Studies on the pathogenesis of hepatitis C virus

Thesis submitted for the degree of Doctor of Philosophy

by

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Abstract

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To facilitate the structural and functional analysis of hepatitis C virus (HCV), a system for in vitro propagation of hepatitis C virus-like particles expressed from a molecular clone would be desirable. Virus protein expression, localisation and action could then be studied using standard molecular techniques. This system would also allow analysis of novel antiviral compounds and vaccines. In order to facilitate the construction of such an in vitro propagation system, work was carried out to generate molecular clones spanning the polyprotein coding region of the HCV genome. A fragment cloning technique was employed to amplify short regions of the genome that could be sequentially pieced together for the construction of a molecular clone of HCV. However, none of the clones obtained were found to contain the products of HCV genomic amplification.

Studies on the pathogenesis of HCV continued, investigating part of the immune response mounted against HCV within infected cells. Studies on the response of HCV patients to interferon-alpha therapy have proven that the majority of patients who undergo treatment, which is both expensive and associated with many severe side effects, do not respond. The search for patient and virus factors that may be used to predict the response of an HCV infected patient to treatment would be of great benefit in allowing patients and doctors to make informed choices about undertaking interferon-alpha treatment.

The double-stranded RNA-induced protein kinase (PKR) is a major downstream effector of the interferon-alpha immune response. Expression levels of PKR may therefore play some role in determining the effectiveness of interferon-alpha treatment. Studies were therefore undertaken to investigate the role of PKR as a predictive factor for HCV patient response to interferon-alpha treatment. Assays were developed to measure expression levels of PKR mRNA and PKR protein extracted from liver biopsy tissues of patients infected with HCV. The aim was to analyse and correlate pre-treatment expression levels of PKR with patient response to interferon-alpha treatment. A quantitative competitive RT-PCR assay was successfully developed to measure PKR mRNA in cellular RNA extracts. A quantitative western blot assay was also developed for the quantitation of PKR protein in cellular protein extracts. The work carried out here forms the basis for future experiments in which the analysis and quantitation of specific proteins from cell culture or tissue extracts can be achieved.
ACKNOWLEDGEMENTS

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A big thanks goes to Little Al for showing me the ropes all these years (and the silly talks). Also to Dr Will Irving at Queens Medical Centre, Nottingham for his enthusiastic supply of samples, Dr. Martin Wiselka, Maggie Nicholls and the nurses at the LRI ultrasound.

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Abbreviations

A Absorbance value
aa Amino acid
Ab Antibody
Ac Acetate
amp Ampicillin
AMV Avian myeloblastosis virus
APS Ammonium persulphate
ATP Adenosine triphosphate
bp base pairs
BSA Bovine serum albumin
Cm Chloramphenicol
cpm Counts per minute
CTP Cytosine triphosphate
DEPC Diethyl pyrocarbonate
ddNTP (Di)deoxynucleoside triphosphate
DMEM Dulbecco’s modified eagle medium
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP Deoxynucleoside triphosphate
DTE Dithioerythritol
DTT Dithiothreitol
ECL Enhanced chemiluminescence
E.coli Escherichia coli
EDTA Ethylenediaminetetraacetic Acid
eIF Eukaryotic initiation factor
EtBr Ethidium bromide
F Faradays
FCS Foetal calf serum
GTP Guanosine triphosphate
HBV Hepatitis B virus
HCV Hepatitis C virus
HCC Hepatocellular carcinoma
HIV Human immunodeficiency virus
HVR Hypervariable region
IFN Interferon
<table>
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<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthio-β-D-galactoside</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ISDR</td>
<td>Interferon sensitivity determining region</td>
</tr>
<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDalton</td>
</tr>
<tr>
<td>l</td>
<td>Litres</td>
</tr>
<tr>
<td>M</td>
<td>Moles per litre</td>
</tr>
<tr>
<td>m</td>
<td>Milli (x10^-3)</td>
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<tr>
<td>mA</td>
<td>Milli amps</td>
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<td>μ</td>
<td>Micro (x10^-6)</td>
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<tr>
<td>MW</td>
<td>Molecular weight</td>
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<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
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<tr>
<td>NS</td>
<td>Nonstructural</td>
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<td>Ohms</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>p</td>
<td>Pico (x10^-12)</td>
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<td>PIPES</td>
<td>Piperazine-N,N'-bis[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PTB</td>
<td>Polypyrimidine binding protein</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA dependent RNA polymerase</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Ribonuclease</td>
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<td>rNTP</td>
<td>Ribonucleoside triphosphate</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>RT-PCR</td>
<td>Reverse transcription - polymerase chain reaction</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>TAE</td>
<td>Tris/acetate/EDTA buffer</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/boric acid/EDTA buffer</td>
</tr>
<tr>
<td>Abbreviation</td>
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<tr>
<td>TE</td>
<td>Tris/EDTA buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
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<tr>
<td>TLCK</td>
<td>Na-p-Tosyl-L-lysine chloromethyl ketone</td>
</tr>
<tr>
<td>Tm</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>TMT/SS</td>
<td>PBS/milk/tween 20/lamb serum buffer</td>
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<td>TNMAT</td>
<td>Tris/sodium chloride/milk/tween 20 buffer</td>
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<td>Tris</td>
<td>2-Amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>TTP</td>
<td>Thymidine triphosphate</td>
</tr>
<tr>
<td>U</td>
<td>Units of enzyme</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine triphosphate</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>v/v</td>
<td>Volume by volume</td>
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<td>V</td>
<td>Volts</td>
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<td>W</td>
<td>Watts</td>
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<td>w/v</td>
<td>Weight by volume</td>
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Chapter 1

Introduction
1.1 History of hepatitis C virus

The monotypic genus hepatitis C virus (HCV) is a member of the \textit{Flaviviridae} family of viruses, which also includes the flavivirus and pestivirus groups. In 1985 the members of this family, similar in their genomic structures and replicative mechanisms, were classified as a separate and new group (Westaway \textit{et al}, 1985). Since then there have been over 68 groups associated with the flaviviruses (Calisher \textit{et al}, 1989) which include many agents causing encephalitis and haemorrhagic fever. Three groups have been identified within the pestivirus genera (Moennig \textit{et al}, 1992) that target animals. More recently, HCV was identified by Choo \textit{et al} (1989) as the major cause of viral non A non B hepatitis (i.e. caused by neither hepatitis A virus or hepatitis B virus) and was included in the \textit{Flaviviridae} family. Table 1.1 shows a breakdown of the genera within the \textit{Flaviviridae} family with some associated diseases.

The causative agent of Yellow Fever was the first virus of the \textit{Flaviviridae} to be identified. Flavus (meaning yellow) was therefore used to name this family. Though pestiviruses and flaviviruses are both arthropod borne, HCV is not but is transmitted by direct contact with infected blood and blood products.

Recently two new hepatitis viruses have been identified, hepatitis G virus (HGV) and a transfusion transmitted virus (TTV) (Nishizawa \textit{et al}, 1997), though no disease has yet been associated with either of these viruses. A virus isolate termed GB virus isolate C (GBV-C) is known to have 85%-95% sequence homology to HGV (Simons \textit{et al}, 1995; Linnen \textit{et al}, 1996). HGV/GBV-C possesses a genome similar to those of the \textit{Flaviviridae} and has therefore been associated with this family of viruses.

The disease caused by HCV is recognised as a major health care problem. It causes a range of problems in an infected patient stemming from damage to its target organ, the liver. The initial infection with HCV may sometimes remain acute and self-limiting though more often, it will progress to a chronic stage that may remain silent for up to 30 years. Chronic infection of the liver leads to long term damage that may result in death of the patient from liver failure.
Table 1.1 Members of the *Flaviviridae* family

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<tr>
<th>Genus</th>
<th>Group</th>
<th>Some viral members</th>
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<td>Flaviviruses</td>
<td>Yellow Fever virus</td>
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<td>Dengue (DEN)</td>
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<td>Tick-borne encephalitis</td>
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<td>Pestiviruses</td>
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<td>Classical swine fever virus</td>
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<td>Hepacivirus</td>
<td>Hepatitis C virus</td>
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1.2 Structure of the HCV virus

Though the constituent genera of the *Flaviviridae* family cause quite dissimilar diseases, they are all structurally similar and as a result are believed to function and replicate in similar fashions. All members of the *Flaviviridae* family are enveloped viruses that consist of a lipid bilayer containing surface glycoproteins surrounding a nucleocapsid which itself contains a single stranded, positive sense ribonucleic acid (RNA) molecule. Immunoelectron microscopic studies have shown viral particles in plasma from HCV infected patients and sections from Daudi cells inoculated *in vitro* with HCV from infected plasma (Kaito *et al*, 1994; Shimizu *et al*, 1996). These reports indicate that HCV virus particles are 50 to 65 nm in diameter, having an inner core expected of an enveloped virus. Viral particles reportedly have a buoyant density in sucrose gradients between 1.09 and 1.11 g/ml (Bradley *et al*, 1991). Kaito *et al* (1994) also reported surface projections of 6 nm visible from the surface of the virus, indicative of envelope glycoproteins. This viral envelope is also sensitive to sodium dodecyl sulphate (SDS) and proteinase K treatment indicating its protein-rich nature.

The HCV genome is encoded by a positive sense RNA which is around 9500 nucleotides (nt) in length. This encodes untranslated regions at both the 5' and 3' termini of the genome flanking a single open reading frame (ORF). The large ORF is translated to a single polyprotein 3010 to
3033 amino acids (aa) long (Kato et al, 1994; Takamizawa et al, 1991; Okamoto et al, 1994) that is cleaved to its constituent proteins by a combination of host and viral proteases. Figure 1.1 shows the organisation of the HCV genome.

The structural proteins include the capsid (C) and two envelope glycoproteins E1 and E2 which are cleaved and processed by a host signal peptidase located within the lumen of the host cell endoplasmic reticulum (ER) (Selby et al, 1993). The non-structural proteins are encoded in the order NS2, NS3, NS4a, NS4b, NS5a and NS5b. Sequence similarities of HCV proteins with comparable proteins in other virus members of the Flaviviridae have been identified, leading to the identification of HCV protein functions.

**Figure 1.1** The HCV genome

The process of virus replication which involves the production of viral proteins, replication of the viral genome through a negative sense full length RNA intermediate and progeny virion assembly occurs within the host cell cytoplasm. Through the use of molecular techniques, the functions of some of these viral proteins and their cellular localisation and action have been elucidated.
1.2.1 Structural proteins of HCV

i) Core protein

The core protein is the first protein encoded by the genome. It is a 22 kDa protein encoded by 570 nt, a high proportion of which (20%) translate to residues Arg and Lys with 12% encoding Pro (Houghton et al., 1991; Choo et al., 1991). There is a large amount of sequence conservation between different viral genotypes, suggesting the function of this core protein is essential. Due to its highly conserved nature, this protein may be an important factor in devising an immunisation strategy against infection by HCV. Lagging et al. (1995) created recombinant full length core protein and elicited both an antibody and a cytotoxic T lymphocyte immune response in BALB/c mice when challenged, suggesting that core protein-encoding DNA may be used as a vaccine.

The C-terminal 20 aa of core protein are thought to act as the sequence identified by a host signal peptidase which cleaves the core protein at residue 174 (Santolini et al., 1994) whilst the protein is within the lumen of the cellular ER (Farci et al., 1994). The mature core protein is then directed back across the membrane of the ER into the cytoplasm (Santolini et al., 1994; Selby et al., 1993). Santolini also describes that core protein remains associated with the ER membrane whilst some core protein has been located to the cell nucleus (Shih et al., 1993).

The core protein also contains an RNA binding domain located to residues 1-75, the RNA binding capacity acting to complex core with progeny genomes in the formation progeny virion nucleocapsid. A ribosomal binding capacity is located to the same region and binds the 60S subunit of host ribosomes in vitro (Santolini et al., 1994). Matsumoto et al. (1996) suggested that in order to complex with the RNA genome (so forming the protective nucleocapsid structure) core protein must form multimers of itself. In vitro studies identified a hydrophilic domain at residues 1-115 that are thought to be responsible for homotypic interactions of the core protein. The C terminal portion of core protein covering residues 116-191 is associated with localisation of the protein to the ER membrane though distinct regions have not been recognised.
Shih et al (1995) identified two serine residues at positions 99 and 116 that are phosphorylated by both protein kinase A and C. In this study, the phosphorylated core protein is thought to suppress the replication of hepatitis B virus (HBV) which may be an important mechanism employed by HCV to subjugate co-infecting viruses, though the mechanism by which this may occur has not been reported.

ii) E1 and E2
These two proteins constitute the envelope glycoproteins that are responsible for the attachment of HCV to the CD81 receptor on its host cell (Pileri et al, 1998). E1 and E2, consecutive in the viral polyprotein, are cleaved at residue Gly383/His384 of the E1/E2 junction.

E1 is encoded by residues 173-383 of the polyprotein, the first 18 aa acting as the recognition sequence for a host encoded signal peptidase. This signal directs the 35 kDa protein to the lumen of the ER where it is cleaved, leaving E1 attached to the ER membrane. There is a hydrophobic domain in the C-terminal region of E1 from residues 348-370 (Matsuura et al, 1992; Kohara et al, 1992) thought to act as an anchor for the attachment of the protein within the ER membrane. Similarly, E2 is thought to be anchored to this membrane via a C-terminal domain as yet unidentified. Evidence of C-terminal truncated E2 proteins being secreted by a baculovirus expression system for the structural protein shows the presence of a membrane anchor for this protein (Matsuura et al, 1994). The anchoring of these two structural proteins to the ER membrane, with the main body of the protein in the ER lumen correlates well with the replicative model that progeny virion nucleocapsids bud into the lumen of membrane-bound vesicles within the host cell, collecting a surrounding envelope with attached glycoproteins that would now be turned onto the outer surface of new virions.

E2 is a 70 kDa protein encoded by residues 384-755. It is very similar in structure to the E2 structural envelope glycoprotein of pestiviruses and the NS1 protein of flavivirus, though it is now certainly more like the former. E2 contains areas of glycosylation also found in the HCV E1 protein. HCV E1 contains 5-6 glycosylation sites while HCV E2 possesses 9-11 sites (Hijikata et al, 1991). A 7 kDa protein (p7) has also
been associated with E2 and is found at its C-terminal between residues 746-809 (Lin et al, 1994). This may be associated with E2 in a precursor form. Cleavage of p7 may result in the mature form of the E2 protein.

E1 and E2 proteins are believed to possess a heterodimeric nature (Choo et al, 1994). Bound complexes of E1 and E2 have also been proposed though evidence suggests this occurs without the formation of disulphide bridges (Grakoui et al, 1993; Ralston et al, 1993).

