISH was successful for genomic and antigenomic HDV RNA detection, the copy number of these RNA molecules was 0.5x 10^6 and 2.5x 10^4 genomes per cell, respectively. HCV RNA is likely to be at a low level since viraemia in infected individuals is low (Weiner *et al.*, 1990; Fong *et al.*, 1991). Furthermore, radioactive ISH detected 140 HCV genomes per mononuclear cell and HCV RNA was not detected in hepatocytes. Amplification by antibodies (analogous to autoradiography to detect bound radiolabelled probes) also failed to detect HCV RNA:RNA hybrids in infected liver tissue.

A number of groups have detected HCV RNA in formalin-fixed and frozen liver tissue using non-isotopic methods with oligonucleotide and RNA probes corresponding to the 5' UTR or the C region. In one study, virus RNA was detected in a widespread number of hepatocytes in acute phase chimpanzee samples (Negro *et al.*, 1992), and in others a few randomly distributed hepatocytes were positive in samples from chronic carriers (Lamas *et al.*, 1992; Haruna *et al.*, 1993; Yamada *et al.*, 1992; Nouri Aria *et al.*, 1993; Tanaka *et al.*, 1993; Yamada *et al.*, 1993). Hence, this chapter clearly demonstrates the necessity for a sensitive ISH assay for the detection of HCV RNA. This is consistent with an unconfirmed report in which nearly 100% of hepatocytes contained HCV RNA following RT-PCR/ISH (Nuovo *et al.*, 1993).

CHAPTER 7

PCR ANALYSIS OF HCV RNA WITHIN HCV-INFECTED LIVER SAMPLES

7.1 INTRODUCTION

The inability to successfully propagate HCV *in vitro* has hindered progress in determining the strategy of HCV replication. However, the classification of HCV in the Flaviviridae has led to the postulation that HCV may adopt a RNA replication strategy closely related to that of Flaviviruses (Westaway, 1987; Chambers *et al.*, 1990a). The steps involved in the replication of Flaviviruses are described in detail in section 1.6. Briefly, input viral RNA is translated to produce the polyprotein; this is cleaved into the individual polypeptides followed by virus RNA replication through a negative strand intermediate, virion assembly and release from the infected cell (Figure 1.2). Although several reports have described the detection of negative sense HCV RNA in infected liver tissue by conventional RT-PCR using a sense primer for RT (Fong *et al.*, 1991; Shibata *et al.*, 1991; Takehara *et al.*, 1992; Horiike *et al.*, 1993; Muller *et al.*, 1993a; Sherker *et al.*, 1993), the authenticity of these studies has been questioned by four reports (Willems *et al.*, 1993; Gunji *et al.*, 1994; Lanford *et al.*, 1994; McGuinness *et al.*, 1994). Furthermore, although the detection of the negative RNA strand in tissue samples indicates active viral replication, it provides no information about the configuration of the HCV RNA, such as the presence of RF. Hence, the work described in this chapter aimed to study the nature of HCV RNA in liver tissue from naturally-infected individuals.

7.2 EXPERIMENTAL DESIGN

Liver tissue was collected from two anti-HCV positive patients who had undergone liver transplantation for HCV-related end stage chronic liver disease. Specimens of the explanted livers were immediately frozen in liquid nitrogen and stored at -70ºC. The samples were kindly provided by Professor G Farrell, Westmead Hospital, Sydney. Liver samples from HCV-negative patients, obtained during surgery, were snap frozen as above and served as negative controls. RNA was extracted from liver tissue (section 2.3.1) and HCV-specific RNA detected by RT-PCR using primers designed to amplify a 324bp fragment from the 5' UTR (section 2.4; Figure 2.2). The products were analysed by agarose gel electrophoresis (section 2.1.4) and the specificity confirmed by Southern blot hybridisation using a ^{32}P-end-labelled internal oligonucleotide (sections 2.5.4 and 2.6; Figure 2.2). In addition, each set of RT-PCR examined HCV RNA-negative liver tissue and substitution of the RNA component with DDW.

A number of approaches were undertaken to determine if RF exist in naturally-infected liver tissue.

(1) A RT-PCR assay was developed to detect negative sense HCV RNA following 3' end chemical modification of total liver RNA samples (section 2.3.4).

(2) Total liver RNA was subjected to either no heat, 65ºC or 100ºC for 5 min prior to RT-PCR and during the subsequent PCR, 10µl samples were collected after 25, 30 and 35 cycles.

(3) RNase A digestion of liver RNA was performed in DDW or high salt (0.75M NaCl) at 37ºC or 100ºC (section 2.3.2) and RT-PCR performed on denatured or native products of these reactions.

(4) Liver RNA preparations were adjusted to 2M LiCl in a precipitation step designed to separate ssRNA and dsRNA (section 2.3.3). RT-PCR was then performed on the pellet and supernatant.

Following RT-PCR in approaches 1-4, the intensity of the resultant ethidium bromide stained HCV-specific products was compared after agarose gel electrophoresis.

(5) Immunostaining for dsRNA (sections 2.2.2 and 2.2.5) was performed on frozen and formalin-fixed, wax-embedded liver tissue from chronic carriers of HCV.

7.3 RESULTS

7.3.1 Detection of HCV RNA

Initially, attempts were made to detect HCV RNA by Northern blotting (section 2.6.1) followed by hybridisation with specific negative sense ssRNA probes representing 80% of the viral genome (section 2.6.2; Figure 6.9). HCV RNA was not detected in liver samples from the HCV-infected individuals, presumably due to either the low copy number or instability of HCV RNA. Liver RNA preparations were shown to be of good quality, as assessed by the detection of intact 18s and 28s rRNA following denaturing agarose gel electrophoresis (section 2.5.6) and ethidium bromide staining. However, HCV RNA was amplified from 1μg and 10μg of total RNA extracted from both liver samples (Figure 7.1A). The specificity of these products was confirmed by Southern blot hybridisation using an internal ^{32}P-end-labelled oligonucleotide (Figure 7.1B). Liver tissue from case 1 was chosen for further study as this contained a higher level of HCV RNA (Figure 7.1A).

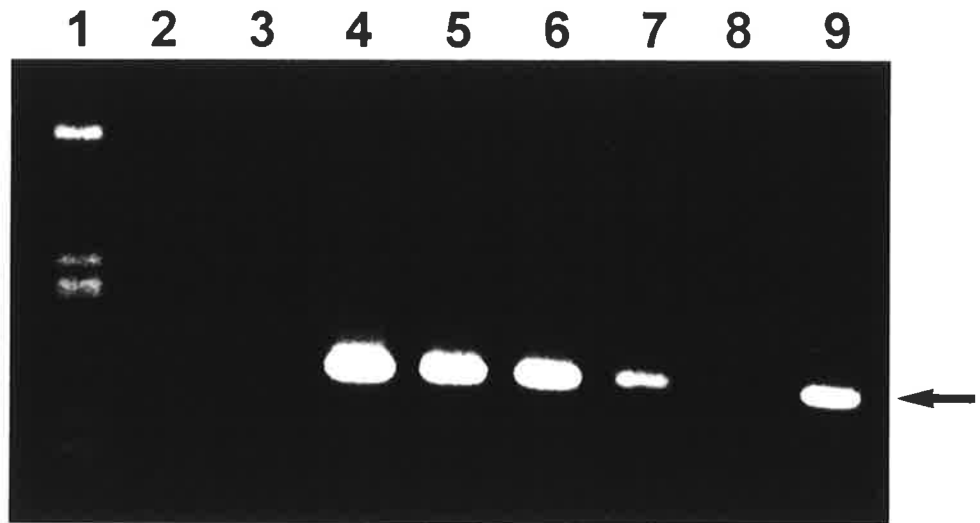
RT-PCR was performed on serial dilutions (500ng to 0.1ng) to determine the concentration of total RNA for use in further studies. HCV sequences were amplified and detected from 500ng to 1ng of liver RNA, but not 0.1ng (Figure 7.2). The intensity of the cDNA products amplified from 500ng, 100ng and 50ng of liver RNA were identical, although a gradual reduction in the cDNA yield was

Figure 7.1 Agarose gel electrophoresis (A) and Southern blot hybridisation (B) analyses of RT-PCR products generated from liver tissue from cases 1 and 2.

Lane 1	λ DNA marker digested with Pst I
Lane 2	Blank
Lane 3	DDW negative control
Lanes 4 and 5	10μg and 1μg case 1 liver RNA, respectively
Lanes 6 and 7	10μg and 1μg liver RNA from case 2, respectively
Lane 8	10μg RNA from a HCV-negative liver tissue
Lane 9	10pg *in vitro* transcribed 5' UTR RNA

The position of the 324bp product is shown by an arrow and is consistent with the molecular weight marker (lane 1).