The HCV E2 glycoprotein possesses two hypervariable regions (HVR) located to its N-terminal (HVR1) at residue 384-411 and a second region (HVR2) at residue 474-481 (Hijikata et al, 1991). These two regions are believed to be the locations for surface antigen epitopes recognised by host antibodies. Sequence analysis of HVR1 revealed similarities with the V3 loop of the human immunodeficiency virus (HIV) gp120 protein (Weiner et al, 1992). This has been identified as a hypervariable region involved in viral evasion of the immune response. By comparison, HVR1 of HCV E2 has been proposed to act in a similar fashion in evading recognition by host antibodies previously raised against variant surface epitopes of HCV. HVR1 has been proven to induce antibody responses (Kato et al, 1993) indicating that E2 is indeed a viral surface glycoprotein that is recognised by host immune B-cells. Zibert et al (1997) further defined this recognition by host antibodies to the C-terminal of E2 HVR1. Mutations within the two hypervariable regions are believed to contribute to the high level of genetic variation within a viral population. As these regions may be the main viral epitopes recognised by host antibodies, mutations within E2 HVRs would account for the high rate of immune escape by HCV though an infected individual will still show the presence of anti-HCV antibodies. Such antibodies will be circulating but will be unable to recognise the now mutated epitopes on the surface of the virus (Shimizu et al, 1994).
**1.2.2 Non-structural proteins of HCV**

i) **NS2**

No defined biological function has yet been identified for this protein though it is known to act in association with the N-terminal region of NS3 in HCV polyprotein cleavage. NS2 is encoded between residues 810 and 1026 within the viral polyprotein giving a 23 kDa product (Grakoui *et al*, 1993). The N-terminal cleavage of NS2 is thought to be mediated by a host signal peptidase while cleavage at its C-terminal, at the junction with NS3, occurs through the action of a viral protease. It is believed that the NS2 and NS3 proteins remain associated during transcription and cleavage of the polyprotein. This NS2/NS3 protein complex then acts autocatalytically in cleaving its own NS2/NS3 junction. Santolini *et al* (1995) described an NS2/NS3 precursor protein directed to the cytoplasmic ER where cleavage of the two results in NS2 insertion into the ER membrane. The purpose of this is unknown. There does not seem to be any similar protein to NS2 found in the flavivirus and pestivirus members of the *Flaviviridae* family and the lack of an *in vitro* expression system for NS2 has hampered the understanding of this protein.
It is known that the NS2/NS3 protease is stabilised by zinc ions at positions His 952 and Cys 993. Neddermann et al (1997) proposed two models to this protease Zn$^{2+}$ binding, the first involving the His and Cys residues acting as a thiol-protease catalytic dyad. The second model involves the dimerisation of two NS2/NS3 protein complexes through the formation of a tetrahedral Zn$^{2+}$ binding site. How such protein complexes function has not been determined. The current understanding of NS2 is that it forms a metallo-proteinase with the N-terminal of NS3 that acts in cis to cleave itself from NS3 and is then directed to the ER membrane within the host cell. Further investigation is required in order to understand the function of this first non-structural protein of the HCV genome.

ii) NS3
This 70 kDa protein encoded from residues 1027-1657 has been extensively studied as one of the major proteins of the hepatitis C virus. NS3 has been shown to possess three different enzymic functions. The N-terminal third of NS3 acts as a serine proteinase in conjunction with either NS2 or NS4a proteins to cleave the non-structural regions of the viral polyprotein. This domain contains the conserved residues His 1083, Asp 1107 and Ser 1165 forming what has been identified as a chymotrypsin-like serine proteinase motif (Tomei et al, 1993; Hahm et al, 1995) found in serine proteinases in other viruses within the same family. The action of this proteinase is described in section 1.2.2.i and 1.2.2.iii.

The C-terminal two-thirds of NS3 contain the functional regions for an RNA helicase and a nucleoside triphosphatase (NTPase). The region encoded by these two contains conserved sequence motifs recognised as NTP-binding proteins found in the DEAD superfamily of RNA helicases. These are believed to act in conjunction with the NS5b RNA-dependent RNA-polymerase in replication of the viral RNA genome. Dong-Wook et al (1997) examined the functional domains of the RNA helicase and NTPase enzymes within NS3 and identified them to residues 1209 - 1608 in the polyprotein. This gives a 400 aa protein that possesses both functions and is consistent with the lack of any evidence that this region of NS3 is cleaved into the two enzymes separately.
Recombinant NS3 protein has been shown to bind tightly to added poly U tracts. This results in an increase in both the protease and NTPase enzymic activities, though RNA helicase was not affected (Morgenstern et al, 1997).

Given this evidence, a model was proposed for the activity and role of the NS3 NTPase and proteinase in viral transcription and therefore replication. The binding of NS3 to a poly U tract in the 3'UTR is thought to destabilise the NTPase and RNA helicase activities while increasing the activity of its proteinase region. This in turn may enhance the proteolytic processing of the NS5b polymerase, even localising it to the 3'UTR where viral RNA replication can occur. The NTPase and RNA helicase must also play some role in the destabilisation of RNA secondary structure and the separation of positive and negative strands of RNA during genomic replication to facilitate the binding and function of the RNA-dependent RNA polymerase.

A conserved sequence has also been identified within NS3 that is similar to a functional domain on the cellular protein kinase A (PKA) (Borowski et al, 1997). It has been proposed that this sequence (RRGRTGRGRRGITR) on the viral protein binds to cellular PKA and inhibits the translocation of PKA to the cell nucleus, inhibiting action of the protein. In an infected cell, this may have an effect of inhibition of host anti-viral mechanisms other than the inhibition of PKR by the viral NS5a (section 1.2.2.v). Viral replication would not be inhibited by the host cell so allowing HCV to survive.

iii) NS4a

Little is known about this 4 kDa non-structural protein. It is encoded from residue 1658 -1711 of the viral polyprotein. NS4a is thought to act as a co-factor for the action of the N-terminal portion of NS3, the serine protease. This association acts to cleave the viral polyprotein at the junctions between NS3 and NS4a in cis at a Thr residue and between NS5a/NS5b, NS4a/NS4b and NS4b/NS5a in trans at Cys residues in this order (Grakoui et al, 1993; Pizzi et al, 1994). The complex formed between NS4a and NS3 is detergent-stable (Neddermann et al, 1997)
with the interaction between the two proteins believed to involve a 30 aa region at the N-terminal of NS3 and a 14 aa region in NS4a near its N-terminal. Similar to the theory for NS2 insertion into the ER membrane, NS4a is thought to act as an anchor for the attachment of NS3 to the membrane of intracellular vesicles (Tanji et al, 1995). Lin et al (1995) described a highly hydrophobic region at the N-terminal of NS4a that may form a helix used as a trans-membrane anchor. What purpose this may serve is unknown but it may have been a by-product of the in vitro expression system used.

iv) NS4b
Analyses of proteolytic cleavage and processing of the HCV genome have lead to the identification of this NS4b protein encoded from residue 1712 to 1972 giving a 27 kDa product (Selby et al, 1993; Grakoui et al, 1993; Bartenschlager et al, 1994). This coincides with similar proteins found in other viruses of the Flaviviridae family, notably the p30 protein of the pestiviruses (Grakoui et al, 1993). Kinetic studies performed by Bartenschlager et al (1994) showed there to be stable intermediates in the polyprotein processing containing complexes of NS4a/4b and NS4a/4b/5a. The relatively slow cleavage of NS4b from these precursors indicates it is not an essential protein for viral protein synthesis. Whether these non-structural protein complexes act functionally or are simply intermediates in the polyprotein cleavage is unknown. NS4b has also been shown to accumulate in the endoplasmic reticulum along with NS4a (Selby et al, 1993).

This mainly basic protein seems to have little, if any, similarities with other major protein sequences known to date. There can be no comparisons made by structural analysis with other proteins and therefore its function remains unknown.

v) NS5a
The non-structural protein 5a is encoded between residues 1973 and 2419 of the polyprotein. This gives a 56 kDa product which has also been found as a 58 kDa protein upon hyperphosphorylation within the region aa 2197 - 2204. Interaction of NS5a with NS4a seems to enhance
phosphorylation and expression of the larger 58 kDa protein. Phosphorylation of both the 56 and 58 kDa versions of NS5a have been correlated with residues 2200 - 2250 and an area at the C-terminus of NS5a (Tanji et al, 1995). Reed et al (1997) demonstrated that phosphorylation occurs at Ser and Thr residues.

The precise function of this protein has not been elucidated though some roles have been suggested. One of these is the action of NS5a as a cofactor for NS5b and NS3 as the major viral proteins involved in viral replication. Significant studies have centred on the role of NS5a as an anti-immune response protein employed by HCV to combat the cytotoxic host immune response. These studies have focused on determining why certain HCV genotypes seem to confer a higher incidence of resistance to interferon (IFN) alpha therapy. Infection with types 1a and 1b has been generally accepted as one of the more important factors in determining patient resistance to IFN (Gale Jr. et al, 1997 & 1998; Duverlie et al, 1998; Polyak et al, 1998; Fukuma et al, 1998). Enomoto et al (1995) carried out an extensive study on the sequence diversity between different isolates of HCV in patients undergoing IFN therapy. A significant number of isolates from non-responding individuals shared a conserved sequence within the NS5a region and patients who responded to IFN and whose HCV isolates were therefore sensitive, commonly possessed mutations from the conserved sequence. This region has been termed the interferon sensitivity determining region and is located to residues 2209 - 2248. The presence of this region has been verified by Chayama et al (1997) who confirmed that substitutions within this region confer less resistance to IFN therapy than isolates with the prototype sequence taken from HCV genotype 1b. An important single substitution that seems to switch HCV from being IFN-resistant to IFN-sensitive is commonly found at residue 2218 though other residue substitutions have also been observed at 2171, 2187 and 2413 (Enomoto et al, 1995).

Gale Jr. et al (1997) suggested a mechanism by which the NS5a protein directly interacts with protein kinase R (PKR), a double-stranded RNA binding protein induced by IFN that plays an important role in arresting viral protein synthesis and therefore viral replication (section 1.7.4). There is evidence that NS5a binds to residues 244 - 366 of the
PKR protein, the dimerisation region essential for its functional activity. By occupying this site in PKR, NS5a inhibits dimerisation, preventing PKR activation and therefore function of the kinase domain. Protein synthesis within the infected cell is not inhibited and thus HCV protein synthesis occurs resulting in viral replication. Studies by Gale Jr. et al. (1998) confirmed the binding of NS5a to the dimerisation region of PKR in a similar fashion to the p58IPK protein of influenza virus (Lee et al., 1994).

In light of this anti-PKR mechanism, a role for NS5a has also been suggested in the development of hepatocellular carcinoma (HCC) (Gale Jr. et al., 1997), a condition suffered by some HCV patients that is linked with their viral infection. As well as acting as an anti-viral protein, PKR is thought to play a role in cell apoptosis. If the action of PKR is inhibited, the cell may continue functioning and over time, may become immortalised so beginning a cancer in the individual. This is still only a theory but there is some correlation between long term chronicity of HCV and the development of HCC over a period of tens of years.

vi) NS5b
This 65 kDa protein is the final protein encoded within the HCV polyprotein, from residue 2421 to the UGA stop codon. It has a highly conserved sequence that contains the GDD motif at residues 2737-2739 (Yuan et al., 1997), a recognised motif conserved in RNA virus RNA polymerases. NS5b has therefore been identified as an RNA-dependent RNA-polymerase (RdRp) and is responsible for the replication of the viral RNA genome. Yuan et al. (1997) carried out expression studies of NS5b protein using a bacterial in vitro expression system of a full length NS5b clone. They demonstrated that this protein acts as an RNA polymerase in the presence of poly A template. This was proven by the work of Behrens et al. (1996) who reported the activity of NS5b in replicating a variety of added RNAs. The RdRp enzyme is Mg^{2+} dependent with an optimum activity at pH 7.5. The production of RNA dimers as products of the RdRp has also been demonstrated (Behrens et al., 1996). The model given for the action of RdRp is that the C-terminus of genomic RNA acts as a template for the priming of the
polymerase enzyme by looping back on itself to a complementary sequence. The polymerase then transcribes negative sense RNA from this primed position, forming an RNA hairpin duplex. Though other groups have found these duplex RNAs during in vitro replication of RNA by various viral RNA-dependent RNA-polymerases (Cho et al, 1993; Neufeld et al 1991) and in in vivo systems (Young et al, 1985) this has not yet been proven in an in vivo study of HCV and is therefore a proposed model for HCV RdRp action based on other viruses.

The low levels of RdRp activity reported by both Behrens et al (1996) and Yuan et al (1997) correlate with low levels of similar RdRp's found in flavi- and pestiviruses which may indicate a low stability of the enzyme. It has been suggested that NS5b may require an additional cofactor for efficient activity. NS5a has been put forward for this role though this is purely speculative until the true nature of the NS5a protein is understood.

1.2.3 Untranslated regions of the HCV genome
i) 5'UTR
The 5' untranslated region (UTR) of HCV spans the first 341 nt of the RNA genome up to the AUG start codon used for transcription of the viral polyprotein (figure 1.1). It contains a highly conserved sequence with over 98% homology between different genotypes of HCV. For this reason, detection of the 5'UTR by the reverse-transcription polymerase chain reaction technique is often used as a diagnostic test for the presence of HCV. Oligonucleotide primers used in this detection method would be able to recognise and anneal to the 5'UTR of most HCV genomes circulating in a given patient.

The 5'UTR sequence contains 3 to 5 AUG codons, many of which are not translated directly in viral protein synthesis. Various stem-loop structures have been predicted within this region (Fukushi et al, 1997; Brown et al, 1992; Honda et al, 1996) which, by comparison to similar 5'UTR regions of pestiviruses and picornaviruses, has lead to the identification of an internal ribosome entry site (IRES) around the 3' most portion of this non-coding sequence. This IRES allows direct binding of host ribosome to the viral genomic RNA upstream of the
initiator start codon. This results in cap-independent translation of the viral polyprotein, unlike flaviviruses. Various groups have examined this IRES region to identify its functional domains and have so far proven that almost the whole region is important for the IRES function (figure 1.3). Reynolds (1995) showed that the 3' boundary of this IRES is found 14-31 nt downstream of the initiator AUG codon and is therefore within the N-terminal region of the HCV core protein. In vitro analysis of deletions within the 5'UTR cloned into an expression vector by Kamoshita et al (1997) identified the 5' boundary of the HCV IRES to 28-45 nt of the 5'UTR.

Figure 1.3 Predicted structure of the HCV IRES region

Key:
- AUG codon
- HCV RNA genome

Adapted from Brown et al, 1992

Nomoto et al (1994) identified motifs in the HCV IRES similar to those found in the picornavirus IRES (Pilipenko et al, 1992) in which certain sequences are complementary to the 18S ribosomal subunit. It has therefore been suggested that the 18S subunit of the ribosome binds directly to the IRES of HCV from which transcription of the polyprotein proceeds.
Three poly-pyrimidine tracts have also been identified within the 5'UTR (Ali et al, 1995) that have been proven to support binding to cellular poly-pyrimidine tract binding proteins (PTBs). These are located in the regions 1-84 nt, 126-134 nt and 135-291 nt. Fukushi et al (1997) stated that PTBs are unlikely to play a major role in the action of the HCV IRES as addition of cellular PTBs to a PTB-deficient translation system that included the HCV IRES sequence did not restore the function of this expressed IRES. Fukushi identified a cellular 25 kDa protein that binds to the α-branch in the second stem-loop domain of the 5'UTR. This does not contain a PTB binding site suggesting that other cellular factors may play a role in the function of the HCV IRES. However, Ito et al (1998) reported the enhancement of HCV translation from the IRES in the presence of PTBs bound to the 3'X tail of the 3'UTR genomic region. A definite role of PTB binding to the 5'UTR and 3' UTR regions of HCV has therefore yet to be determined.

ii) 3'UTR
This region extends from the stop codon of the HCV polyprotein for 200 to 220 nt. There is an initial stretch of 28-42 nt that has a high variability between different viral isolates (Neddermann et al, 1997), a poly U stretch of variable length and a 98 nt sequence which has been termed the 3'X tail (Tanaka et al, 1995). Tanaka et al described the polyU stretch as being 60-80 nt long which is interspersed at various points by mainly C and sometimes G nucleotides.

The presence of the 3'X tail was verified by the work of Kolykhalov et al (1996) in which a 98 - 100% conserved 98 nt sequence was identified. Models of the secondary structure of this tail have predicted a stable stem-loop structure which Kolykhalov et al (1996) identified to the last 46 nt (figure 1.4). This is not an uncommon occurrence in single stranded RNA viruses, though previous work on the 3' end of HCV could only identify a poly U tract as the 3' terminus. The flavi- and pestiviruses have also been found to possess initial unconserved sequences at the amino terminal of their 3'UTR that are followed by long single-nucleotide stretches 225 nt in length in pestiviruses and of varying length and base composition in flaviviruses depending on the
species. Like HCV, these two other genera of the Flaviviridae have a region at their extreme 3' end which in flaviviruses is around 100 nt long but as yet not defined for pestiviruses. The fact that this type of conserved sequence is found in viruses indicates that it serves some function in the viral life cycle. A role of this conserved tail has been described for some RNA viruses in the initiation of viral genomic packaging into new virions through trans or cis interactions with host or viral proteins. The poly U tract has also been identified in some viruses as having binding motifs for polypyrimidine-tract binding proteins (PTB) which may interact with other factors in the replication of viral RNA (Singh et al, 1995).

It is believed that the highly conserved sequence downstream of the poly U tract in HCV is the definitive 3' terminus of the genome.

**Figure 1.4** Predicted structure of the 3'X tail region of the HCV genome. Taken from Tanaka et al (1995)
1.3 Replication of the HCV virus

The replication of flaviviruses has been used as a model for the replication of HCV, though this is still a relatively early picture. Figure 1.5 shows the general life cycle of HCV. It is believed that virus particles enter a host cell through attachment of the E1 and E2 glycoproteins to, as yet, unidentified receptors on the surface of the target cell. The virus is taken into the cell by endocytosis where, under low pH conditions, viral and cellular membrane fusion occurs resulting in the release of the viral nucleocapsid into the host cell cytoplasm. Entry by direct membrane fusion between viral and cellular plasma membranes has also been reported for flaviviruses (Hase et al, 1989a; 1989b) though whether HCV uses these mechanisms is unclear.

The viral RNA genome acts as a species of mRNA and is transcribed and processed through the direction of host ribosomes to the IRES element in the 5'UTR. The structural and non-structural proteins are translated in a single polyprotein which is initially cleaved by host peptidases. Once cleaved, virus proteins act to further cleave the polyprotein. They also act in genomic RNA replication and packaging of progeny virions. Genomic RNA associates with core protein which then buds into membrane bound cytoplasmic vesicles already embedded by the two envelope glycoproteins. The RNA genome acts as a template for the synthesis of intermediate negative strand RNA through the actions of the NS5b RNA dependent RNA polymerase and the C terminal region of NS3 (NTPase and helicase). These negative sense RNA molecules than act as templates for the synthesis of positive strand RNAs which are then either used as templates for further RNA replication, protein synthesis or as genomic RNA for progeny nucleocapsid formation and virion packaging.

Rice et al (1990) put forward the theory that the cytoplasmic core protein of flaviviruses interacts with positive sense RNA molecules to form nucleocapsid precursors in the cell cytoplasm. These bud into the lumen of a membrane bound vesicle, originating from the cellular endoplasmic reticulum (ER), collecting its envelope from the vesicle membrane already embedded with envelope glycoproteins. Within the host cell are now intracellular vesicles containing forms of progeny virions. This theory continues in that some further processing occurs.
within these vesicles to modify the envelope glycoproteins to their mature state and is supported by evidence of cleavage of the flavivirus prM protein by a host furin-like enzyme which leaves the virions released from the cell with a mature M protein (Shapiro et al, 1972). Processed mature virions then leave the cell by exocytosis of the vesicle involving fusion of the vesicle membrane with the cell plasma membrane so releasing new virus.

Shimizu et al (1996) described immunoelectron microscopic evidence that virus like particles can be detected within cytoplasmic vesicles. This would suggest that HCV replication has similarities with the acknowledged method for the assembly and maturation of flaviviruses. As the replicative mechanism of HCV is still relatively unknown, the model given by the flaviviruses may be taken as the model for HCV replication until an alternative process is described.
The life cycle of HCV

1) Attachment of viral glycoproteins to host cell membrane receptors and uptake of virus by endocytosis.
2) Viral and cellular membrane fusion releases viral genomic RNA.
3) Transcription of viral genomic RNA to polyprotein.
4) Cleavage of polyprotein by host signal peptidases and viral proteinases.
5) Replication of viral genomic RNA by NS5b RNA dependent RNA polymerase through synthesis of a negative strand RNA intermediate.
6) Envelope glycoproteins targeted to cytoplasmic vesicle membrane. Genomic RNA forms nucleocapsid with core protein by association, buds into membrane bound vesicle, collecting membrane so forming new virions.
7) Exocytosis of vesicle resulting in release of progeny virions.
1.4 HCV genetic variability

Studies to sequence various HCV isolates have lead to the understanding that there are significant sequence differences found within certain regions of the viral genome. Ogata et al (1991) showed that over a period of 13 years, the sequence of extracted virus from a single patient varied by up to 82% in certain regions of the genome. The highest rate of variation seemed to occur within a hypervariable region at the C-terminus of E2. Two HVR regions within the envelope glycoproteins have now been identified and have been attributed to epitope variation by the virus resulting in host antibody evasion (section 1.2.1.ii).

Sequence variation within a single stranded RNA genome is recognised as a common occurrence due to the lack of any proof-reading ability by the viral RdRp enzyme during RNA replication. Therefore, a natural variation will occur as the virus undergoes multiple replication cycles. Some of these will produce deleterious mutational changes in the genome and some will be beneficial (antibody evasion for example). It has been generally accepted that the relatively high sequence variation that occurs in the C and NS5 regions of the genome are sufficient to identify one particular isolate from another. Comparisons of a 222 bp sequence within either the NS5 and core regions are used to identify different virus isolates and are reported as sufficient to group these isolates into different viral genotypes. Over time, the further isolation of many different isolates of HCV has lead to the classification of 6 viral genotypes with over 30 sub-types (DeLamballerie et al, 1997). Enomoto et al (1990) suggested that a 33% sequence difference is sufficient to class isolates as different genotypes whilst only a 20% difference classes them into sub-types. Chan et al (1992) suggested viruses of different genotypes should differ by 48-62% while subtypes should only differ by 23-25%.

There is much discussion in the literature over which sequences should be used in developing a standard sequence for genotype identification. Mellor et al (1995) use 222 bp sequences from the NS5 region of the viral genome, whilst Bukh et al (1993) use a similar stretch but from the E1 region. Tokita et al (1994) suggests using the whole genomic sequence to identify different viral genotypes or sub-types. Having to sequence an entire genome (9.5 kb) to identify one genotype from another would be extremely difficult in a clinical diagnostic laboratory.
There have also been a range of different nomenclatures assigned to the genotypes of HCV (Okamoto et al, 1992; Houghton et al, 1991; Simmonds et al, 1993). Simmonds et al (1994) proposed all groups use a single nomenclature used by his group which is the system presently in use. Epidemiological studies have identified many different subtypes associated with specific geographical regions. Table 1.2 shows the different HCV genotypes (using the system devised by Simmonds et al, 1993) with some sub-types and their geographical areas of prevalence.

Phylogenetic analysis of genotype sequences by Simmonds et al (1993) has identified the presence of a common ancestral genomic sequence from which the various genotypes and sub-types found today are related (figure 1.6).

It must be noted that an infected individual may, over time, develop a population of different viral sub-types. They may have become infected more than once (if receiving unscreened blood products) with different viral genotypes. As the virus replicates and mutations arise within progeny genomes, a quasispecies will develop within a single patient, one or more of which genotypes predominates. This is seen especially in patients undergoing interferon therapy in which interferon-sensitive forms of the virus will be affected by the cytokine (section 1.2.2.v). Over time, if a patient carries types 1a or 1b HCV within a quasispecies, these will predominate as non-type 1 genotypes are removed from the population.

Table 1.2 HCV genotypes by the Simmonds system

<table>
<thead>
<tr>
<th>Predominant genotype</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>World-wide</td>
</tr>
<tr>
<td>1b</td>
<td>World-wide</td>
</tr>
<tr>
<td>2a</td>
<td>World-wide</td>
</tr>
<tr>
<td>2b</td>
<td>World-wide</td>
</tr>
<tr>
<td>3a</td>
<td>Pakistan, Thailand</td>
</tr>
<tr>
<td>3b</td>
<td>Bangladesh, India, Thailand</td>
</tr>
<tr>
<td>4a</td>
<td>Middle East and Africa</td>
</tr>
<tr>
<td>5a</td>
<td>South Africa</td>
</tr>
<tr>
<td>6a</td>
<td>Hong Kong</td>
</tr>
</tbody>
</table>
Figure 1.6 Phylogenetic analysis of NS5 sequences from different HCV isolates shows six major genotypes with some sub-types

Taken from Simmonds et al (1993)

1.5 HCV infection

1.5.1 Sites of infection

HCV targets and infects the liver. No other organs of an infected patient have been proven to support viral replication. Though Cribier et al (1995) reported the existence of HCV particles in peripheral blood mononuclear cells (PBMCs), the low level of negative strand genomic RNA detected would indicate a very low level of replication. This was verified by the work of Mellor et al (1997) in which PBMCs were also studied for HCV replication. It was found that the level of HCV found in PBMCs of infected patients support this idea that HCV replicates in PBMCs, but at low levels. Mellor et al's report also discounted the idea that PBMCs act as a reservoir for the HCV virus in the role of infection chronicity.
1.5.2 \textit{In vivo and in vitro} models of HCV replication

To date, there has only been one \textit{in vivo} model found that supports HCV virus replication, the chimpanzee. Various problems arise from this in that only one inoculation per single chimpanzee can be carried out in any experimental trial which makes this a very expensive system for virus propagation. A useful system would be the development of a mammalian cell culture proven to support the long term replication of HCV. This would lead to a more accessible way in which to study the virus, using cell extraction techniques and \textit{in situ} analysis of protein transcription, processing, localisation, viral genome replication and eventual virion assembly. A fully competent \textit{in vitro} system would also be of great importance in assaying the effects of novel anti-viral therapies.

Various groups have attempted to propagate HCV in cell lines, using HCV from infected sera or by cloning the virus and promoting expression in cell lines. Yoo \textit{et al} (1995) and Mizuno \textit{et al} (1995) both reported virus replication in Huh7 and HeLa G cell lines respectively, but these two groups used an expression vector encoding a full length clone of HCV with a poly U terminus. Tanaka \textit{et al} (1995) later reported the existence of the 3'X tail, a 98 nt region at the C-terminus of the 3'UTR (section 1.2.3.ii). This region was not included in either of the previous full length clones and while virus proteins were shown to be expressed by the clones, as the self-priming 3' end of the genome was missing, genomic RNA replication could not have taken place.

HCV taken from infected sera has been used to infect cell lines \textit{in vitro} and so propagate the virus, setting up a long-term expression system. Ito \textit{et al} (1996) dissociated liver cells from an HCV infected patient and propagated these primary hepatocytes. Assays for the presence of HCV replication by negative strand specific RT-PCR showed a very low level of negative strand HCV genomic RNA present. However, Lanford \textit{et al} (1995) discussed the reliability of such assays as insignificant in that the negative strand of genomic RNA detected may well occur as a result of the original inoculum still present. There is also some doubt over the specificity of the RT-PCR used, suggesting that mis-priming may result in detection of positive as well as negative strands of the viral genome. Sequential dilution of passaged virus through a cell line and detection of increased viral titres would prove increasing viral levels. Also, detection of HCV
non-structural proteins extracted from within cells would prove replication is occurring. Structural proteins detected may originate from extracellular virus used as the inoculum.

Recently, Kolyakhov et al (1997) reported the successful expression of a full length HCV clone. All regions of the viral genome known to date were encoded including the 3’X tail. RNA transcribed \textit{in vitro} from this clone was proven to cause infectious hepatitis upon inoculation into a naive chimpanzee. Yanagi et al (1997 & 1998) also described the construction of full length clones of HCV that caused hepatitis in inoculated chimpanzees and resulted in recovery of HCV virus from the animal.

Aizaki et al (1998) and Moradpour et al (1998) both described the development of cell cultures in which cloned HCV genomic sequences resulted in the expression of virus RNA and functional proteins. These reports will allow groups to study HCV \textit{in vitro} in much greater detail, studying virus processes as well as providing an initial screening system for novel anti-viral compounds, something that could not be achieved when using chimpanzees.

1.6 Pathogenesis of HCV
Hepatitis is defined as inflammation of the liver and can be caused by a number of different agents. Hepatitis C virus is the major pathological agent responsible for cases of transmissible hepatitis (Alter et al, 1989a; Choo et al, 1990). The virus is usually transmitted parenterally, primarily by direct contact with infected blood or blood products. Power et al (1995) reported that a single batch of HCV infected anti-D immunoglobulin resulted in 100% of recipients becoming sero-positive for HCV. It is also believed that 50-100% of intravenous drug users are infected with the HCV virus (Bell et al, 1990).

Weiner et al (1993) reported incidences of transmission of HCV \textit{in utero}. Vertical transmission from infected mother to child during birth has also been reported by Lin et al (1994), Thomas et al (1998) and is reviewed by Chang (1996). Perinatal transmission is thought to occur in less than 10% of cases where the mother is HCV positive. Lin et al (1994) and Thomas et al (1998) suggest that low viraemia of HCV positive mothers accounts for
the low rate of transmission, only children born of mothers with very high HCV titres become infected with the virus. Rice et al (1993) reported cases of HCV infection where the likely route of transmission was sexual, whilst Saltoglu et al (1998) reported cases of HCV transmission between family members but at a very low rate. Evidence for sexual and vertical transmission of HCV is still largely inconclusive, though cases have been identified in which babies become infected from their mothers. Parenteral transmission therefore remains the most recognised form of HCV transmission.

It should be noted that some patients infected with HCV have no apparent risk factors associated with infection. This indicates that all the routes of HCV transmission may not be known.