A



B

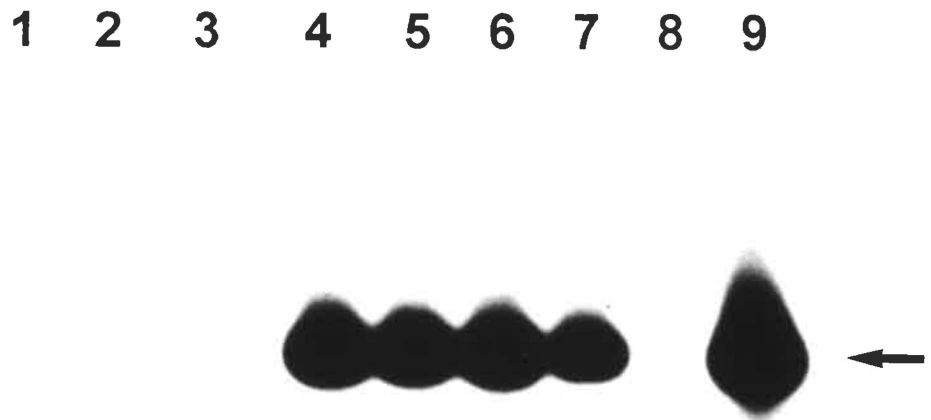


Figure 7.2 Agarose gel electrophoresis analysis of the 324bp HCV-specific cDNA products generated from serial dilutions of case 1 liver RNA.

Lane 1	500ng
Lane 2	100ng
Lane 3	50ng
Lane 4	10ng
Lane 5	5ng
Lane 6	1ng
Lane 7	0.1ng
Lane 8	λ DNA digested with Pst I

1
2
3
4
5
6
7
8

observed with 10ng to 1ng of liver RNA. Hence, 50ng was chosen for subsequent analyses since this was the lowest concentration of liver RNA to produce an intense product.

7.3.2 Detection of negative sense HCV RNA

To determine if replication was occurring in HCV-infected liver tissue, a strand specific RT-PCR assay was developed to detect negative sense HCV RNA.

(i) *In vitro* transcribed 5' UTR RNA

Initially, the specificity of strand specific RT-PCR was determined using positive and negative sense 5' UTR RNA synthesised separately by *in vitro* transcription. All RT-PCR assays were performed in the presence of hepatic cellular RNA from normal liver to mimic the conditions used to detect HCV RNA in RNA extracts from infected liver samples. Initially, a RT-PCR assay was employed that used only one primer during cDNA synthesis, followed by inactivation of the RTase and amplification of the cDNA by PCR with both primers present. However, this technique lacked strand specificity in that positive and negative sense 5' UTR were amplified when the incorrect primer for cDNA synthesis was added (Figure 7.3A, lanes 3 and 6). Furthermore, a PCR product was amplified in the absence of a primer during RT when the two primers were subsequently added for PCR amplification (Figure 7.3A, lanes 4 and 7). Control reactions without RTase, performed in parallel, were negative, indicating that the false products originated from the RNA templates (Figure 7.3A, lane 8). The lack of strand specificity may be due to false priming, in which fragmented HCV RNA and/or cellular RNA molecules act as nonspecific primers for RT as described in other reports (Willems *et al.*, 1993; Gunji *et al.*, 1994; Lanford *et al.*, 1994; McGuinness *et al.*, 1994).

Figure 7.3 Agarose gel electrophoresis analysis of the cDNA products following strand specific RT-PCR.

A. Amplification of *in vitro* transcribed 5' UTR RNA by conventional RT.

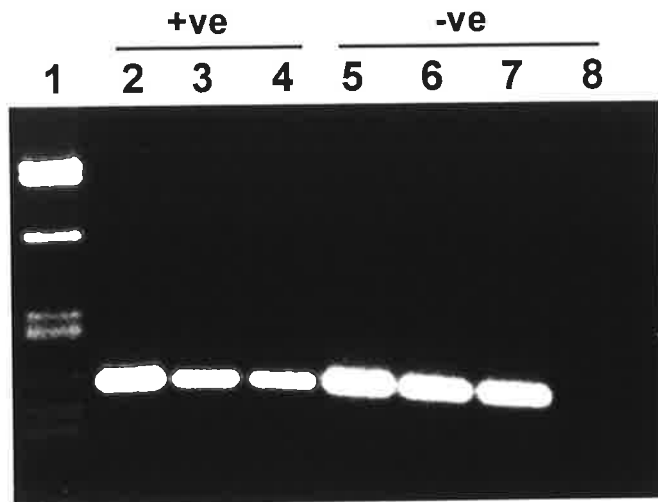
Lanes 2-4 and 5-8 represent the products after RT-PCR of *in vitro* transcribed positive and negative strand HCV RNA, respectively. Lanes 2 and 6 show the results after RT (followed by PCR) using primer 154 (antisense), lanes 3, 5 and 8 the results using primer 153 (sense), and lanes 4 and 7 the results without the addition of any primers during RT. Lane 8 represents the product resulting from RT without the addition of the enzyme.

B. Amplification of *in vitro* transcribed HCV RNA and HCV RNA from total cellular RNA following 3' end chemical modification.

Lanes 2 and 3, lanes 4-7, lanes 8-11 and lanes 12 and 13 represent positive sense 5' UTR RNA (+ve), negative sense 5' UTR RNA (-ve), case 1 liver RNA (HCV +ve), and liver RNA from a HCV-negative individual (HCV -ve), respectively. Lanes 2, 5, 8 and 12, and lanes 3, 4, 7, 9, 11 and 13, contain either 154 or 153 during cDNA synthesis, respectively. Lanes 6 and 10 represent cDNA synthesis without primer, but both primers added for PCR. Lanes 7 and 11 represent the products resulting from RT without the addition of RTase.

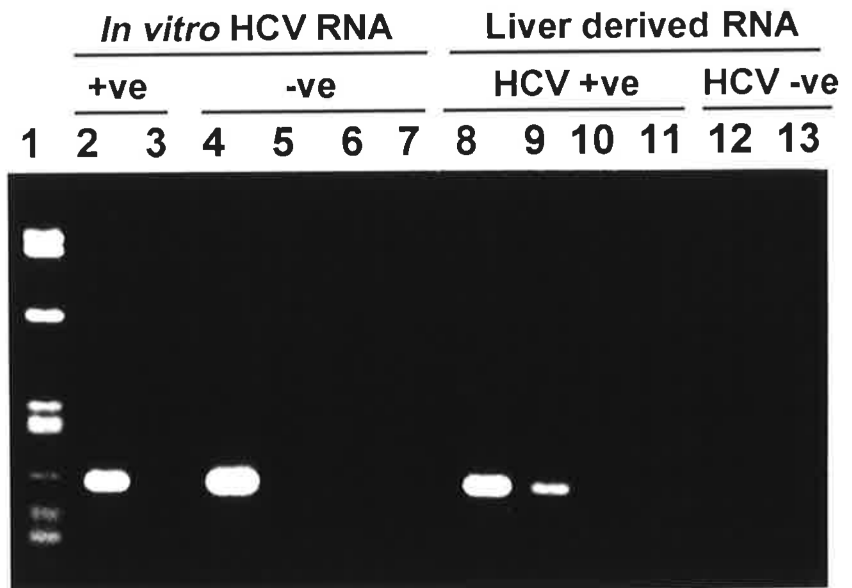
Lane 1 λ DNA digested with Pst I

A



Primer for RT 154 153 - 153 154 - 153

B



Primer for RT 154 153 153 154 - 153 154 153 - 153 154 153

To address the question of whether fragmented RNA molecules are actually acting as primers during RT, the RNA samples were modified at the 3' end by chemical agents (section 2.3.4) prior to cDNA synthesis. This method converts the 3' terminal nucleotide of RNA to a dialcohol and as a result, RNA with modified 3' ends cannot act as primers. One chemical modification of the RNA samples did not result in complete strand specificity. However, following a second chemical modification of the RNA samples, positive and negative strands of the *in vitro* transcribed 5' UTR RNA were specifically amplified using the antisense (154) and sense (153) primer, respectively for RT (Figure 7.3B, lanes 2 and 4). In addition, omission of the primer (Figure 7.3B, lane 6) or addition of the incorrect primer (Figure 7.3B, lanes 3 and 5) during cDNA synthesis, failed to generate a cDNA product. Hence, two chemical modifications circumvented the amplification of falsely primed cDNA products and as a result strand specificity was established.

(ii) Liver RNA

Liver RNA from case 1 was chemically modified at the 3' end and strand specific RT-PCR performed as described above. In addition to the positive sense HCV RNA, the negative strand was also detected in the liver RNA preparation (Figure 7.3B, lane 9). From ethidium bromide staining, it was estimated that the ratio of positive:negative strands was 10:1 (Figure 7.3B, lane 8 and 9). Furthermore, no cDNA products were observed when both primers were omitted from the RT step (Figure 7.3B, lane 10), when case 1 liver RNA was replaced by RNA from a HCV-negative individual (Figure 7.3B, lanes 12 and 13), and when RT was performed without the addition of RTase (Figure 7.3B, lane 11).