Coinfection of HCV with other hepatotropic viruses is often found. To date, there has been no adverse affects on the course of HCV infection when patients are coinfected with hepatitis G virus (Goldstein et al, 1997; Cacopardo et al, 1998; Lin et al, 1998), which confirms reports that no specific disease is associated with HGV/GBV-C infection. Coinfection of HCV with hepatitis A virus (HAV) (Vento et al, 1998), hepatitis B virus (HBV), hepatitis delta virus (HDV) (Weltman et al, 1995; Zignego et al, 1997) and human immunodeficiency virus (HIV) (reviewed in Zylberberg & Pol, 1996; Cribier et al, 1997; Sabin et al, 1997) all lead to an increase of the severity of HCV infection with reduced time of progression to disease symptoms for the patient. HIV coinfection with HCV makes no distinction between genotypes of HCV in increasing disease progression (Cribier et al, 1997). Weltman et al (1995) also suggested that HBV infection is promoted during interferon therapy of patients coinfected with HCV. As HCV viraemia is reduced during therapy (depending on genotype) any competition between the two viruses is removed, promoting HBV infection.

HCV is known to infect PBMCs (Cribier et al, 1995) though hepatocytes in the liver are recognised as the target host cells. The majority of cases occur in haemophiliacs, intravenous drug users and organ transplant recipients. There has been some correlation observed between the initial dose of virus and the severity of resultant hepatitis, patients who contract HCV from an infected organ transplantation are seen to develop a more severe
disease in a shorter time than patients who are infected with HCV via. a needle stick injury (Pereira et al, 1991 & 1992).

It has been estimated that 1 - 1.5% of the Western population is infected with HCV (van der Poel, 1994) whilst parts of the developing world show a much higher prevalence that is supposed due to inefficient sterilisation of needles and other surgical instruments. The highest reported incidence of HCV has been found within the Egyptian population with 19.2% of blood donors sero-positive for the virus (Hibbs et al, 1993).

Up to 20% of those infected with HCV may develop a self-limiting acute hepatitis with fever-like symptoms and in some cases, the development of jaundice. Virus clearance occurs and the body shows the presence of HCV antibodies whilst previously raised alanine aminotransferase (ALT) levels normalise and RT-PCR studies show the patient to be negative for circulating virus. However, 80-85% of infected individuals go on to develop a chronic infection (Powell, 1997). This may follow either an initial acute or more commonly, an insidious phase where no symptoms are suffered by the patient, that may last from weeks to years. Chronic HCV may persist asymptomatically in a patient for up to 20-30 years from the initial infection. The mechanism by which the virus achieves this state is not fully understood. It is known that mutations occurring during HCV replication result in the generation of variable epitopes in the surface glycoproteins at HVR1 and HVR2 (section 1.2.1.ii). These are able to escape detection and therefore elimination by the host's humoral immune response. The suggestion by Zignego et al (1992) that PBMCs act as a reservoir for viral replication has been discounted by several groups (Cribier et al, 1995; Mellor et al, 1997).

A common manifestation of this chronic phase is the presence of fluctuating alanine aminotransferase (ALT) levels that are used to monitor infection severity. As the virus invades and attacks the liver, damage is done to the hepatic architecture. The severity of this damage gives an indication of the severity of the disease and thus the viral infection. Histological studies are performed on liver biopsies to assess the extent of fibrosis and necrosis. Four areas of liver cell architecture are examined, the extent of damage in each region is given a score, the total of which is classed a the Knodell score (Knodell et al, 1981). A Knodell score
over 6 indicates severe chronic hepatitis and the patient will be considered for interferon treatment.

Chronic hepatitis will progress either to a state of chronic persistent hepatitis or chronic active hepatitis in which necrosis of the infected hepatocytes and surrounding portal tracts results in severe damage and loss of function of the liver. Long term chronic disease in 20% of patients has been associated with post-necrotic cirrhosis (Alter et al, 1989b) in which infected and damaged hepatocytes are replaced by functionally deficient regenerative cells. Loss of liver function eventually results in patient death. There has been some association of long term HCV infection with the development of hepatocellular carcinoma (HCC) (Tsukuma et al, 1993). A definite mechanism has not yet been defined for this phenomenon though cirrhosis has been ruled out as a pre-requisite for the development of HCC (El-Refaie et al, 1996). The fact that HCV is a single stranded RNA virus discounts carcinogenic mechanisms used by other viruses such as integration of double stranded DNA viral genomes by retroviruses. There may be some relationship between the interaction of the NS5a protein of HCV with cellular PKR in the progression of HCC (section 1.2.2.v) (Gale Jr. et al, 1997).

Symptoms other than those directly related to liver dysfunction have been reported by HCV patients (Dolan, 1996). These include indigestion, joint pains, mental fatigue, abdominal pains and spider naevi, some of which may be related to secondary effects of liver damage.

1.7 Host immune responses

1.7.1 Anti-viral response
Once HCV enters its host cell, a number of immune mechanisms are initiated and mounted against the virus. The humoral immune response is mediated by antibodies directed against specific epitopes within the viral surface glycoproteins and the E3 protein (Lok et al, 1993). Epitopes on both the structural and non-structural viral proteins within the infected cell are also recognised by major histocompatibility complex (MHC) class I CD8+ cytotoxic lymphocytes found associated with infected hepatocytes (Koziel et al, 1992 & 1993). Hayata et al (1991) observed a correlation between
decreasing ALT levels and decreasing numbers of CD8+ cells within HCV infected livers, suggesting the presence of these lymphocytes plays a role in reducing viral load and so the extent of hepatic damage. MHC class II CD4+ lymphocytes have also been observed in infected hepatocytes (Minutello et al, 1993; Zhang et al, 1997; Tsai et al, 1997). Tsai et al (1997), showed HCV infected PBMCs possess T-lymphocyte helper type 1 and 2 (Th1 and Th2) immune cells. Th1 responses are thought to act through the production of various cytokines such as interleukin 2 (IL-2) and IFN-γ in removing the virus. These are known to play a significant role in hepatitis B viral clearance (Romagnani, 1994; Barnaba et al, 1994). Th2 cells are believed to respond in a reciprocal fashion by down-regulating the effects of Th1 through the production of IL-10 cytokines. Zhang et al (1997) indicated that this CD4+ anti viral response is significantly active, even after the cellular immune response has itself been activated by IFN-α and -β.

Antibodies may also act in conjunction with the complement system in the recognition and direct lysis of the enveloped virus and infected host cells. In these ways, antibodies contribute to hepatocyte lysis and therefore liver damage.

1.7.2 Interferons

The earliest and main host immune response is the induction of IFN as part of the host's innate immunity. These cytokines can be grouped into type I IFNs (IFN-α and IFN-β) and type II IFNs (IFN-γ). IFN-α is produced by leukocytes (Mantei et al, 1980) whilst IFN-β is produced by fibroblasts (Taniguchi et al, 1980), both are induced by viral infections. IFN-γ is stimulated by the humoral immune response and is produced by T-lymphocytes in the MHC class II system and Natural killer (NK) cells. The type I interferons are very similar in their secondary structures that explains their use of a common receptor on cell surfaces of all host cells (Tovey et al, 1987). Type II interferon utilises a different receptor.

The inducement of type I interferon is thought to be initiated by double stranded RNA (dsRNA) found in virally infected host cells (Field et al, 1967). Control of the production of interferons occurs at a transcriptional level with both positive and negative regulatory domains located to the 5' regions of the genes involved (reviewed in Taniguchi, 1989). IFN-β is
additionally regulated by the nuclear factor kappa-B (NFκ-B) acting in trans at the positive regulatory domain (PRD) II site on the IFN-β gene.

The main actions of interferons are summarised in figure 1.7. The anti-viral state of host cells are largely induced by type I IFNs that induce host proteins 2'5'oligoadenylate synthetase, double-stranded RNA-activated protein kinase (PKR) and the Mx protein (known to confer resistance to the influenza virus, Lindenmann, 1962).

**Figure 1.7** Major anti-viral effects of interferon

![Diagram](image)

1.7.3 2'5'A-synthetase

2'5'A-synthetase, induced by type I IFN, plays an important role in the inhibition of viral replication. The protein has been found in four different forms, as p40, p46, p69/70 and p100, each thought to act in a different manner in the overall action of 2'5'A synthetase (Chebath *et al*, 1987). The p40 and p46 forms are encoded by the same gene but are products of differential splicing between the 5th and 6th exons, yielding proteins different only at their C-terminals (Saunders *et al*, 1985; Hovanessian *et al*, 1988). p69 and p70 are identical between amino acids 1-683 (Hovanessian *et al*, 1987) and are associated with the host cell
and p46 (Hovanessian et al, 1988). The p100 form of this protein is thought to be ribosome associated (Sperling et al, 1991) and as a result, believed to act in the splicing of RNA.

The functional form of 2'-5' A synthetase is a dsRNA binding protein that actively catalyses the synthesis of oligomers, up to 12 adenylate residues in length, linked by 2'-5'-phosphodiester bonds (2'-5'A). These oligos then bind to and activate the dimerisation of RNaseL, a latent ribonuclease that acts in cleaving single stranded RNA (ssRNA) within the cell (Cole et al, 1996). 2'-5'A oligos are cleaved back to ATP and AMP by a 2'-5' phosphodiesterase (Jaramillo et al, 1995). Nilson & Baglioni (1979) described the action of this active RNaseL in the preferential cleavage of mRNA bound to dsRNA. The mechanism for this recognition is unknown though in HCV infected cells, RNaseL may recognise species of RNAs produced when genome replication takes place by the RdRp enzyme, yielding associated positive and negative ssRNA molecules. The cleavage of mRNA results in the cessation of translation within the cell so terminating viral replication. Figure 1.10 summarises the action of the IFN induced 2'-5'oligoadenylate synthetase.

1.7.4 PKR
PKR is a cellular serine-threonine kinase that is induced by type I interferon (Laurant et al, 1985) and is activated in the presence of double-stranded RNA. It has been identified in both the cell nucleus (localised to the nucleolus) and the cytoplasm (associated with ribosomes) (Jeffrey et al, 1995). The theoretical molecular weight of this 551 amino acid protein is 62 kDa but is actually observed as a 68 to 72 kDa protein (Laurant et al, 1985) due to a high degree of phosphorylation. Hannash et al (1993) mapped PKR to the 2p21-22 region of the human chromosome. The protein itself is homologous to other protein kinases present in rat, mouse and rabbit cells (Clemens & Elia, 1997).

Figure 1.8 shows an overview of the PKR protein which contains two main domains, the regulatory domain in the first half and the catalytic domain in the second half of the protein. The regulatory domain contains two double stranded RNA binding motifs (RBM). RBM1 has been proven as both sufficient and necessary for PKR activation (McCormack et al,
A dimerisation domain is encoded between residues 244 and 296 which acts in the folding of the protein to allow autophosphorylation to occur. The catalytic domain encodes the kinase region of the protein, encoded by 11 domains typical of this family of kinases (Hanks et al, 1988).

**Figure 1.8** Overview of the PKR protein

![Diagram of PKR protein](image)

KEY:
- dsRNA binding domain (RBM)
- Dimerisation domain
- Kinase domains I - XI
- numbers = amino acid positions

**i) Activation of PKR**

PKR is generally activated by dsRNA though it may also be activated by heparin and dextran sulphate. Binding of the latter two to the RBMs has not been proven and may therefore bind to different areas of the protein. dsRNA able to bind PKR would be present in virally infected cells as the virus genomes replicate through RNA intermediates. The minimum length dsRNA found to activate PKR is 11 nt (Scmedt et al, 1995) whilst the optimum length is thought to be 85 nt (Manche et al, 1992). Whilst low levels of dsRNA are known to induce PKR, at high concentrations, PKR activation is inhibited.

The current model for PKR activation was described by Mathews (1996). PKR is believed to act as a dimer, each molecule trans-phosphorylating the other bound PKR. dsRNA binds to each RBM, bringing about a conformational change in the protein that exposes and activates the kinase domain of PKR.
ii) Action of PKR

PKR has been implicated in the regulation of the transcription factor nuclear factor kappa-B (NFκB) via the phosphorylation of NFκB inhibitor I-κB (Kumar et al, 1994). Wong et al (1997) reported the involvement of PKR with regulation of the interferon regulatory factor IRF-1 and the Stat1 component of the ISGF-3 transcription factor complex activated by interferon. The role of PKR in tumour suppression has also been studied widely and is reviewed in Proud (1995).

PKR is better known as an anti-viral effector of type I interferon and is the subject of study in chapters 4 and 5. Similarly to 2'5'oligoadenylate synthetase, one of the functions of PKR is to inhibit protein synthesis within a virus infected cell. The substrate for PKR is the small (α) subunit of the eukaryotic initiation factor (eIF-2α). eIF-2α is phosphorylated by PKR at position Ser51 (Samuel, 1979). In the synthesis of proteins, eIF-2α forms the translation initiation complex with met-tRNA and the 40S ribosomal subunit (figure 1.9). After translation has been initiated, eIF-2α is recycled by eIF-2β, a guanine exchange factor (GEF). Phosphorylated eIF-2α has a higher affinity for GEF that unphosphorylated eIF-2α and forms a stable complex. This results in sequestering of eIF-2α. Further translation initiation complexes cannot be formed as eIF-2α-GDP is not recycled to eIF-2α -GTP and so virus as well as cellular protein synthesis comes to a halt.
Figure 1.9 Translation initiation pathway

Adapted from Hershey, 1993
1.7.5 Cellular inhibitors of PKR
PKR is usually present in very low concentrations and in an inactive state. It is believed the cellular protein p58 binds directly to PKR, inhibiting possible dimerisation and autophosphorylation events. This binding is reversible, for example under heat shock conditions where the heat shock protein hs40 preferentially binds to p58 liberating bound PKR (Melville et al, 1997). Cellular proteins TER-BP, p67, dRF and the La antigen have also been implicated in the binding to and inhibition of PKR (see Clemens & Elia, 1997 for a review).
Table 1.3 Anti-PKR mechanisms used by different viruses (adapted from Gale Jr. & Katze, 1998)

<table>
<thead>
<tr>
<th>Virus</th>
<th>Effector</th>
<th>Role in PKR inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>VAI RNA</td>
<td>Competitive inhibitor of RBMs - sequesters PKR</td>
</tr>
<tr>
<td>Epstein-Barr virus (EBV)</td>
<td>EBER RNA</td>
<td>Competitive inhibitor of RBMs - sequesters PKR</td>
</tr>
<tr>
<td>Human immunodeficiency virus (HIV)</td>
<td>TAR RNA</td>
<td>Competitive inhibitor of RBMs. Targets TAR RNA-binding protein to PKR</td>
</tr>
<tr>
<td></td>
<td>TAR RNA-binding protein</td>
<td>Sequesters dsRNA</td>
</tr>
<tr>
<td></td>
<td>Tat protein</td>
<td>Pseudosubstrate for PKR</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>E3L protein</td>
<td>Sequesters dsRNA</td>
</tr>
<tr>
<td></td>
<td>K3L protein</td>
<td>Pseudosubstrate for PKR</td>
</tr>
<tr>
<td>Reovirus</td>
<td>σ3 protein</td>
<td>Sequesters dsRNA</td>
</tr>
<tr>
<td>Hepatitis C virus (HCV)</td>
<td>NS5a protein</td>
<td>Inhibits PKR dimerisation</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>NS1 protein</td>
<td>Sequesters dsRNA</td>
</tr>
<tr>
<td></td>
<td>p58IPK</td>
<td>Inhibits PKR dimerisation</td>
</tr>
<tr>
<td>Poliovirus</td>
<td>Unknown protease</td>
<td>Degrades PKR</td>
</tr>
<tr>
<td>Herpes simplex virus (HSV)</td>
<td>γ1 34.5 protein</td>
<td>Dephosphorylates eIF-2α(P)</td>
</tr>
<tr>
<td>SV40</td>
<td>T antigen</td>
<td>Rescue of eIF-2α(P)</td>
</tr>
</tbody>
</table>
1.7.6 Inhibition of PKR by viruses
Much work has been done over recent years in identifying various mechanisms employed by viruses to inhibit the action of PKR, allowing cellular protein synthesis (and therefore synthesis of virus proteins) to persist. Table 1.3 shows the main PKR-inhibitory mechanisms developed by viruses. These can be grouped into 5 main mechanisms described below (see Gale & Katze, 1998 for a full review).