7.3.3 Effect of heat on HCV RNA detection

To determine whether RF are present in HCV-infected liver, prior to the RT step, the extracted RNA was heated to 65°C and 100°C for 5 min and the results

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compared with results from samples without heat treatment. These temperatures were chosen as 65°C was considered to be high enough to remove secondary structures without denaturing the RNA, while 100°C was expected to completely denature double-stranded RNA forms. A comparison of the intensity of the ethidium bromide stained HCV-specific products after 25, 30 or 35 cycles in the PCR, respectively found no differences regardless of the heat treatment (Figure 7.4). Furthermore, the final yield of cDNA after 35 cycles was similar in duplicate tubes, from which 10μl samples were removed after 25 and 30 cycles, compared with tubes which were not sampled in this manner (Figure 7.4), suggesting that sampling did not affect amplification. Hence, denaturation of liver RNA prior to RT did not increase the yield of cDNA, suggesting HCV RNA was single-stranded.

7.3.4 RNase A digestion of HCV RNA

Single-stranded RNA has been reported to be digested specifically by RNase in high salt leaving double-stranded (RNA:RNA) hybrids intact (Pardue, 1985; Harper *et al.*, 1986). Hence, the sensitivity of HCV RNA to RNase A in DDW and high salt was assessed. The RNase A digestions were performed not only at the optimal reaction temperature of 37°C, but also at 100°C to ensure that all dsRNA would be fully denatured during digestion and thus, this step would constitute a negative control. Positive controls included the omission of RNase A from the digestion reactions.

(i) *In vitro* transcribed 5' UTR RNA

RNase A was titrated in DDW and high salt to determine the lowest concentration required to completely digest full-length *in vitro* transcribed ssRNA from the 5' UTR of the HCV genome. This was assessed by the absence of a 324bp cDNA product after RT-PCR. As a positive control, the 5' UTR RNA was amplified from digestions performed at 37°C and 100°C in the absence of RNase A (Figure 7.5, lanes 1 and 3). The 5' UTR RNA was not amplified following digestion with

Figure 7.4 Agarose gel electrophoresis analysis of HCV-specific cDNA products following heat treatment at 65°C and 100°C prior to RT-PCR.

Lanes 1-4, lanes 5-8 and lanes 10-13 represent 100°C, 65°C and no heat treatment, respectively prior to RT-PCR. Lanes 1, 5 and 10, lanes 2, 6 and 11 and lanes 3, 7, and 12 represent 10μl samples collected at PCR cycles 25, 30 and 35, respectively. Lanes 4, 8 and 13 represent the resultant product when no samples were taken during 35 cycles of PCR.

Lane 9 λ DNA digested with Pst I

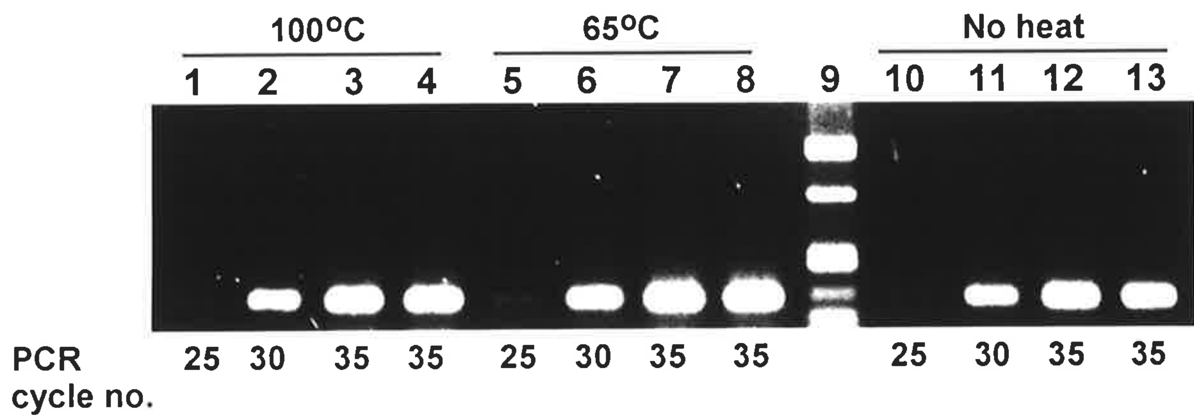

100°C
65°C
No heat
1 2 3 4 5 6 7 8 9 10 11 12 13
PCR cycle no.
25 30 35 35 25 30 35 35 25 30 35 35

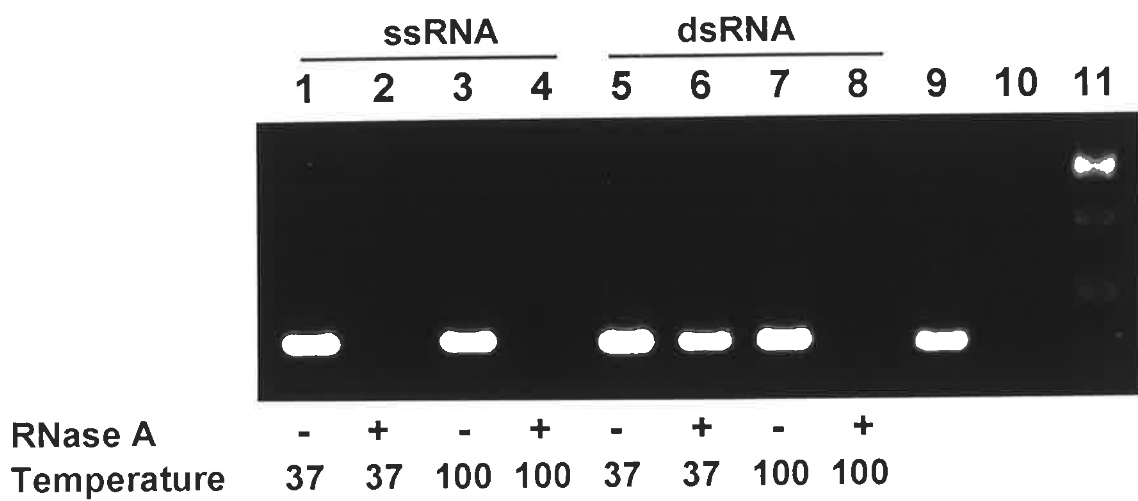
Figure 7.5 Amplification of *in vitro* transcribed single-stranded and double-stranded 5' UTR RNA following RNase A digestion at 37°C and 100°C in high salt.

Lanes 1-4 and lanes 5-8 represent RNase A digestions of ssRNA and dsRNA derived from the 5' UTR, respectively. Lanes 1, 2, 5 and 6 and lanes 3, 4, 7 and 8 represent RNase A digestions performed at 37°C and 100°C, respectively. Lanes 1, 3, 5 and 7 and lanes 2, 4, 6 and 8 represent RNase A digestions without (-) and with (+) 50μg/ml RNase A, respectively.

Lane 9 1pg 5' UTR RNA

Lane 10 DDW negative control

Lane 11 λ DNA digested with Pst I



ssRNA
dsRNA
1 2 3 4 5 6 7 8 9 10 11
RNase A
- + - + - + - +
Temperature
37 37 100 100 37 37 100 100

RNase A at 50μg/ml at 37°C and 100°C (Figure 7.5, lanes 2 and 4). However, digestion with concentrations <50μg/ml resulted in amplification of the 5' UTR (data not shown). Hence, RNase A at 50μg/ml was used in subsequent experiments. Double-stranded HCV RNA, prepared by annealing *in vitro* transcribed positive and negative 5' UTR strands, was digested with 50μg/ml of RNase A at 37°C and 100°C in DDW and high salt, and then denatured prior to RT-PCR to determine if dsRNA was protected from RNase degradation in high salt. The 5' UTR RNA was amplified following RNase A digestion of the dsRNA preparation at 37°C in high salt (Figure 7.5, lane 6). In addition, digestion of dsRNA at 37°C and 100°C in the absence of RNase A resulted in amplification of the 5' UTR (Figure 7.5, lanes 5 and 7). In contrast, no cDNA products were observed after RNase A digestion of dsRNA at 100°C in high salt (Figure 7.5, lane 8) or after digestion at 37°C and 100°C in DDW (data not shown). Hence, these preliminary experiments proved that dsRNA was protected from RNase A degradation in high salt, but not in DDW.