1) Virus RNA binds to the RBMs of PKR, blocking binding of dsRNA, preventing PKR activation.
2) Virus protein binding to dsRNA within an infected cell, preventing dsRNA binding to PKR and therefore inhibiting PKR activation.
3) Virus protein binds to the dimerisation region of PKR preventing PKR dimers forming and therefore preventing PKR autophosphorylation and activation.
4) Virus proteins act as a pseudosubstrate for PKR blocking kinase activity and preventing phosphorylation of eIF-2α.
5) Virus proteins act to dephosphorylate eIF-2α ensuring this can be recycled by GEF and translation initiation complex can be formed.

The NS5a protein of HCV contains a 65 aa region (ISDR) thought to act in preventing dimerisation of PKR (section 1.2.2.v). This mechanism has been implicated in resistance of certain genotypes of HCV to the host immune response and in particular, to interferon alpha therapy given.

1.8 Anti-HCV therapies
Given that such a large proportion of HCV infections go on to cause chronic disease, the need to find an effective therapy is important. Since the virus and its mode of transmission have been identified, methods for detection and prevention of spread have been used in monitoring blood products, reducing the incidence of infection. Much of this has been achieved through the use of blood screening and correct sterilisation techniques employed in health care. However, there are still a large number of individuals becoming infected and also a large proportion of presently unidentified infected people who will develop severe hepatitis from which they may die.
1.8.1 **Anti-HCV vaccines**

The majority of HCV infections can be detected by the presence of antibodies to HCV. Trials using chimpanzees inoculated with recombinant forms of the E1 and E2 viral proteins conferred protection against challenge with same-strain virus in 70% of the animals (Choo *et al.*, 1993). This indicates the benefits of using recombinant viral proteins as a vaccine to elicit a humoral immune response. However, other studies have shown that rechallenge of protected chimpanzees resulted in a high proportion becoming reinfected with the virus (Farci *et al.*, 1992; Prince *et al.*, 1992). The major drawback to the use of attenuated HCV or recombinant envelope glycoproteins is the virus' high genetic variability (section 1.2.1.ii). Protection conferred by one sub-type of HCV will not necessarily confer protection against another sub-type. The development of a multi-strain vaccine would require a great deal of investigation which has no guarantee of success as populations of HCV quasispecies continuously evolve.

HCV is known to elicit a cytotoxic immune response. Class I cytotoxic T-lymphocyte (CTL) responses have been observed against the envelope and core proteins (Koziel *et al.*, 1992 & 1993). These proteins are subject to mutational change and probably of little use in protective immunity. Other proteins of the HCV genome are highly conserved to ensure viral replication occurs. These include the RNA-dependent RNA polymerase (NS5b) and the main viral proteinase NS3. The initiation by vaccination of CTL recognition epitopes against these proteins may confer a more stable immune response upon subsequent infection.

The CTL immune response is responsible for the recognition and destruction of infected host cells. Cells expressing a DNA vaccine would therefore be destroyed, reducing the impact of the vaccine dose. If vaccination succeeded in producing HCV recognising CTL response cells, a heightened immune response would result in destruction of infected hepatocytes. This would result in significantly increased overall liver damage. Inducing such a CTL response may be used as a therapeutic system by inducing and therefore enhancing the host's own immune response. Destruction of infected cells will cause further liver damage but may be considered worthwhile to attain total clearance of virus.
It may be some time before a vaccine against HCV is developed. The major problem being the virus’s high mutation rate. However, if one were to be developed, the world-wide rate of infection by HCV and its impact on health care required for chronic hepatitis patients would be significantly reduced.

1.8.2 Interferon-α
Interferon-α is the main beneficial anti-HCV therapy and has been approved for use as a general anti-viral drug. IFN-β and IFN-γ have also been examined for their anti-viral activities (Saez-Royuela et al, 1991; Ohnishi et al, 1989; Takano et al, 1994). While IFN-γ has no effect at reducing viral load or decreasing hepatic damage, IFN-β shows some anti-viral effects.

It is believed that IFN-α acts to enhance the patient’s own immune response, inducing higher levels of PKR and 2′5′A-synthetase for the direct inhibition of viral replication. Several randomised trials have proven IFN-α treatment of chronic HCV patients causes viral RNA levels and biochemical markers such as ALT to decrease, proving that some significant anti-HCV effect occurs.

IFN-α is currently available both as an extract from human lymphoblasts and as a recombinant product in the form IFN-α 2a or IFN-α 2b. Trials comparing the efficacy of these two forms have shown them to act in a similar fashion, lymphoblastoid IFN resulting in an overall increased sustained response (Bardelli et al, 1995; Farrell et al, 1997).

Trials have been carried out to look at the efficacy of using combination therapies. IFN-α has been used in conjunction with a number of other anti-viral drugs such as ribavirin (DiBisceglie et al, 1994 & 1995; Brillanti et al, 1994; Bodenheimer Jr. et al, 1997), indomethacin (Andreone et al, 1994), thymosin-α1 (Sherman et al, 1994 & 1998) and glycyrrhizin (Naito et al, 1995). Recently, ribavirin was approved for use in combination with IFN-α for the treatment of HCV after several phase III clinical trials proved it’s increased effectiveness in clearing virus from patients who previously relapsed or did not respond to IFN-α monotherapy (Poynard et al, 1998; Davis et al, 1998; McHutchison et al, 1998). In these reports, combination
therapy resulted in twice as many patients clearing HCV after 24 weeks therapy and over twice as many responders occurring after 48 weeks therapy. Combination therapy with ribavirin was proven to have an effect on type 1 HCV, known to be a resistant genotype to IFN-α monotherapy, though response times were generally later into therapy than is observed with IFN-α alone.

Currently, interferon therapy is given at 1-6 million units (MU), 1-3 times weekly for up to 12 months. The dose and time period are largely dependent on the individual patient. Factors in deciding the treatment regime include patient age, viral load and severity of disease. The standard method of deciding when to start therapy uses histological results of a liver biopsy. The severity of necrosis and thus hepatic damage is an indication of the extent of viral infection (section 1.6 and section 5.12). Patients are considered for therapy when Knodell scores over 6 are reached. Many groups treating HCV patients consider treatment at an earlier stage results in a better outcome for the patient in terms of viral clearance. Treatment may cease early if the patient suffers too many side effects and wishes to stop, if viral clearance occurs for a sustained period soon after therapy is started, or if viral load and liver function tests show no decrease, or even an increase after several months of therapy and therefore no response to the interferon-α given. Combination therapy is considered for those patients who either relapse after, or do not respond to IFN-α monotherapy.

During, and for a period up to two years after treatment, patients are monitored for HCV RNA levels, liver function tests and a liver biopsy is performed 1 year after treatment cessation. If all tests show viral clearance and extent of liver disease has been reduced, or even eliminated, then treatment for HCV with IFN-α is deemed successful. Those patients who clear the virus are deemed to be responders. Patients may initially show viral clearance and improved liver functions but upon cessation of treatment, their viral load returns (sometimes to higher levels than before treatment began) and are therefore known as partial responders or relapsers. Patients may show no improvement to IFN-α treatment and are therefore classed as non-responders. For these partial and non-responders, the inability to clear the virus will eventually result in progression of liver damage and eventual death from liver failure.
1.8.3 Problems associated with IFN-α

Despite the findings that IFN-α therapy plays a positive role in boosting the host's immune system to clear HCV, there are a number of problems associated with this treatment that have to be addressed by both the patients and doctors. Though interferon acts in infected cells to halt viral replication, host protein synthesis is also stopped, killing those cells. Together with host cell attack by the cytotoxic T-lymphocytes, this leads to severe problems for the patient as their liver undergoes attack by the immune system. Side effects known to occur with this treatment can be summarised in table 1.4. Quite often, these side effects become so severe that the patient will stop treatment altogether.

A second factor is the cost of such treatment. In 1992, the cost of giving a chronic HCV patient a course of IFN-α at 3 MU 3 times a week for 6 months was estimated at $US 4000 (Wright, 1992). Since then, costs have increased and if a regime uses a higher dose of IFN for a longer period of time, each patient will cost national health budgets a considerable amount. This may be tolerated if the outcome of therapy was 100%. It has been estimated that only 5-25% of patients completely respond (Davis et al 1989; Shindo et al 1992). Even with ribavirin combination therapy, only 50% HCV patients are predicted to respond fully and clear the virus. Ribavirin itself has been observed to cause severe haemolysis and reduce haemoglobin concentrations. Patients have also reported increased depression, fatigue and nausea with ribavirin and interferon combination therapy than occurred when taking interferon alone (personal communication J. James, Leicester Royal Infirmary, Leicester), despite reports that side effects of ribavirin are no worse than those of IFN-α (Bodenheimer Jr. et al, 1997; Davis et al, 1998).

The outcome of treatment and secondary problems the drug causes the patient are both serious factors that will influence both the doctor, in approving the treatment, and the patient in taking it.
Table 1.4 Side effects of IFN-α treatment

<table>
<thead>
<tr>
<th>Frequent effects</th>
<th>Infrequent effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depression</td>
<td>Delirium</td>
</tr>
<tr>
<td>Stomach and bowel pains</td>
<td>Impotence</td>
</tr>
<tr>
<td>Weight loss</td>
<td>Visual disturbances</td>
</tr>
<tr>
<td>Hair loss</td>
<td>Numbness</td>
</tr>
<tr>
<td>Fever</td>
<td>Trembling</td>
</tr>
<tr>
<td>Fatigue</td>
<td>Psychosis</td>
</tr>
<tr>
<td>Bone marrow suppression</td>
<td>Reduced blood cell counts</td>
</tr>
<tr>
<td>Heartburn</td>
<td></td>
</tr>
</tbody>
</table>

Taken from Dolan (1996) and Hirschman (1995).

1.8.4 Alternative therapies

Many patients chronically infected with HCV try both allopathic and alternative therapies to combat the virus and alleviate symptoms suffered. Alternative remedies are aimed at the latter and include Traditional Chinese Medicine, homeopathy, herbal medicines and the use of vitamins and minerals (Dolan, 1996). There has been no significant proof that these aid in reducing viral levels but patients do report an alleviation of their symptoms.

1.9 Predictive factors

As IFN-α treatment is very expensive and so few chronic HCV patients respond to therapy, there is a need to find ways of predicting potential responders. This is increasingly important as health budgets are decreasing. Being able to predict responders will also benefit non-responders, as they may choose not to undergo therapy and its unfavourable side effects that will be of no benefit.

To date, some factors associated with HCV infection and the host's immune response have been assessed in relation to infection outcome after interferon therapy. Some factors have been statistically proven to bear no relation with patient response, these include quantitative assays for β2-microglobulin (Malaguarnera et al, 1995), serum levels of

Many groups have analysed the response of HCV patients to interferon therapy in relation to physical and biochemical factors such as age, sex, duration of infection, genotype and HCV RNA serum levels. It has been reported that the route of infection is significant to the patient in that the larger the dose of HCV contracted (eg. by organ transplant rather than needlestick injury) the more severe the disease suffered and the less likely a patient’s response to therapy.

One of the most significant factors used to date has been the quantitation of HCV RNA. Decrease of genomic RNA is observed as patients respond to therapy, clearance of virus indicated by a zero score for RNA titres. However, quantitation of HCV RNA is carried out on patient serum which is easily obtainable, allowing the patient to be frequently monitored over a period of months or even years. Haydon et al (1998) reported the presence of HCV in the livers of patients, despite serum samples testing negative for virus by RT-PCR. This study indicated that HCV may be present in the serum of patients below the sensitivity level of the test used. Therefore, patients cannot necessarily be classed as HCV negative (and therefore responders to therapy) if their serum tests are negative for the virus.

The genotype of HCV is by far the most reliable predictor of response used. Recent studies have identified an interferon sensitivity determining region located within the NS5a protein (section 1.2.2.v) that is thought to inhibit the action of PKR (section 1.7.6). Patients carrying type 1a or 1b HCV do not respond to interferon alpha therapy, though recent studies indicate type 1 HCV can be cleared using a combination of interferon alpha and ribavirin (section 1.8.2).
1.10 Aims and objectives
Section 1.5.2 described the lack of a proven cell culture based system for the propagation of HCV. Such a system would allow for more extensive analysis of the virus than is currently possible. The initial aims of this project were to develop a cell culture system for the propagation of non-infectious HCV. This would be based on the virus like particle system developed by Haddrick et al (1996) in which HIV particles were propagated in mammalian cell culture that were morphologically similar to wild type virus but did not contain full length genomic RNA and were therefore considered non-infectious.

If this were to be successful, then further mutagenesis analysis would be performed on HCV virus like particles in mammalian cell culture. Any changes to virus protein expression and function brought about by changes to the genomic sequence would be observed. Development of a cell culture system would therefore greatly enhance the ability to study HCV in terms of its structure and function.

The identification of a factor that could be used to predict the response of HCV patients to interferon alpha therapy will also be investigated. Section 1.9 described various factors that have already been analysed in relation with patients eventual response to therapy. Until the time the experiments described in chapters 4 and 5 were carried out, pre-treatment levels of PKR had not been analysed as a possible predictive factor. In view of this, the aims of this particular project were two-fold.

i) To develop a quantitative system by which to measure the level of PKR expression, both the level of mRNA and protein present in the cells of an HCV infected patient.

ii) To correlate the expression level of PKR in HCV infected patients from tissue samples taken before therapy began with other patient data such as biochemical test scores (ALT levels and Knodell scores), virus data (genotype and titre of HCV) and patient data (age, sex, plus any factors that become apparent as the investigation progresses). The outcome of any interferon alpha therapy taken by the patients in this study will be noted.
The objective of this study will be to determine if pre-treatment levels of PKR in HCV infected patients correlate with eventual clearance of the virus and thus recovery of the patient. If there is some degree of correlation, then PKR may be proven a predictive factor to interferon response. If there is no correlation found then PKR cannot be considered a predictive factor and would therefore be one less part of the immune response to be considered when looking for a specific and effective factor predictive of treatment response in HCV infected patients.
Chapter 2

Materials and Methods
2.1 Suppliers

1) All chemical reagents were purchased from the following as high purity grade reagents,

BDH-Merck Ltd. Lutterworth, Leicestershire, UK
Fisher Scientific UK, Loughborough, Leicestershire, UK
ICN Biomedicals Inc. Aurora, Ohio, USA
Sigma-Aldrich Company Ltd. Poole, Dorset, UK

2) Molecular biological products were purchased from the following sources,

Amersham Life Scienc Ltd. Little Chalfont, Buckinghamshire, UK
BioRad Laboratories Ltd. Hemel Hempstead, Hertfordshire, UK
Boehringer Mannheim UK (Diagnostics & Biochemicals) Ltd. Lewes, East Sussex, UK
Burkard Scientific Ltd. Uxbridge, Middlesex, UK
Clontech Laboratories Inc. Palo Alto, California, USA
Dako Ltd. High Wycombe, Buckinghamshire, UK
Gibco-BRL Life Technologies, Paisley, Scotland, UK
Harlan Sera-Lab, Loughborough, Leicestershire, UK
Imperial Laboratories Ltd. Andover, Hampshire, UK
MRC-AIDS directed program reagent project, Medical Research Centre, London, UK
Molecular Research Centre Inc. Cincinnati, Ohio, USA
Nalge Nunc International, Naperville, Illinois, USA
National Diagnostics, Hessle, Hull, UK
NEN Life Science Products, Boston, Massachusetts, USA
New England Biolabs, Hitchin, Hertfordshire, UK
Novagen, Madison, Wisconsin, USA
Oxoid Ltd. Basingstoke, Hampshire, UK
Pharmacia Biotech, St.Albans, Hertfordshire, UK
Promega UK, Southampton, UK
Ribogene Inc. Hayward, California, USA
Stratagene Ltd. Cambridge, UK

3) Equipment used was originally purchased from,

Agfa-Gevaert Ltd. Brentford, Middlesex, UK
Beckman Instruments Inc. Palo Alto, California, USA
DuPont (UK) Ltd. Stevanage, Hertfordshire, UK
Invitrogen BV, NV Leek, The Netherlands
MSE, Leicester, Leicestershire, UK
Packard Instrument Company Inc. Downers Grove, Illinois, USA
Polaroid (UK) Ltd. Wheathampstead, Hertfordshire, UK
Savant Instruments Inc. Farmingdale, New York, USA
Techne (Cambridge) Ltd. Duxford, Cambridge, UK
UVP Ltd. Cambridge, UK
2.2 Buffers used

Table 2.1 Common buffers at 1x concentration

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Content at 1x concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA loading</td>
<td>40% sucrose, 0.25% bromophenol blue</td>
</tr>
<tr>
<td>KlenTaq</td>
<td>40 mM Tricine-KOH (pH 9.2), 15 mM K-OAc, 3.5 mM Mg-OAc, 75 µg/ml BSA</td>
</tr>
<tr>
<td>Oligonucleotide protection buffer</td>
<td>50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine</td>
</tr>
<tr>
<td>PBS</td>
<td>0.14 M NaCl, 2 mM KCl, 5 mM Na₂H₂PO₄, 2 mM NaH₂PO₄</td>
</tr>
<tr>
<td>PBS-T</td>
<td>1x PBS, 0.1% (v/v) Tween-20</td>
</tr>
<tr>
<td>Pfu DNA polymerase</td>
<td>20 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 0.1 mg/ml BSA</td>
</tr>
<tr>
<td>PKR 1-170</td>
<td>50 mM Tris-HCl (pH 8.0), 100 mM NaCl</td>
</tr>
<tr>
<td>RNA hybridisation buffer</td>
<td>0.2 M NaCl, 40 mM PIPES (pH 4.6), 1 mM EDTA</td>
</tr>
<tr>
<td>RNA denaturing</td>
<td>95% (v/v) deionised formamide, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 0.02% (w/v) xylene cyanol blue</td>
</tr>
<tr>
<td>RNase protection digestion buffer</td>
<td>10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 200 mM NaOAc</td>
</tr>
<tr>
<td>1xSDS</td>
<td>50 mM Tris-HCl (pH 8.0), 1% (w/v) SDS</td>
</tr>
<tr>
<td>2xSDS</td>
<td>50 mM Tris-HCl (pH 8.0), 20% (w/v) glycerol, 2% (w/v) SDS, 5% (v/v) 2-6 mercaptoethanol, 0.25% (w/v) bromophenol blue</td>
</tr>
<tr>
<td>SDS-PAGE running buffer</td>
<td>25 mM Tris-HCl, 200 mM glycine, 0.1% (w/v) SDS</td>
</tr>
<tr>
<td>Single tube RT</td>
<td>50 mM Tris-HCl (pH 8.8), 10 mM KCl, 2.5 mM MgCl₂</td>
</tr>
<tr>
<td>ST</td>
<td>50 mM Tris-HCl (pH 8.0), 25% (w/v) sucrose</td>
</tr>
<tr>
<td>STET</td>
<td>50 mM Tris-HCl (pH 8.0), 8% (w/v) sucrose, 50 mM EDTA, 0.5% (v/v) Triton X100</td>
</tr>
<tr>
<td>T4 DNA Ligase</td>
<td>50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (w/v) polyethylene glycol-8000</td>
</tr>
<tr>
<td>T4 Kinase Forward</td>
<td>70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 100 mM KCl, 1 mM 2-8 mercaptoethanol</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris-HCl, 1 mM EDTA, 0.57% (v/v) glacial acetic acid</td>
</tr>
<tr>
<td>Taq DNA polymerase</td>
<td>10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (v/v) Triton X-100</td>
</tr>
</tbody>
</table>
### Buffer Content at 1x concentration

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Content at 1x concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq Extender DNA polymerase mix</td>
<td>20 mM Tris-HCl (pH 8.0), 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% (v/v) Triton X-100, 0.1 mg/ml BSA</td>
</tr>
<tr>
<td>TBE</td>
<td>89 mM Tris-HCl, 2.5 mM EDTA, 89 mM boric acid</td>
</tr>
<tr>
<td>TE</td>
<td>10 mM Tris-HCl (pH 7.5), 1 mM EDTA</td>
</tr>
<tr>
<td>TEN</td>
<td>10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 25 mM NaCl</td>
</tr>
<tr>
<td>TMT/SS</td>
<td>90% (v/v) PBS, 3% (w/v) skimmed milk powder, 0.1% (v/v) Tween-20, 10% (v/v) sheep serum</td>
</tr>
<tr>
<td>TNM/AT</td>
<td>50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (w/v) skimmed milk powder, 0.05% (v/v) Tween-20</td>
</tr>
<tr>
<td>Triton-Lysis</td>
<td>50 mM Tris-HCl (pH 8.0), 1.5% (v/v) Triton X-100, 50 mM EDTA</td>
</tr>
<tr>
<td>Vent DNA polymerase</td>
<td>20 mM Tris-HCl (pH 8.8), 10 mM KCl, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.1% (v/v) Triton X-100</td>
</tr>
<tr>
<td>Western transfer buffer</td>
<td>25 mM Tris-HCl, 190 mM glycine, 20% (v/v) methanol</td>
</tr>
</tbody>
</table>

### 2.3 Oligodeoxynucleotides used

All oligodeoxynucleotides were synthesised by the Protein and Nucleic Acid Chemistry Laboratory at the University of Leicester. Oligodeoxynucleotides were stored at -20°C.

Oligodeoxynucleotide molar concentration was calculated by measuring the spectrophotometric absorbance at 260nm of a suitably diluted sample in the following formula, where n is the number of each nucleotide.

Molar concentration = \( \frac{A_{260\text{nm}} \times \text{dilution factor} \times 1000}{(\text{pmol/} \mu\text{l or } \mu\text{M})} \sum [ (An \times 15.4) + (Cn \times 7.3) + (Gn \times 11.7) + (Tn \times 8.8) ] \)

The melting temperature (Tm) of an oligodeoxynucleotide was calculated using the formula,

\[ Tm \ (°C) = \sum [ 2 \times (A_n + T_n) ] + 4 \times (C_n + G_n) \]
Table 2.2 Oligonucleotide sequences (endonuclease restriction sites in bold type)

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sense/Antisense</th>
<th>Sequence 5' to 3'</th>
<th>Site encoded</th>
<th>Primer position (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV 1 Sense</td>
<td>GCAACAAGACGCACTCCATGAA</td>
<td>-</td>
<td>-</td>
<td>326-350</td>
</tr>
<tr>
<td>HCV 2 Antisense</td>
<td>GACTCCGACTCCAGGACACG</td>
<td>-</td>
<td>-</td>
<td>5322-5300</td>
</tr>
<tr>
<td>HCV 3 Antisense</td>
<td>GGAAGGTTTATCTCCCCGGTTCAG</td>
<td>-</td>
<td>-</td>
<td>9382-9358</td>
</tr>
<tr>
<td>HCV 3 Not I</td>
<td>GCGAAGCGGGCCCGCCGAGTTTATTCTCCCCGGTTCATG</td>
<td>Not I</td>
<td>9396-9358</td>
<td></td>
</tr>
<tr>
<td>HCV 4 Sense</td>
<td>TTAAGCTTGGCAACATGGACACTCCTAAAACC</td>
<td>Hind III</td>
<td>336-359</td>
<td></td>
</tr>
<tr>
<td>HCV 5 Sense</td>
<td>GAAACTGCAAGCTGGTCAACAC</td>
<td>Pst I</td>
<td>1566-1588</td>
<td></td>
</tr>
<tr>
<td>HCV 6 Antisense</td>
<td>GTGTTGACCAGCTGGACTTTC</td>
<td>Pst I</td>
<td>1588-1566</td>
<td></td>
</tr>
<tr>
<td>HCV 7 Sense</td>
<td>CGGCTGGTGACTTGGTACGC</td>
<td>Kpn I</td>
<td>2652-2669</td>
<td></td>
</tr>
<tr>
<td>HCV 8 Antisense</td>
<td>GCTACCAAGGTACCGGCGC</td>
<td>Kpn I</td>
<td>2669-2652</td>
<td></td>
</tr>
<tr>
<td>HCV 9 Antisense</td>
<td>CCAAGAAGGCCTCGAGTCTTCG</td>
<td>Xho I</td>
<td>3480-3459</td>
<td></td>
</tr>
<tr>
<td>Nest 5 Sense</td>
<td>GGTGCAATCAGTCATGGTTATG</td>
<td>-</td>
<td>1449-1470</td>
<td></td>
</tr>
<tr>
<td>Nest 6 Antisense</td>
<td>CAGTACTGGATATGGAACGAGAC</td>
<td>-</td>
<td>1615-1591</td>
<td></td>
</tr>
<tr>
<td>Nest 7 Sense</td>
<td>GAAACCTGGTACAGCTGGACTTACGC</td>
<td>-</td>
<td>2602-2622</td>
<td></td>
</tr>
<tr>
<td>Nest 8 Antisense</td>
<td>CAAAGACCCGCTACGGTGTAG</td>
<td>-</td>
<td>2798-2778</td>
<td></td>
</tr>
<tr>
<td>Nest 9 Antisense</td>
<td>CTCAAGGACTGCAAGTCTAC</td>
<td>-</td>
<td>3509-3489</td>
<td></td>
</tr>
<tr>
<td>209 Antisense</td>
<td>ATACTCGAGTGACCGGTCTACGAGACCT</td>
<td>-</td>
<td>319-347</td>
<td></td>
</tr>
<tr>
<td>211 Antisense</td>
<td>CACTTGGAGGCCCTACCCGCTCTAG</td>
<td>-</td>
<td>286-311</td>
<td></td>
</tr>
<tr>
<td>939 Sense</td>
<td>CTGTTAGGAACACTGCTTCTT</td>
<td>-</td>
<td>43-62</td>
<td></td>
</tr>
<tr>
<td>940 Sense</td>
<td>TTCACGAGAAGCCGGGTCTAG</td>
<td>-</td>
<td>61-80</td>
<td></td>
</tr>
<tr>
<td>2'S A 1 Sense</td>
<td>GGAAGGCGACGCTCACGAAGAC</td>
<td>-</td>
<td>924-944</td>
<td></td>
</tr>
<tr>
<td>2'S A 2 Antisense</td>
<td>CTTTATGGAAATGAAATCTTG</td>
<td>-</td>
<td>1323-1302</td>
<td></td>
</tr>
<tr>
<td>B-Act 2 Sense</td>
<td>CTGTTGCACTGGGAGGAG</td>
<td>-</td>
<td>1522-1504</td>
<td></td>
</tr>
<tr>
<td>B-Act 3 Antisense</td>
<td>GTGGAATCGTCCGAAAGGACC</td>
<td>-</td>
<td>900-918</td>
<td></td>
</tr>
<tr>
<td>PKR 1 Sense</td>
<td>GCCTTTCTATCCTAATGGGAATTC</td>
<td>-</td>
<td>1082-1104</td>
<td></td>
</tr>
<tr>
<td>PKR 2 Antisense</td>
<td>GAAATCTGTGTGAAGGGCTCTAG</td>
<td>-</td>
<td>1382-1362</td>
<td></td>
</tr>
<tr>
<td>huGAPDH Probe olio</td>
<td>AAAAAACCTTATCCTCTCTTCAGTCAATTGGAATTCTCTACGACTGCAAAGCGGGGAAGGTGAGAAGGGTGAGGTGGTGATGGTGGTGCTATGG</td>
<td>-</td>
<td>38-65</td>
<td></td>
</tr>
<tr>
<td>GAPDH2 Probe olio</td>
<td>CCAATACGGCAAAAATCCGAGACTCCGGACTCCCTACCTCCCCGGCTTCTGAAGTGCTAAGTAGATCCTACTTTGGAAGGATAGTGAAGGTTT</td>
<td>-</td>
<td>38-65</td>
<td></td>
</tr>
<tr>
<td>huPKR Probe olio</td>
<td>GTTCTGGACGGTTAGTTAAATCAATCCTTCCAGGAGAAGACTCTCGTGAAGCTCAACCCGGCTTGGTACCTAGGAGAAGGATAGTGAAGGTTT</td>
<td>-</td>
<td>20-70</td>
<td></td>
</tr>
<tr>
<td>T3 Sequencing</td>
<td>AATTAAACCTCTACATAAAGGG</td>
<td>-</td>
<td>793-772</td>
<td></td>
</tr>
<tr>
<td>T7 Sequencing</td>
<td>TAATACGACTCAGTATA</td>
<td>-</td>
<td>625-646</td>
<td></td>
</tr>
</tbody>
</table>
2.4 Plasmids used
Plasmids used were obtained from laboratory glycerol stocks.