(ii) Liver RNA

Using the conditions described above, a RNase A digestion step was performed on liver RNA prior to RT-PCR. No cDNA product was detected when the RNase A digestion was performed at either 37°C (Figure 7.6, lanes 3 and 4) or 100°C (Figure 7.6, lanes 7 and 8) in DDW. Similarly, HCV RNA was not amplified when RNase A digestion was performed at 37°C or 100°C in high salt, irrespective of the inclusion of a denaturation step prior to RT-PCR. However, a product was amplified from RNA samples digested at 37°C and 100°C, in the absence of RNase A, in DDW and high salt (Figure 7.6, lanes 1, 2, 5 and 6), serving as a positive control and showing that RNase A was responsible for RNA degradation. Furthermore, the specificity of the HCV RNA detection was confirmed by the absence of a product from RNase A-digested RNA, extracted from HCV-negative liver tissue (Figure 7.6, lane 9), and when the RNA component was substituted with DDW (Figure 7.6, lane 11). Hence, RNase A digestion of liver RNA

Figure 7.6 Agarose gel electrophoresis analysis of HCV-specific RT-PCR products generated from RNase A-digested liver RNA at 37°C or 100°C in DDW or high salt.

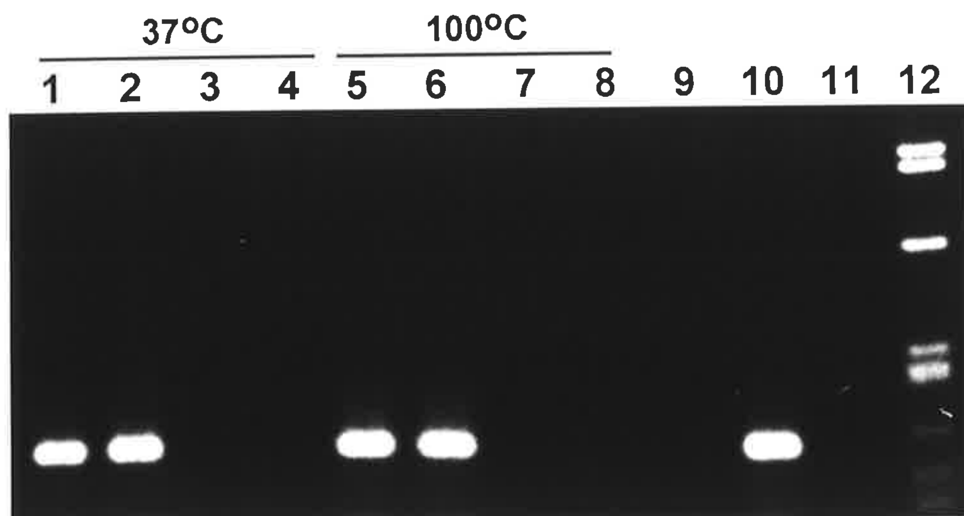
Lanes 1-4 and lanes 5-8 respresent RNase A digestions performed at 37°C and 100°C, respectively. Lanes 1, 2, 5 and 6 and lanes 3, 4, 7 and 8 represent digestions without (-) and with (+) 50μg/ml RNase A, respectively. Prior to RT-PCR, RNA samples were either added directly to the reaction (-) (lanes 1, 3, 5 and 7) or denatured at 100°C (+) (lanes 2, 4, 6, and 8).

Lane 9 RNase A-digested liver RNA from a HCV-negative individual

Lane 10 50ng case 1 liver RNA

Lane 11 DDW negative control

Lane 12 λ DNA digested with Pst I


37°C
100°C
1 2 3 4 5 6 7 8 9 10 11 12
RNase A - - + + - - + +
Denaturation - + - + - + - +

suggested that dsHCV RNA could not be amplified and thus, the viral RNA was single-stranded (see sections 7.4 and 8.3).

7.3.5 LiCl fractionation of HCV RNA

LiCl precipitates ssRNA and partially ssRNA (LiCl-insoluble) leaving dsRNA in the supernatant (LiCl-soluble). Separation of Flavivirus RI from RF by LiCl precipitation has been successfully applied to the study of the Flavivirus RNA replication cycle (Westaway, 1987) and to facilitate cloning of the BVDV genome (Collett *et al.*, 1988). Hence, it was considered that this approach may confirm the single-stranded nature of HCV RNA in infected liver tissue as demonstrated by RNase A digestion above.

(i) ^{32}P-labelled 5' UTR RNA

Initially, the efficiency of LiCl precipitation was monitored by precipitation of ^{32}P-labelled single-stranded 5' UTR RNA. The resultant pellet and supernatant, following precipitation, were analysed by counting 1μl samples and determining the percentage recovery of ^{32}P-labelled ssRNA in the pellet. Initially, LiCl precipitation of ^{32}P-labelled ssRNA was inefficient; only 63% of ^{32}P-labelled ssRNA was recovered in the LiCl-insoluble fraction (Table 7.1). The recovery of ^{32}P-labelled ssRNA in the LiCl-insoluble fraction was increased to 75% when tRNA was added to the LiCl precipitation to a final concentration of 1mg/ml (Table 7.1). However, the addition of total cellular RNA, extracted from the human hepatoma-derived cell line Hep G2, to 1mg/ml in the LiCl precipitation, was required for successful precipitation of ssRNA; ≥98% of ^{32}P-labelled ssRNA was consistently recovered in the LiCl-insoluble fraction (Table 7.1). Hence, these optimal conditions were used in the LiCl precipitation experiments to follow.

Table 7.1 LiCl precipitation analysis of ^{32}P-labelled single-stranded 5' UTR RNA

	-		+tRNA		+HepG2 RNA	
	P	S	P	S	P	S
Counts (cpm/μl)	8901	5227	10915	3638	14027	331
Proportion (%)	63	37	75	25	98	2

P = pellet (LiCl-insoluble fraction)
S = supernatant (LiCl-soluble fraction)

(ii) *In vitro* transcribed 5' UTR RNA

To further evaluate the efficiency of LiCl precipitation, *in vitro* transcribed, full-length positive sense, single-stranded 5' UTR RNA was precipitated by LiCl using the conditions determined above. The LiCl-soluble and -insoluble fractions were then analysed by RT-PCR. A HCV-specific product was observed in the LiCl-insoluble fraction (Figure 7.7, lane 1), but not in the LiCl-soluble fraction (Figure 7.7, lane 2). Hence, the inability to amplify the 5' UTR RNA from the LiCl-soluble fraction further demonstrates the successful precipitation of ssRNA by LiCl. Furthermore, a dsRNA preparation, made from *in vitro* transcribed positive and negative 5' UTR strands, was fractionated with 2M LiCl and RT-PCR performed as described above. Prior to LiCl fractionation, the dsRNA preparation was digested with 50μg/ml RNase A in high salt at 37°C (as described in section 7.3.4) to ensure that all RNA molecules were completely double-stranded. Positive sense 5' UTR RNA was amplified from the LiCl-soluble fraction (Figure 7.7, lane 4), however no product was observed in the LiCl-insoluble fraction (Figure 7.7, lane 3). Hence, these experiments showed that double-stranded 5' UTR RNA could be amplified from the LiCl-soluble fraction.

(iii) Liver RNA

Total liver RNA was fractionated with 2M LiCl and RT-PCR performed on the LiCl-soluble and -insoluble fractions as described above. HCV RNA was detected in the LiCl-insoluble fraction (Figure 7.7, lane 5), but no cDNA product was observed in LiCl-soluble forms (Figure 7.7, lane 6). Furthermore, the same result was observed when case 2 liver RNA was precipitated by LiCl. The specificity of RT-PCR for HCV RNA was confirmed by the absence of a cDNA product in LiCl precipitated liver RNA extracted from a HCV-negative individual (Figure 7.7, lanes 7 and 8). Hence, LiCl precipitation showed that HCV RNA in the liver is either single-stranded or partially double-stranded and single-stranded (ie. RI).

Figure 7.7 Agarose gel electrophoresis analysis of HCV-specific cDNA products following fractionation with 2M LiCl.

Lanes 1, 3, 5 and 7 represent the LiCl-insoluble fractions from precipitation of single-stranded 5' UTR RNA, double-stranded 5' UTR RNA, case 1 liver RNA and HCV-negative liver RNA, respectively. Lanes 2, 4, 6 and 8 represent the LiCl-soluble fractions following precipitation of single-stranded 5' UTR RNA, double-stranded 5' UTR RNA, case 1 liver RNA and HCV-negative liver RNA, respectively.

Lane 9 DDW negative control

Lane 10 λ DNA digested with Pst I

1 2 3 4 5 6 7 8 9 10

7.3.6 Immunohistochemical detection of double-stranded RNA

Formalin-fixed and frozen liver sections were examined by indirect immunofluorescence for the presence of dsHCV RNA using a rabbit antibody (a gift from Dr. R Francki; section 2.2.2) specific for dsRNA. Vero cells infected with YFV were included as a positive control. dsRNA was detected within the cytoplasm of 100% of YFV-infected Vero cells 4 days after infection (Figure 7.8A), while staining was absent in uninfected Vero cells (Figure 7.8B). Furthermore, the fluorescent signal was abolished when the antiserum was preadsorbed against dsHCV RNA, transcribed *in vitro* from the 5' UTR of the genome, prior to immunostaining. On the other hand, preadsorption of the antiserum against single-stranded 5' UTR RNA did not abolish the signal. Although this antibody was shown to be specific for dsRNA, dsRNA was not detected in liver sections from anti-HCV positive individuals using this antibody preparation (Figure 7.8C).