Table 2.3 Plasmid vectors used

<table>
<thead>
<tr>
<th>Plasmid used</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBCCX</td>
<td>mammalian expression from SV40 promoter site, bacterial expression from T7 promoter site.</td>
<td>A.J. Cann, University of Leicester</td>
</tr>
<tr>
<td>pBluescript SK(+)</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt; Cm&lt;sup&gt;r&lt;/sup&gt; His Tag upstream of cloning site.</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET16b</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt; T&lt;sub&gt;6&lt;/sub&gt; and T7 polymerase transcription initiation sites and promoters.</td>
<td>Novagen</td>
</tr>
<tr>
<td>pGEM-1</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt; T&lt;sub&gt;6&lt;/sub&gt; and T7 polymerase transcription initiation sites and promoters.</td>
<td>Promega</td>
</tr>
<tr>
<td>pSport</td>
<td>NotI / SalI digested (Superscript cDNA synthesis system Gibco-BRL)</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>pUC18</td>
<td>Amp&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Lab stocks</td>
</tr>
</tbody>
</table>

Cm<sup>r</sup> - resistance to Chloramphenicol  
Amp<sup>r</sup> - resistance to ampicillin

Table 2.4 Plasmid constructs used

<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET16b/FL-PKR</td>
<td>Full length PKR sequence cloned into BamH1 to Nde1 sites of pET16b vector</td>
</tr>
<tr>
<td>pNB 92/008</td>
<td>Full length PKR sequence cloned into BamH1 to Pst1 sites of pGEM-1 vector</td>
</tr>
<tr>
<td>pNB 92/020</td>
<td>PKR1-170 cloned into Bam HI to Pst I sites of pGEM-1 vector</td>
</tr>
<tr>
<td>pJF001</td>
<td>84 bp piece of pBluescript SK(+) cloned into Afl II site of pNB 92/008</td>
</tr>
<tr>
<td>pJF002</td>
<td>Deletion of pJF001 sequence 729-1174.</td>
</tr>
</tbody>
</table>

pET16b/FL-PKR, pNB92/008 and pNB92/020 were all a gift from Dr. S Heaphy, University of Leicester.
Figure 2.1 pBCCX plasmid vector based on the reported vector pBC12/CMV/IL2 (Cullen, 1986) with a Hind III to Sma I polylinker site substituted for IL2 gene (personal communication and plasmid stock from A. Lau, University of Leicester)

Figure 2.2 pBluescript SK(+) plasmid vector (Alting-Mees, 1989)
Figure 2.3 pET16b plasmid vector (Dubendorff & Studier, 1991)

Figure 2.4 pGEM-1 plasmid vector (Promega UK)
Figure 2.5 pSPORT plasmid vector (Gibco-BRL)

![pSPORT plasmid vector diagram](image)

Figure 2.6 pUC18 plasmid vector (Vieria et al, 1982)

![pUC18 plasmid vector diagram](image)
2.5 Bacterial cells and growth media
All bacterial strains were obtained from laboratory stocks stored at -80°C in 15% glycerol.

Table 2.5 Bacterial strains used

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Characteristics</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AG1</td>
<td>Standard host strain for cloning vectors</td>
<td><em>recA1, endA1, gyrA96, thi-1, hsdR17( r^k, m^k), supE44, relA1</em></td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>Protein expression strain carries regions of <em>lacUV5, lacI, lacZ</em> and T7 genes</td>
<td>F-, <em>ompT, hsdSB, (r^B, m^B), dcm, gal</em>, λ(DE3)</td>
</tr>
<tr>
<td>DH5α</td>
<td>Standard host strain for cloning vectors</td>
<td>Δ80ΔlacZΔM15, *recA1, endA1, gyrA96, thi-1, hsdR17(r^k, m^k), supE44, relA1, deoR, Δ(lacZYA-argF) U169</td>
</tr>
<tr>
<td>JM110</td>
<td><em>dam</em> mutation of adenine methylase</td>
<td><em>rpsL, thr, leu, thi, hsdR17(r^k, m^k), lacY, galK, galT, ara, tonA, tsx, dam, supE44Δ(lac-proAB)</em></td>
</tr>
</tbody>
</table>

Table 2.6 Growth media used

<table>
<thead>
<tr>
<th>Media</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>2TY broth</td>
<td>1.6% (w/v) Bacto-tryptone (Oxoid)</td>
</tr>
<tr>
<td></td>
<td>1.0% (w/v) Yeast extract (Oxoid)</td>
</tr>
<tr>
<td></td>
<td>0.5% (w/v) NaCl (Fisons)</td>
</tr>
<tr>
<td>2TY agar</td>
<td>2TY broth plus 1.5% (w/v) agar (Lab M, Amersham)</td>
</tr>
</tbody>
</table>

Media was sterilised at 121°C, 15psi for 20 minutes. 20mls agar media was poured for each plate and once set, stored at +4°C. Plates were dried prior to use.
2.6 Eukaryotic cells and growth media

2.6.1 Media used
All media were purchased from Gibco-BRL as 1x stocks.

RPMI - 1x RPMI 1680 medium with additional 100 U/ml penicillin, 100 U/ml streptomycin, 200 mM glutamine and 10% Fetal Calf Serum (FCS).

DMEM - 1x DMEM medium with additional 100 U/ml penicillin, 100 U/ml streptomycin, 1% nonessential ammino acids (NEAA) and 10% FCS.

Trypsin/EDTA - 0.5 mg/ml trypsin plus 0.2 mg/ml EDTA (pH 8.0)

2.6.2 Maintenance of cell lines
Cell line maintenance was carried out in a category III containment area with all safety practices adhered to. T cell lines were obtained from frozen laboratory stocks stored in RPMI media with additional 10% FCS and 10% dimethsulphoxide (DMSO) under liquid nitrogen in 2 ml cryofreezer vials. T-cells were originally obtained from the MRC AIDS directed program. The Chang cells were a gift from Joseph Lu, University of Leicester, originally obtained from Imperial Laboratories, UK.

Cells were maintained in RPMI or DMEM media (table 2.7) in Nunclon cell culture flasks at 37°C under 5% CO₂. T25 and T80 were used for non-adherent cells whilst larger, T175 flasks were used for adherent Chang cells.

Chang cells were grown up and seeded every 3 to 4 days. Medium was removed and discarded from the flask. The cell monolayer was washed with 1x PBS to remove trace amounts of serum and a trypsin/EDTA solution added with incubation at 37°C for 5 minutes. An equal volume of RPMI medium was added to inhibit further trypsin action. Cells were removed from the flask to a 50 ml tube and a viable cell count performed. Cells were pelleted by centrifugation at 1250 rpm, 20°C in a Mistral 3000i centrifuge (MSE) for 5 minutes, waste media removed and the cells resuspended in an appropriate volume of pre-warmed media to a seeding density of 2-4 x10⁴ cells/cm² of flask.
Non-adherent cells (T-cells) were grown up and seeded every 3 to 4 days. Cells were removed from the flask to a 15 or 50 ml Falcon tube and a viable count performed. Cells were pelleted by centrifugation at 1250 rpm for 5 minutes. Waste medium was removed and cells resuspended in an appropriate volume of pre-warmed medium to a seeding density of 2-3 x 10^5 cells/ml.

Table 2.7 Eukaryotic cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Type of cell</th>
<th>Media</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8166</td>
<td>Human T-cell</td>
<td>RPMI</td>
<td>Salahuddin et al, 1983</td>
</tr>
<tr>
<td>CEM4</td>
<td>Human T-cell</td>
<td>RPMI</td>
<td>Foley et al, 1965</td>
</tr>
<tr>
<td>Chang</td>
<td>Human tertiary hepatocytes</td>
<td>DMEM</td>
<td>Chang, 1954</td>
</tr>
<tr>
<td>Hut78</td>
<td>Human T-cell</td>
<td>RPMI</td>
<td>Gazder et al, 1980</td>
</tr>
</tbody>
</table>

2.6.3 Viable cell count
To 200 μl of 0.2% trypan blue solution (Gibco-BRL) was added to 200 μl of cell suspension and the mix applied to a haemocytometer. Viability was determined by the ratio of viable (white) to non-viable (blue) cells counted in all four quadrants. Cells were seeded and used if the viability exceeded 80%. Total viable cell concentration was calculated using the formula,

\[
\text{Cells/ml} = \text{viable cells in 4 quadrants} \times \text{dilution factor} \times \frac{\text{quadrants/ml}}{2500}
\]

2.6.4 Preparation of cells for protein and RNA extractions
Cells were pelleted and waste medium removed as described above. Cells were washed in two changes of 1x PBS to remove serum proteins, with pelleting of cells between washes. Cells were either resuspended in a desired volume of 1x PBS for further dilutions or extracted directly from the pellet.
2.7 General techniques

2.7.1 Nucleic acid quantitation and quality determination
DNA and RNA were quantified in accordance with the method given by Sambrook et al. (1989). The absorbance (A) at 260 nm of nucleic acid samples was determined using an Ultraspec III spectrophotometer (Pharmacia). The absorbance readings were used to determine the concentration using the following formulae,

\[
d_{\text{ds DNA concentration (\(\mu g/\text{ml}\)}} = A_{260 \text{ nm}} \times \text{dilution factor} \times 50 \mu g/\text{ml} \quad (1)
\]

\[
d_{\text{ssDNA concentration (\(\mu g/\text{ml}\)}} = A_{260 \text{ nm}} \times \text{dilution factor} \times 33 \mu g/\text{ml} \quad (2)
\]

\[
d_{\text{ss RNA concentration (\(\mu g/\text{ml}\)}} = A_{260} \times \text{dilution factor} \times 40 \mu g/\text{ml} \quad (3)
\]

Glasel (1995) proved the acknowledged method of nucleic acid purity determination by measuring the ratio of A$_{260}$:A$_{280}$ is flawed. It is generally accepted that a ratio between 1.8 and 2.0 is indicative of pure nucleic acid. An A$_{260}$:A$_{280}$ ratio of 2.0 was proven to indicate pure nucleic acid but values below this indicate protein contamination of the sample. Any DNA contamination of RNA was observed by agarose gel electrophoresis of the RNA sample denatured in 0.2N NaOH (section 2.9.2). It was assumed that little protein contamination of cell RNA extracts occurred due to the nature of the extraction procedures (section 2.9.2). In vitro transcribed RNA (section 2.9.8) should also be assumed to contain trace amounts of protein at best.

2.7.2 Nucleic acid precipitations
i) Precipitation in ethanol
Nucleic acids in solution were precipitated by the addition of 0.1 volume 3 M NaOAc (pH 5.2) and 2 to 2.5 volumes 100% ethanol. 7 M NH$_4$OAc was used when the nucleic acid was to be phosphorylated by T4 kinase. This was stored on dry ice for 20 to 30 minutes and the nucleic acid pelleted by centrifugation at 13,000 rpm for 15 minutes in a bench-top microcentrifuge. The supernatant was discarded and the pellet washed in 500 µl 70% ethanol with a further spin for 3 minutes. The final pellet of nucleic acid was air dried and resuspended in the desired volume of TE buffer or H$_2$O.
ii) Precipitation in isopropanol
To the nucleic acid in solution was added an equal volume of isopropanol (propan-2-ol). This was incubated at room temperature or on ice for 10 to 20 minutes. Nucleic acids were pelleted by centrifugation, washed in 70% ethanol and resuspended in TE buffer or H₂O as described above.

2.7.3 Phenol chloroform extraction of nucleic acids
An equal volume of phenol (equilibrated in 0.1 M Tris-HCl (pH 8.0) for DNA and 0.1 M citrate saturated (pH 4.3) for RNA) (Sigma) was added to the nucleic acid sample, mixed by vortexing and phases separated by centrifugation for 5 minutes at 13,000 rpm. The upper aqueous phase was transferred to a fresh tube and extracted with an equal volume phenol:chloroform (50:50 v/v) with phase separations until the interface was clear. The final aqueous phase was extracted with an equal volume of chloroform, the nucleic acid precipitated with ethanol from the aqueous phase, resuspended in TE buffer and concentration determined spectrophotometrically.

2.7.4 Nucleic acid separation by agarose gel electrophoresis
DNA and RNA nucleic acids were visualised by electrophoretic separation through agarose gels and stained with ethidium bromide. 0.8 to 2% molten agarose (ICN) in 1x buffer (TAE or TBE) containing 0.5 mg/ml ethidium bromide was poured into a mini-gel cast (8 cm x 8 cm) taped at both ends. A 14 well comb was inserted and the gel left to set. When ready for use, the comb and tape was removed and the gel run in 1x gel buffer (TAE or TBE).

To DNA products an equal volume of DNA loading buffer (40%(w/v) sucrose, 0.25% (w/v) bromophenol blue) was added and this loaded into the gel wells. An appropriate DNA ladder was applied to one well and run alongside the test DNA. The gel was run at 8-9 V/cm.

To RNA products, an equal volume of RNA denaturing formamide loading buffer (95% deionised formamide, 10 mM EDTA, 0.02% (w/v) each xylene cyanol blue and bromophenol blue) was added, boiled for 2 minutes and placed directly on ice. This was spun down to collect the
sample and loaded onto the gel. Again, an appropriate marker ladder was run and an electrical current passed at 6-7 V/cm. Nucleic acids were visualised under ultraviolet (UV) light on a dual intensity UV transilluminator (UPV Inc.) and a permanent record taken using a polaroid MP-4 land camera (Polaroid).

**DNA markers used**
- 0.5 µg λ/Hind III (Gibco-BRL)
- 0.25 µg φX174 / Hae III (Gibco-BRL)
- 0.25 µg pBluescript SK(+) / Sau3A I

**RNA markers used**
- 5 µg RNA molecular weight marker III (Boehringer Mannheim)

**Table 2.8 Sizes of marker bands observed on 0.8% to 2% agarose gels**

<table>
<thead>
<tr>
<th>pBluescript SK(+) / Sau3AI (bp)</th>
<th>φX174/Hae III (bp)</th>
<th>λ/Hind III (bp)</th>
<th>RNA marker III (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1030</td>
<td>1351</td>
<td>23130</td>
<td>1600</td>
</tr>
<tr>
<td>734</td>
<td>1078</td>
<td>9416</td>
<td>1000</td>
</tr>
<tr>
<td>341</td>
<td>872</td>
<td>6557</td>
<td>400</td>
</tr>
<tr>
<td>258</td>
<td>603</td>
<td>4361</td>
<td>300</td>
</tr>
<tr>
<td>192</td>
<td>310</td>
<td>2322</td>
<td></td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>2027</td>
<td></td>
</tr>
<tr>
<td></td>
<td>271</td>
<td>546</td>
<td></td>
</tr>
<tr>
<td></td>
<td>234</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**2.7.5 Denaturing polyacrylamide gel electrophoresis (PAGE)**
A stock solution of 40% polyacrylamide gel mix was prepared in 500 ml H₂O by dissolving 190 g acrylamide (BDH) and 10 g bis-acrylamide (BDH). This was deionised with 5 g mixed bed resin (BDH) and the resin removed by gravity filtration through 3MM Whatman filter paper prior to storage at +4°C.

8% denaturing gel mix was made by the addition of 50 ml 10x TBE buffer, 240 g urea (Fisher Scientific), 400 µl APS (Fisher Scientific) and 10 µl TEMED (Sigma) to 100 ml stock 40% PAGE mix.
20% denaturing gel mix was prepared by the addition of 3 ml 10x TBE buffer, 5.63 g urea, 200 µl APS and 80 µl TEMED to 15 ml 40% stock PAGE mix.

6% top solution was prepared by the addition of 25 ml 10x TBE, 230 g urea and 75 ml 40% stock PAGE mix to a final volume of 500 ml with H₂O.

6% bottom solution was prepared by the addition of 50 ml 10x TBE, 92 g urea, 50 ml H₂O, 30 ml 40% stock PAGE mix and 0.02% (w/v) bromophenol blue.

Gradient gels were prepared from two stocks of 6% PAGE gel mixes of differing TBE content. These were deionised and gravity-filtered through 3MM Whatman filter paper with storage at room temperature in the dark. Briefly, to 40 ml 6% top solution was added 200 µl 10% APS and 12 µl TEMED. To 10 ml 6% bottom solution, 50 µl 10% APS and 3 µl TEMED was added. Into a 25 ml glass pipette, 13 ml top solution was taken up followed by 10 ml bottom solution. Three air bubbles were drawn up through the solution mix forming the gradient. This mix was poured into the glass plates, topping the gel up with top solution.