7.4 CONCLUSIONS AND DISCUSSION

Although the exact mechanism involved in HCV replication in infected individuals is still unknown, it is assumed that negative stranded HCV RNA will represent the presence of RI (Fong *et al.*, 1991; Shibata *et al.*, 1991; Takehara *et al.*, 1992; Horiike *et al.*, 1993; Muller *et al.*, 1993a; Sherker *et al.*, 1993; Gunji *et al.*, 1994). Hence, the detection of negative strand HCV RNA and RF in HCV infected liver, described in this chapter, was perfomed to further our understanding of HCV RNA replication. Initially, a strand specific RT-PCR assay was developed to permit authentic detection of the negative strand of HCV. Modification of the RNA at the 3' end allowed discrimination between the two strands of HCV RNA. Only positive sense HCV RNA was detected by strand specific RT-PCR in serum samples from 6 HCV-positive patients chosen at random, suggesting negative sense HCV RNA detected in the liver is not from contaminating serum. Hence, strand specific RT-PCR confirmed that negative sense HCV RNA was present in the liver sample and thus, this was likely to represent the presence of RI.

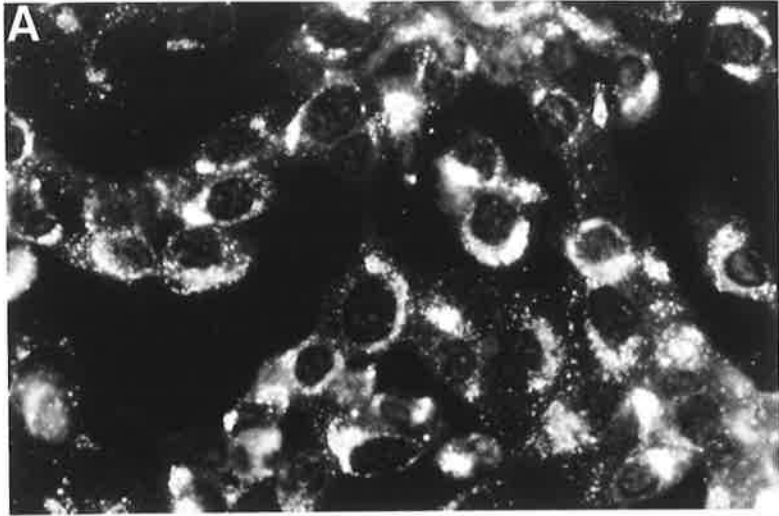
Figure 7.8 Immunostaining for dsRNA.

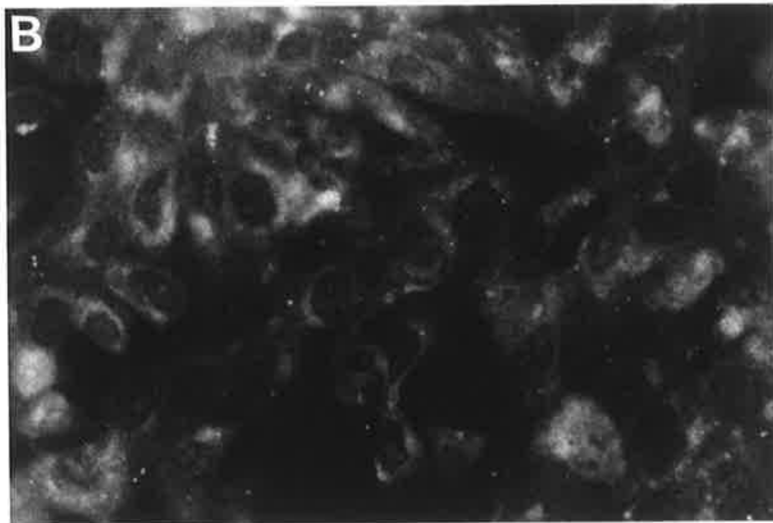
A. YFV-infected Vero cells (Day 4)

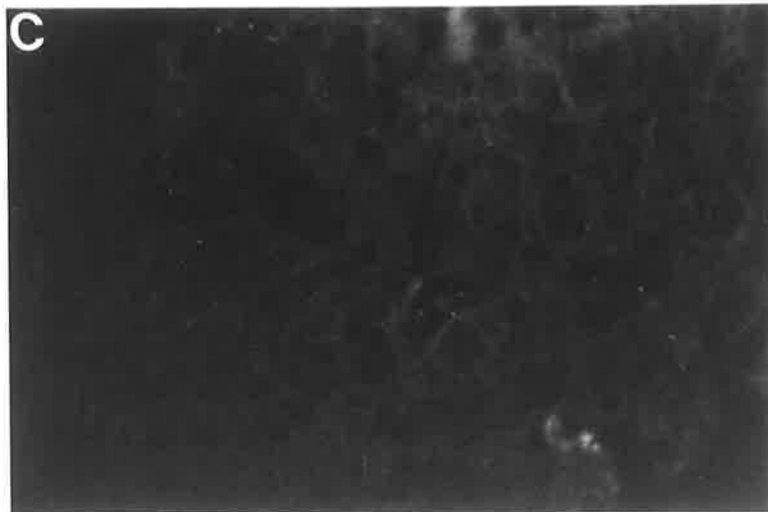
B. Uninfected Vero cells

C. HCV-positive liver tissue (frozen section)

Original magnification x400.


A


B


C

Following the specific detection of positive and negative sense HCV RNA, the remaining work in this chapter focussed on determining whether these two strands form a RF during HCV RNA replication. Collectively, the results suggest that ssHCV RNA is the predominant species in the infected liver tissues studied, as expected for any positive strand RNA virus infection.

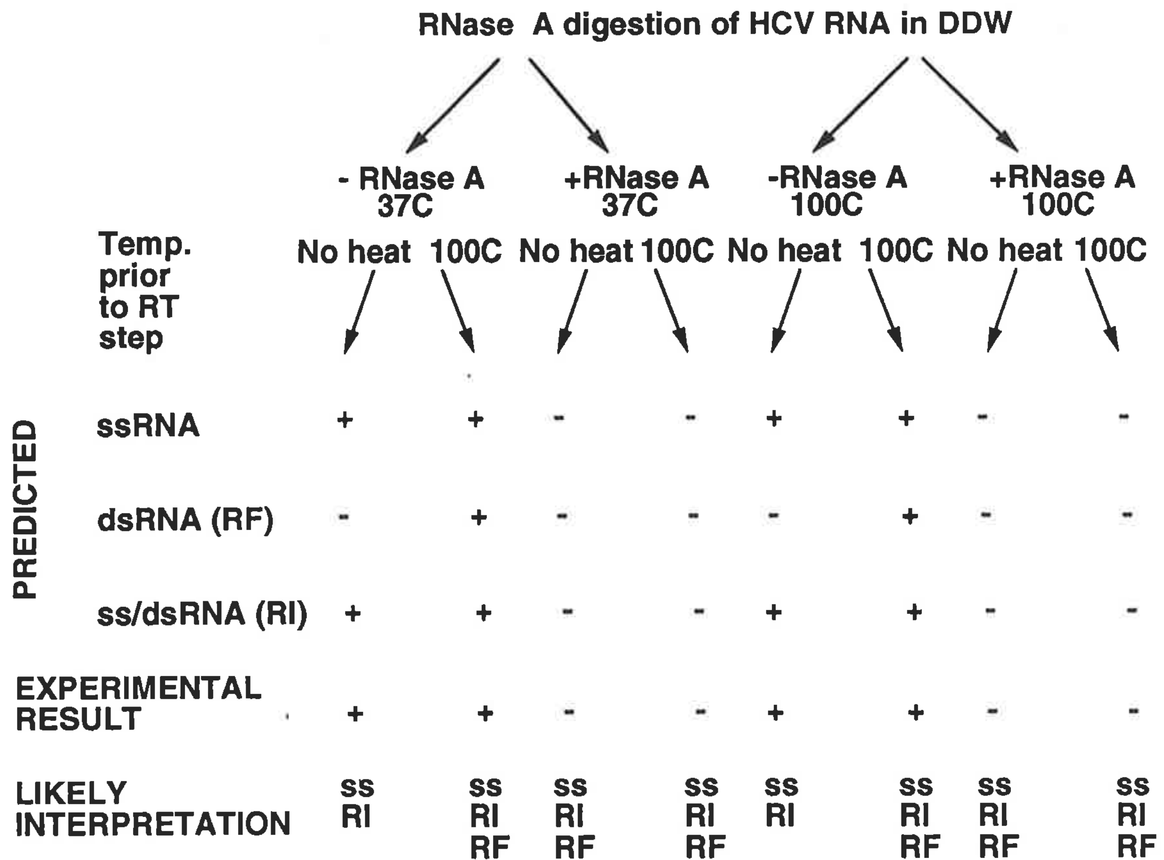
RT-PCR of RNase A-digested liver RNA showed that HCV RNA was RNase A-sensitive in DDW. Comparison of this data with the predicted results if either ssRNA or dsRNA or both were present in infected liver tissue, suggested that the HCV RNA was only single-stranded or was present as both single-stranded and double-stranded forms (RI) (Figure 7.9A). Similarly, RNase A-resistant HCV RNA was not detected following digestion at 37°C in high salt. Since double-stranded (RNA:RNA) hybrids are protected from degradation in high salt (Pardue, 1985; Harper *et al.*, 1986), this result suggests that dsRNA is not present in infected liver tissue (Figure 7.9B) (but see below).

In addition to the results achieved following RNase A digestion, evidence to suggest that HCV RNA is single-stranded in naturally-infected liver tissue was also provided by: (i) LiCl fractionation of total liver RNA extracts; (ii) Denaturation of liver RNA prior to RT-PCR that did not increase the yield of HCV-specific cDNA; and (iii) the inability of anti-dsRNA to detect dsRNA in infected liver tissue by immunofluorescence. Thus, (i) RF may represent a minor species in HCV replication and as a result the level of RF is too low for detection; or (ii) the formation of RF is not involved in HCV RNA replication; or (iii) RF may appear in a transient manner. Furthermore, reports describing negative strand specific HCV RNA detection in liver tissue (Gunji *et al.*, 1994) and in *in vitro* culture studies (Lanford *et al.*, 1994) failed to denature RNA samples at 100°C prior to cDNA synthesis. RF have been detected in the replication cycle of flaviviruses, but have not been described for BVDV or other pestiviruses. Likewise, RF were not

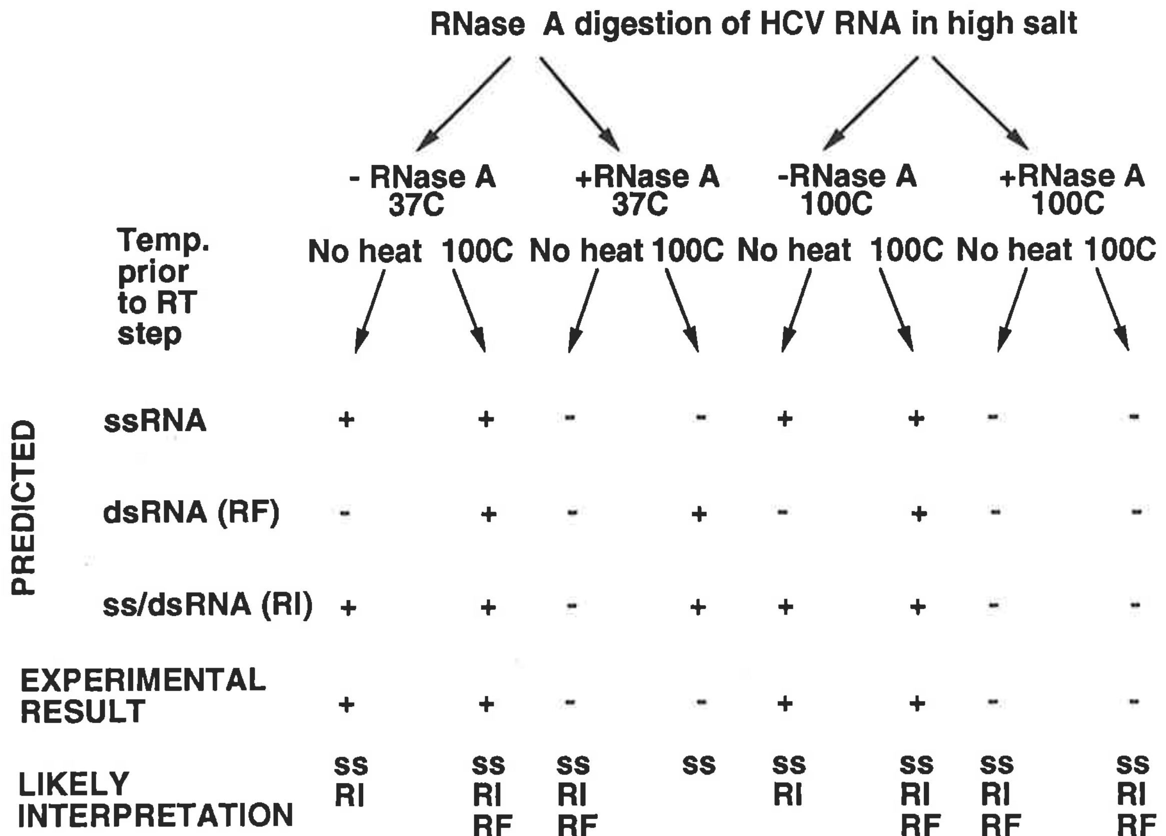
Figure 7.9 Flow charts outlining the predicted vs experimental results when liver RNA was digested with RNase A at 37°C and 100°C in DDW (A) and high salt (B), before RT-PCR to detect HCV RNA.

In this flow chart, + represents a positive RT-PCR result, while - represents a negative result.

A



B



detected in HCV-infected liver tissue in this study, despite the detection of negative sense HCV RNA.

CHAPTER 8

CONCLUDING REMARKS

8.1 Introduction

At the commencement of this thesis, serological assays for anti-HCV were available and PCR was used to detect HCV RNA, but events related to virus replication in the liver were unknown. Specifically, the localisation of HCV-infected hepatocytes in the human liver was unclear. Hence, the experiments described in this thesis examined: (i) the expression and distribution of HCV-specific antigens and RNA in HCV-infected liver tissue. The development of immunohistochemical assays to detect HCV antigens in this study led to an investigation into the relationship between virus antigen expression and liver histology and the cell types infected with HCV; and (ii) the configuration of HCV RNA in naturally-infected liver tissue in an effort to gain an insight into the replication strategy of HCV. The significance of these studies is discussed below.

8.2 HCV antigen and RNA expression in hepatic and extrahepatic cells

The inability to reproducibly culture HCV *in vitro* has hindered efforts to examine the replication cycle of the virus. However, the classification of HCV in the Flaviviridae has promoted the view that HCV is likely to replicate in the hepatocyte cytoplasm by a strategy closely related to that of Flaviviruses and/or Pestiviruses. Hence, it is likely that one of the early steps in the replication cycle of HCV is the translation of the input positive sense RNA genome into a polyprotein which is subsequently cleaved into the individual proteins. The localisation of the S and NS proteins in the cytoplasm of hepatocytes described in this study and in other reports (Infantolino *et al.*, 1990; Hiramatsu *et al.*, 1992; Krawczynski *et al.*, 1992; Sansonno and Dammacco, 1993; Yamada *et al.*, 1993;

Hiramatsu *et al.*, 1994; Tsutsumi *et al.*, 1994; Yap *et al.*, 1994) is consistent with cytoplasmic replication. Furthermore, both positive and negative sense HCV RNA have been localised to the cytoplasm of very few hepatocytes in chronic carriers by ISH (Lamas *et al.*, 1992; Yamada *et al.*, 1992; Nouri Aria *et al.*, 1993; Yamada *et al.*, 1993), suggesting that this represents the site for HCV RNA replication. However, HCV RNA remained undetected by ISH in liver tissue from other chronic carriers (Lamas *et al.*, 1992; Haruna *et al.*, 1993; Nouri Aria *et al.*, 1993; Tanaka *et al.*, 1993; Yamada *et al.*, 1993). The ISH assay to detect HCV RNA described in this thesis demonstrated the presence of the HCV genome in mononuclear cells (see below), but not in hepatocytes within infected liver tissue. The lack of an hepatic signal may be due to low copy number HCV RNA in the liver tissues studied. Support for this hypothesis was provided by Nuovo *et al*, (1993) who rarely detected HCV RNA in hepatocytes in chronically-infected liver tissues by conventional ISH, whereas almost 100% of hepatocytes were shown to be positive following RT-PCR/ISH. These observations suggest that most infected hepatocytes in chronic carriers contain less than 20 copies of HCV RNA per cell, which is reported to be the threshold of detection of conventional ISH (Brahic and Haase, 1978; Nuovo *et al.*, 1991). In contrast, experiments conducted in this thesis estimated that 140 HCV genome copies were found in mononuclear cells. These findings, coupled with the observation that many liver samples are PCR-positive, ISH-negative, support the findings that amplification by PCR is generally required to conclusively identify HCV RNA in liver tissue.

The low copy number of HCV RNA in infected liver samples from the patients in this and other studies may relate to the stage of infection. The detection of HCV RNA in the cytoplasm of a few hepatocytes in chronically-infected liver tissues contrasts with the results in liver samples from acutely-infected humans (Nouri Aria *et al.*, 1993) and chimpanzees (Negro *et al.*, 1992), in which a higher proportion of hepatocytes expressed HCV RNA. Furthermore, Negro *et al*, (1992) observed a rapid decrease in the level of hepatic HCV RNA which became

undetectable by ISH 22 weeks after experimental HCV infection of chimpanzees, despite confirmation of viraemia by PCR. However, this study used oligonucleotide probes for ISH detection that lack sensitivity due to the low representation of the viral genome. Nevertheless, the localisation of HCV RNA in a high proportion of hepatocytes in the acute phase liver tissue by oligonucleotide/ISH suggests that HCV RNA may be detectable in liver and serum by Northern blot analysis. However, HCV RNA detection in liver tissue by Northern blot-hybridisation has not described since the original report of the HCV genome molecular cloning (Choo *et al.*, 1989). In this report, HCV RNA was detected in liver tissue from a chronic NANBH-infected chimpanzee by spot blot- and Northern blot-hybridisation using the clones identified from the cDNA library constructed in bacteriophage λgt11 (see section 1.3) (Choo *et al.*, 1989). Collectively, during acute infection, the higher level of HCV RNA in hepatocytes and low level viraemia, suggest a possible block in virus secretion from infected hepatocytes. In contrast, the level of hepatic HCV RNA in chronic carriers is either low or less than 20 copies per cell (as described above). The reduction in hepatic HCV RNA during the chronic phase may be due to: (i) low level replication; or (ii) similar levels of replication, as observed in acute phase liver tissue, coupled with an increase in the efficiency of assembly and release of HCV virions. Consistent with the latter suggestion is the detection of the S and NS proteins in a high proportion of hepatocytes in chronic carriers with advanced liver disease (Hiramatsu *et al.*, 1994; Tsutsumi *et al.*, 1994), suggesting increased rates of virus replication. Supporting this observation, one study reported that HCV RNA-positive cells were more frequently recognised in patients with advanced histological features (Haruna *et al.*, 1993). Furthermore, the levels of viraemia tended to increase in patients with advanced liver disease (Hagiwara *et al.*, 1993; Kato *et al.*, 1993b; Naito *et al.*, 1994). Alternatively, an extrahepatic site of HCV replication may be capable of supporting continuous viraemia and lymphocytes appear able to support HCV replication *in vivo* (see below) and *in vitro* (Shimizu *et al.*, 1992, 1993).

The studies described in this thesis indicated that T and B lymphocytes can also be infected by HCV and that HCV replication is probably ongoing as judged by the expression of NS3. In fact, this is the first study to describe the detection of HCV antigens in lymphocytes. HCV RNA has also been detected by ISH in infiltrating mononuclear cells (Lamas *et al.*, 1992; Nouri Aria *et al.*, 1993; Yamada *et al.*, 1993), bile duct epithelial cells (Nuovo *et al.*, 1993; Nouri Aria *et al.*, 1993), Kupffer cells (Nuovo *et al.*, 1993) and sinusoidal cells (Nouri Aria *et al.*, 1993) in liver samples from chronic HCV carriers. Negative sense HCV RNA was found in the above cells, except bile duct epithelial cells, suggesting that virus replication was ongoing. Furthermore, positive and negative sense HCV RNA has been detected in isolated PBMC using PCR (Bouffard *et al.*, 1992; Wang *et al.*, 1992a; Zignego *et al.*, 1992; Muller *et al.*, 1993a; Gunji *et al.*, 1994; Muratori *et al.*, 1994) and ISH (Moldvay *et al.*, 1994). Similar findings have been reported for members of the Flaviviridae, dengue virus 2, dengue virus 4 and Japanese encephalitis virus, which have been shown to infect and propagate in human peripheral blood monocytes and haematopoetic cells (Hase *et al.*, 1989; Nakao *et al.*, 1989). Extrahepatic cells may either be capable of supporting virus replication and provide a source of infectious virus to subsequently infect hepatocytes or conversely, may be infected consequential to hepatocyte infection; there is a clear need for more data regarding sites of virus replication at different stages of HCV infection. In any case, the relevance of HCV infection in cells other than hepatocytes to the pathogenesis process is unknown, but one strategy that viruses have evolved in order to develop persistent infections is to infect cells of the immune system (White and Fenner, 1986).

The detection and distribution of HCV antigens in formalin-fixed liver samples differed to that described for fresh frozen liver sections. The punctate staining of HCV-E2/NS1 and -NS3 in hepatocytes and lymphocytes in frozen tissue may represent putative replication complexes, but the immunogenicity of these complexes was destroyed after fixation in formalin and paraffin wax embedding

and consequently, the lymphocytes in formalin-fixed liver samples were negative. This was coincident with the appearance of diffuse cytoplasmic NS4 staining in hepatocytes in formalin-fixed, wax-embedded samples. The discrepancies in the staining pattern observed between frozen and formalin-fixed liver tissues may be due to several factors, including the levels of antigen expression in the tissue or alteration of viral epitopes. I have proposed that the diffuse cytoplasmic staining pattern of NS4 may represent antigen accumulation, as a result of long-term persistent infection. The life span of a human hepatocyte is estimated to be 200-400 days (Fausto, 1992) and consequently, there is likely to be a low frequency of dividing hepatocytes. On the other hand, although the life span of an activated lymphocyte still remains unresolved, some experiments suggest that this can be 14-21 days for B cells, helper and cytotoxic T cells (Gray, 1993). It is postulated that the turnover of lymphocytes is not feasible for antigen accumulation, whereas viral antigen can accumulate in the hepatocytes of chronic carriers. Hence, following formalin fixation and paraffin wax embedding, HCV antigens remain detectable in the hepatocytes, but not in the lymphocytes. Furthermore, NS5 (Tsutsumi *et al.*, 1994) and C, E1 and NS3 (Hiramatsu *et al.*, 1992) were localised in a higher proportion of hepatocytes in advanced stages of HCV-related liver disease that may be a consequence of long term antigen accumulation or increased rates of virus replication (as described above).

8.3 Mechanism of RNA replication

The mechanism of HCV RNA replication has not been studied extensively, although strand specific HCV RNA amplification by PCR has permitted the identification of tissues and cells in which HCV RNA replication is ongoing. Since HCV is related to flaviviruses, it is plausible that all three forms of RNA, viz. single-stranded positive and negative sense genome-length RNA, partially dsRNA (RI) and complete double-stranded RF, would be present in HCV-infected liver tissue. The development of a strand specific RT-PCR assay to distinguish

between positive and negative sense HCV RNA is crucial to determine the mechanism involved in viral replication. Experiments described in this thesis and in other reports (Willems *et al.*, 1993; Lanford *et al.*, 1994; Gunji *et al.*, 1994; McGuinness *et al.*, 1994) demonstrate the inability of conventional RT-PCR to discriminate between *in vitro* transcribed positive and negative HCV RNA strands. Amplification of each strand of HCV RNA by RT-PCR occurred irrespective of the primer used for RT or independent of primer for RT. Hence, primer-independent cDNA synthesis poses a problem in conventional RT-PCR assays. However, strand specificity was achieved by two rounds of chemical modification of the RNA samples at the 3' end as described by Gunji *et al*, (1994). Chemical modification prevents RNA molecules from acting as primers and as a result, the cDNA for each strand of HCV RNA was specifically synthesised during RT only by the addition of a primer complementary to the target strand. Using this strand specific RT-PCR strategy, ssHCV RNA of both polarities was detected in infected liver tissue homogenates. The detection of the negative strand suggests ongoing replication via a RI. Hence, the inability to detect RF may reflect the absence of a RF step in the replication strategy or represent low level replication. Although the HCV genotypes were not determined in the two patients studied, the level of replication may be related to the genotype of HCV (Dusheiko *et al.*, 1994). Thus, further studies of HCV RNA in liver tissue from patients at different stages of HCV infection are required to establish whether RF play a role in RNA replication.

8.4 Mechanism of liver injury

The previous recognition of HCV RNA-positive patients with normal levels of ALT, and a lack of correlation between the degree of hepatocellular damage as assessed histologically or biochemically (eg. ALT), and the prevalence of HCV protein in liver tissue in this study, suggests that HCV replication *per se* is not directly cytopathic. Although there is no direct evidence, it has been proposed

that the host immune response may play a role in hepatocellular injury during persistent infection and result in chronic liver injury (Mosnier *et al.*, 1993; Nouri Aria *et al.*, 1993; Nuovo *et al.*, 1993; Tanaka *et al.*, 1993). The likelihood of immune-mediated damage in chronic HCV infection is supported by studies which identified a population in the infiltrating lymphocytes in the liver as CD8-positive cytotoxic T lymphocytes specific for C and E2/NS1 proteins (Koziel *et al.*, 1993) and E1 and NS2 proteins (Koziel *et al.*, 1992). Furthermore, numerous CD8-positive T cells have been identified in intraportal lymphoid nodules and in the liver lobule of patients with chronic active HCV in this and in another study (Mosnier *et al.*, 1993). Hence, these findings, and the detection of HCV RNA in the liver from healthy carriers (Takeuchi *et al.*, 1990a; Fong *et al.*, 1991; Nouri Aria *et al.*, 1993), suggest that liver damage due to HCV infection is immune-mediated. However, the possibility of some effect associated with viral replication, as seen in flavivirus-infected cells (Westaway, 1987), cannot be ruled out. Finally, the topographical relationship in HCV infection may differ in the acute phase compared with chronic infection (Sansonno and Dammacco, 1993); two distinct immunopathological patterns were observed in that study. In the acute phase, HCV antigen-positive hepatocytes were distributed in the lobule and showed a direct relationship with areas of liver cell necrosis, whereas no relationship between viral antigen and RNA expression and sites of liver cell necrosis was noted in liver tissue from chronic carriers (Sansonno and Dammacco, 1993; Nouri Aria *et al.*, 1993; Nuovo *et al.*, 1993). It is possible that the relatively higher level of HCV RNA replication in the acute phase causes cytotoxicity resulting in cell death, whereas in the chronic phase, when the level of hepatic HCV RNA is lower, a low level of direct cytotoxicity occurs. However, the presence of viral antigen(s) on the infected cell surface may provide an immunological target during persistent infection that results in hepatocyte injury. Furthermore, a recent report suggested that one mechanism of hepatocyte death in HCV-infected liver is apoptosis, particularly in active inflammation of chronic HCV infection (Hiramatsu *et al.*, 1994). Moreover, the mechanism of cytotoxic T

lymphocyte-induced hepatocyte death in viral infections has been shown to be by apoptosis (Wyllie *et al.*, 1980). On the other hand, HBV-carriers, where liver injury is immune-mediated, and anti-HCV positive patients respond differently to interferon treatment. Unlike HBV-carriers, interferon treatment of HCV-positive patients does not result in a flare up of ALT at the onset of treatment, nor is the improved liver function usually maintained at the end of the course. Hence, the response of HCV-carriers to interferon indirectly suggests that HCV liver disease is mediated by virus replication.

The progression of HCV-related liver disease to HCC has been suggested. The localisation of HCV-NS4 in tumorous and nontumorous hepatocytes in this thesis, combined with PCR detection of HCV RNA in RNA homogenates from HCV/HCC tissue (Yoneyama *et al.*, 1990; Gerber *et al.*, 1992; Horiike *et al.*, 1993; Sherker *et al.*, 1993; Sullivan and Gerber, 1994), suggest that HCV replicates in HCC tissue. Although the mechanisms of HCV-related HCC are not fully understood, there are a number of potential mechanisms: (i) Integration of HCV nucleic acid sequences into the host genome in a manner described for HBV-related HCC (Shafritz *et al.*, 1981). However, a HCV-encoded reverse transcriptase activity has not been described and thus, this is a remote possibility; (ii) HCV protein(s), cleaved from the polyprotein, may act as a transactivator(s); or (iii) Repeated hepatocellular necrosis and regeneration due to long term persistent HCV infection leads to liver cirrhosis. Hence, HCV may not be oncogenic *per se*, but rather oncogenesis is a consequence of cirrhosis.

8.5 Future directions

The experiments described in this thesis provided information regarding the pathogenesis of HCV infection, and to the choice of appropriate fixatives and cells that may be used in *in vitro* culture experiments. However, the lack of a suitable *in vitro* culture system for HCV or the inability to sustain infected hepatocytes in long-term culture, coupled with low copy number hepatic HCV RNA and low level viraemia in infected individuals, has hindered detailed studies of: (i) the nature of HCV virions; (ii) the mechanism of HCV replication and pathogenesis; and (iii) potential prevention and treatment regimens for HCV. Hence, further studies are required to clarify these aspects of HCV discussed below.

1. Clarification of the role of HCV-infected PBMC in the aetiology and kinetics of the chronic HCV infection.

2. Further investigation into HCV-related HCC in an attempt to determine: (i) the mechanism of hepatocarcinogenesis in patients with chronic HCV infection; (ii) if HCC development is dependent on HCV replication or gene expression in hepatocytes; or (iii) if the risk is primarily due to HCV-induced cirrhosis.

3. Development of an *in vitro* infection system or of an infectious HCV cDNA clone. Recombinant Vaccinia Virus vectors have been useful for the study of polyprotein organisation and processing. However, the role of the HCV proteins in HCV virion assembly and RNA replication will remain speculative until it becomes possible to examine virus-encoded proteins in authentic HCV-infected cells and virions, and to assess their importance in the life cycle of HCV.

4. *In vitro* cell culture of HCV would lead to: (i) a convenient model to screen a large number of anti-viral agents; (ii) a system with the potential for virus

attenuation that could lead to the development of candidate live virus vaccine strains; and (iii) understanding protective immunity by allowing one to conduct neutralisation tests.

APPENDIX

PREPARATION OF COVERSLIPS AND MICROSCOPE SLIDES

Slides and coverslips were soaked overnight in chromic acid, washed 2-3h under running tap water, and rinsed 5x in DDW. The acid-washed slides were air-dried, baked at 80°C for 2h then dipped in 2% APES (Sigma), rinsed in succession 3x in 100% ethanol, then 3x in DDW, and rebaked at 80°C overnight. These APES-coated slides were used to ensure good adhesion of tissue sections and the autoradiographic emulsion during ISH experiments. 13mm acid-washed coverslips (stored under DDW) were dipped in 1% prosil-28 (PCR Incorporated), rinsed in two changes of DDW then baked for 2h at 80°C in a vacuum oven. These coated coverslips were utilised in ISH experiments after heat sterilisation at 160°C for 3-4h.

BACTERIAL CULTURE MEDIUM

2YT broth

1.6% tryptone (Difco), 1% yeast extract (Difco), 171mM NaCl

pH to 7.4 and sterilise by autoclaving.

2YT agar

2YT agar was prepared by the addition of 2% powdered agar (Difco)

Superbroth

A: 12g tryptone (1.2%), 24g yeast extract (2.4%), 5ml glycerol (0.5%)

B: 11.5g KH_2PO_4 (84.5mM), 62.5g K_2HPO_4 (359mM)

Autoclave solution A and solution B separately. For use, mix 900ml of Part A with 100ml of Part B.

BUFFERS AND DYES

10xMOPS buffer

0.2M MOPS (3-[N-Morpholino] propanesulfonic acid; Sigma)

0.05M NaOAc

Adjust the pH to 5.5-7.0 with NaOH. Store at rt.

Toluene-based liquid scintillant

2.5L toluene, 10g PPO (0.4%; BDH), 1g POPOP (0.04%; Sigma). Dissolve PPO and POPOP in toluene overnight.

Rapid Haematoxylin

50g aluminium ammonium sulphate, 1L DDW, 4g haematoxylin (Sigma), 0.3g sodium iodate, 1.5g citric acid, 75g chloral hydrate. Dissolve components in order in DDW, allow each to dissolve completely in turn. Store at rt.

Alcoholic Eosin

137ml DDW, 2730ml 95% ethanol, 350ml 1% aqueous eosin (Koch-light Laboratories), 35ml 1% aqueous phloxine (Koch-light Laboratories), 14ml glacial acetic acid. Mix in order, store 4°C.

λ/Pst I DNA standard

λ DNA (48502 bp) Digested with Pst I

Fragment Size (bp)	% of Total	ng DNA/Fragment/total λ DNA Loaded			
		125	250	375	500
11502	24	30	60	90	120
5077	10.5	13	26	39	53
4749	10	12.5	25	37.5	50
4507	9	11.3	22.5	33.8	45
2838	5.9	7.4	14.8	22.1	29.5
2556 }					
2459 }	15.4	19.3	38.5	57.8	77
2443 }					
2140	4.4	5.5	11	16.5	22
1959	4.0	5	10	15	20
1700	3.5	4.4	8.8	13.1	17.5
1159	2.4	3	6	9	12
1093	2.3	2.9	5.6	8.6	11.5
805	1.7	2.1	4.3	6.4	8.5
514	1.1	1.4	2.8	4.1	5.5
468 }	1.9	2.4	4.8	7.1	9.5
448 }					
339	0.7	0.9	1.8	2.6	3.5
264	0.5	0.6	1.3	1.9	2.5
247	0.5	0.6	1.3	1.9	2.5
216 }					
211 }	1.2	1.5	3.0	4.5	6.0
200 }					
164	0.3	0.4	0.8	1.1	1.5
150	0.3	0.4	0.8	1.1	1.5
99	0.2	0.3	0.5	0.8	1.0
94	0.2	0.3	0.5	0.8	1.0
87	0.2	0.3	0.5	0.8	1.0
15	0.03	0.04	0.08	0.11	0.15

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