PAGE gels were cast between two cleaned glass plates, the inner surface of one treated with Replicote VS silicone treatment (BDH). Plates were separated with 0.4 mm (thick) or 2 mm (thin) spacers and taped on three edges. Gel mix was poured into the plates, a comb inserted and the gel allowed to set. Prior to use, the tape from the end edge and comb was removed, the plates assembled into the gel tank with a metal heat diffusion plate attached and the wells washed out thoroughly. 1x TBE buffer was applied to top and bottom gel tank reservoirs and the samples loaded.

2.7.6 Column purification of nucleic acids
Unincorporated nucleotides were removed from RNA in vitro syntheses and oligonucleotide end labeling reactions by sephadex gel filtration.

i) Sephadex G25 spin column
Sephadex G25 (Sigma) was pre-swollen in 3 x bed volume TE buffer. In a 10 ml outer tube, a 1 ml syringe was set up with the plunger removed and
1 cm³ polyallomer wool packed to the bottom. The syringe was filled with Sephadex G25 slurry and spun at 1000 rpm in a Mistral 3000i (MSE) centrifuge. The syringe was packed to the 0.9 ml mark by repeated filling with slurry. The column was equilibrated with 300 μl elution buffer (H₂O or TE buffer) with centrifugation at 2500 rpm for 30 seconds. All wash eluates were removed. The nucleic acid sample was applied to the column and spun at 2500 rpm for 1 minute, the eluate collected in a 0.5 ml eppendorf placed under the syringe.

ii) Sephadex G100 gravity drop column
Sephadex G100 (Sigma) was pre-swollen in 3 x bed volume TE buffer. A glass pasteur pipette tip was drawn out over a flame to decrease the tip diameter and the pipette clamped vertically. 1 cm³ polyallomer wool was packed to the bottom of the pipette and Sephadex G100 slurry applied until the column was packed full by gravity. Slurry buffer eluate was discarded and the column equilibrated by the application dropwise of 500 μl elution buffer (H₂O or TE buffer). The nucleic acid sample was applied to the column and collected dropwise. Several washes with elution buffer were applied and collected separately. Nucleic acid content of the different elutions was determined by ethidium bromide staining (section 2.9.4).

2.8 DNA techniques

2.8.1 Oligonucleotide purification
i) Purification by PAGE analysis
Oligonucleotides over 30 nt in length were separated and extracted from 8% denaturing PAGE gels run in 20 x 20 cm glass plates separated by 2 mm thick spacers with a 4 cm well comb.

60 μg oligonucleotide was made up to a final volume of 40 μl with TE buffer. 40 μl of denaturing formamide loading dye was added, the sample boiled and transferred directly to ice. This was loaded onto the gel and run in 1x TBE running buffer at a constant 15 W. The glass plates were separated, spacers removed and the gel wrapped in cling film. Oligonucleotide bands were visualised under UV light against a thin layer chromatography plate and the band positions marked on the film. The piece of gel containing oligonucleotide was excised with a clean blade,
finely diced and incubated in a 1.5 ml eppendorf tube with 1 ml elution buffer (0.5% SDS, 0.3 M NaOAc (pH 5.2), 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA) at 37°C overnight with gentle shaking. The acrylamide was spun out in a microcentrifuge and the supernatant transferred to a fresh 1.5 ml eppendorf tube. The oligonucleotide was precipitated, the final pellet resuspended in 50 µl TE buffer and oligonucleotide concentration determined spectrophotometrically.

ii) Purification by lyophilisation
Oligonucleotides less than 30 nt in length were found to be pure by PAGE analysis. NH₄ was removed from the oligonucleotides by lyophilisation in a SC100 speed vac (Savant). Dried oligonucleotide was resuspended in TE buffer and the concentration measured spectrophotometrically.

iii) Column purification
GAPDH2 oligonucleotide was synthesised with a dimethoxytrityl end label and purified through an oligonucleotide purification column (OPC) containing reverse phase chromatography material (column and reagents supplied by the Protein and Nucleic Acid Chemistry Laboratory, University of Leicester).

The OPC column was prepared by passing 6 ml acetonitrile followed by 5 ml 2 M triethylammonium acetate through it using a 10 ml syringe connected to the column by a male to male luer tip. 10 OD units (263 µg) oligonucleotide in 500 µl ammonia solution (unspecified NH₃ concentration) was diluted with an equal volume H₂O and passed through the OPC at a rate of 1 drop per second. The eluate was collected and passed through the OPC a second time. The OPC was washed through with 5 ml 1.5 M NH₄OH and 10 ml H₂O. Detritylation was achieved by passing 5 ml 3% trifluoroacetic acid through the OPC followed by 10 ml H₂O, 5 ml 1.5 M NH₄OH and 5 ml H₂O. The oligonucleotide was eluted by passing 1 ml 20% acetonitrile through the column. The eluate was collected, oligonucleotide precipitated out, resuspended in TE buffer and its concentration determined spectrophotometrically.
2.8.2 **Oligonucleotide 5' phosphorylation**
The reaction components were added in the following order and incubated at 37°C for 1 hour.

- ssDNA oligonucleotide: 10 pmols
- 5x Forward reaction buffer: 5 μl
- T4 polynucleotide kinase (Gibco-BRL): 10 U
- 50 mM ATP (Sigma): 0.5 μl
- H₂O: to final volume 25 μl

T4 kinase was heat inactivated at 65°C for 10 minutes. Oligonucleotide was extracted by ethanol precipitation with 0.1 volume 10 M NH₄OAc, resuspended in H₂O and concentration determined by A₂₆₀nm in a spectrophotometer.

2.8.3 **Restriction endonuclease digestion of DNA**

i) **Complete DNA digestions**
DNA was digested using 2 U/μg restriction endonuclease in the appropriate buffer used at 1x concentration for 1 hour at 37°C. 5% glycerol content was never exceeded. All enzymes were purchased from Gibco-BRL except Nae I and Mam I (Boehinger Mannheim). Reactions were halted by heating at 65°C for 10 minutes where possible followed by phenol chloroform extraction to remove buffers and enzymes. For immediate visualisation of digestion products, reaction mixes were added to an equal volume DNA loading buffer and run on an agarose gel in 1x TAE buffer. Table 2.1 shows the endonucleases used with appropriate buffers.

ii) **Partial digestions**
Partial digestions were carried out over a time course under ideal reaction conditions. At each time point, the reaction was stopped and nucleic acid recovered by phenol chloroform extraction. Products were analysed by agarose gel electrophoresis and digested DNA species extracted by electroelution (section 2.8.5).
Table 2.9 Restriction endonucleases and their buffers

<table>
<thead>
<tr>
<th>Restriction endonuclease</th>
<th>Reaction buffer</th>
<th>Cleavage site</th>
<th>Heat innactivated?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afl II</td>
<td>REact 1</td>
<td>C/TTAAG</td>
<td>YES</td>
</tr>
<tr>
<td>Bam HI</td>
<td>REact 3</td>
<td>G/GATCC</td>
<td>NO</td>
</tr>
<tr>
<td>Cla I</td>
<td>REact 1</td>
<td>AT/CGAT</td>
<td>YES</td>
</tr>
<tr>
<td>Eco RI</td>
<td>REact 3</td>
<td>G/AATTC</td>
<td>YES</td>
</tr>
<tr>
<td>Hae III</td>
<td>REact 2</td>
<td>GG/CC</td>
<td>NO</td>
</tr>
<tr>
<td>Hinc II</td>
<td>REact 4</td>
<td>GT(T/C)/(A/G)AC</td>
<td>YES</td>
</tr>
<tr>
<td>Hind III</td>
<td>REact 2</td>
<td>A/AGCTT</td>
<td>NO</td>
</tr>
<tr>
<td>Kpn I</td>
<td>REact 4</td>
<td>GGTAC/C</td>
<td>YES</td>
</tr>
<tr>
<td>Mam I</td>
<td>SuRE/Cut H</td>
<td>GATNN/NNATC</td>
<td>YES</td>
</tr>
<tr>
<td>Mlu I</td>
<td>REact 3</td>
<td>A/CGCGT</td>
<td>YES</td>
</tr>
<tr>
<td>Nae I</td>
<td>SuRE/Cut A</td>
<td>GCC/GGC</td>
<td>YES</td>
</tr>
<tr>
<td>Not I</td>
<td>REact 7</td>
<td>GC/GGCCGC</td>
<td>YES</td>
</tr>
<tr>
<td>Pst I</td>
<td>REact 2</td>
<td>CTGCA/G</td>
<td>YES</td>
</tr>
<tr>
<td>Sal I</td>
<td>REact 10</td>
<td>G/TCGAC</td>
<td>NO</td>
</tr>
<tr>
<td>Sau3A I</td>
<td>REact 4</td>
<td>N/GATC</td>
<td>YES</td>
</tr>
<tr>
<td>Sma I</td>
<td>REact 4</td>
<td>CCC/GGG</td>
<td>YES</td>
</tr>
<tr>
<td>Sst I</td>
<td>REact 2</td>
<td>GAGCT/C</td>
<td>YES</td>
</tr>
<tr>
<td>Xba I</td>
<td>REact 2</td>
<td>T/CTAGA</td>
<td>NO</td>
</tr>
<tr>
<td>Xho I</td>
<td>REact 2</td>
<td>C/TCGAG</td>
<td>NO</td>
</tr>
</tbody>
</table>

REact buffers (Gibco-BRL) at 1x concentration:

REact 1 - 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂
REact 2 - 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM NaCl
REact 3 - 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM NaCl
REact 4 - 20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl
REact 7 - 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 50 mM KCl, 50 mM NaCl
REact 10 - 100 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 150 mM NaCl

SuRE/Cut buffers (Boehringer Mannheim) at 1x concentration:

SuRE/Cut A - 33 mM Tris-HCl pH1.9, 10 mM MgOAc, 66mM KOAc, 0.5 mM DTT
SuRE/Cut H - 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 1 mM DTE
2.8.4 Klenow fill-in of 5' DNA overhangs
To generate blunt ends of linearised dsDNA, 5' overhangs were filled in with the large fragment of DNA polymerase I, known as the Klenow fragment. The following components were mixed and incubated at 20°C for 15 minutes.

Linearised DNA 1 μg
REact 2 buffer 3 μl
5 mM each ATP, CTP, GTP, TTP mix (Pharmacia) 1 μl
Large fragment DNA polymerase I (Gibco-BRL) 0.5 U
H₂O to final volume 30 μl

DNA was purified by phenol chloroform extraction followed by ethanol precipitation.

2.8.5 Purification of DNA fragments from agarose gels
i) Low melting point gels
DNA fragments were separated by low melting point (LMP) agarose gel electrophoresis. 1μg DNA was run per cm well of a 8 x 8 cm minigel cast from 1 to 3% LMP agarose (Ultra Pure, Gibco-BRL) in 1x TAE buffer at 6 V/cm. The DNA band was visualised under UV light and the band of interest excised with a clean blade. The gel slice was diced finely and transferred to a 1.5 ml eppendorf tube with 5 x volume TE buffer. Samples were split to multiple tubes when necessary. The agarose was melted by heating to 70°C for 10 minutes, snap frozen on a dry ice/ethanol mix for 10 minutes and thawed gently. DNA was extracted by pelleting the agarose at 13,000 rpm in a microcentrifuge and the supernatant transferred to a fresh tube for ethanol precipitation. Final DNA pellets were resuspended in TE buffer and DNA concentration determined spectrophotometrically.

ii) Gel electroelution
DNA fragments were separated by agarose gel electrophoresis (section 2.7.4) in 1x TAE buffer, the band visualised under UV light and the band of interest excised with a clean blade. This was placed in dialysis tubing with 1 ml 0.1x TAE buffer and electroeluted at 200V for 1 hour in 0.1x TAE running buffer. The current was reversed for 30 seconds, the buffer removed and tubing washed several times with fresh buffer. Isobutanol
extractions reduced the volume to 500 µl and nucleic acid was extracted by phenol chloroform extraction followed by ethanol precipitation. DNA concentration was determined spectrophotometrically.

2.8.6 DNA fragment ligation
Both cohesive and blunt end ligation were carried out using a 3:1 ratio of insert:vector DNA. Typically, 90 fmol insert DNA and 30 fmol linearised vector DNA was incubated at 20°C for 2 hours (or 14°C for 16 hours) in 1x ligation buffer (50 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 1 mM ATP, 1 mM dithiotritol (DTT), 5% polyethylene glycol (PEG) -8000) with 0.5 U T4 DNA ligase (Gibco-BRL).

Blunt end vector re-ligation were carried out as above using 30 fmol linearised vector only.

2.8.7 Bacterial cell electrotransformation
i) Electrocompetent cell preparation
Dedicated plasmid-free flasks and centrifuge tubes were used and all steps carried out on ice.

Laboratory glycerol stocks of plasmid-free cells were subcultured onto 2TY agar medium and incubated at 37°C overnight. A single colony was inoculated into 10 ml 2TY broth and incubated overnight at 37°C with aeration. This was used as a starter culture for inoculation into 500 ml 2TY broth and bacterial cells grown up at 37°C until the absorbance at 550 nm read 0.5 to 0.6, ensuring cells were in mid-log phase of growth. Cells were pelleted by centrifugation at 3500 rpm, 0°C for 15 minutes in a Sorvall RC5B centrifuge GS-3 rotor (DuPont). The cell pellet was resuspended in 250 ml H₂O, pelleted at 1300 rpm, 0°C for 15 minutes, washed again in 150 ml H₂O and pelleted at 1300 rpm, 0°C for 15 minutes. Cells were then resuspended and washed in 10 ml 15% glycerol in H₂O, pelleted at 5800 rpm, 0°C for 15 minutes before final resuspension in 500 µl 15% glycerol. Cells were snap-frozen as 40 µl aliquots on a dry ice/ethanol mix and stored at -80°C.
ii) Bacterial cell electrotransformation
Each aliquot of electrocompetent cells was gently thawed on ice. Up to 1 ng plasmid DNA or 1 to 2 µl ligation mix was added to the cell aliquot and transferred to a pre-chilled 0.1 cm gap electroporation cuvette (BIO-RAD). Cells were electroporated in an Electroporator II (Invitrogen) at 1500V, 50µF, 150 Ω, resuspended in 1 ml 2TY broth and incubated at 37°C with aeration for 1 hour. Cells were pelleted by centrifugation, resuspended in 100 µl 2TY broth and plated out on 2TY agar medium with an appropriate antibiotic at 1%, 10% and 89% volume of cells. Cultures were incubated overnight at 37°C. H₂O was added to an aliquot of cells as a negative control and 1 ng pUC18 plasmid was added to an aliquot of cells and plated out at 1% cell density as a positive control.

2.8.8 Small scale Triton-boil preparation of plasmid DNA
A single colony from the bacterial plate culture was inoculated into 1 ml 2TY broth containing an appropriate antibiotic and incubated overnight at 37°C with aeration. Cells were pelleted by centrifugation at 13,000 rpm in a microcentrifuge for 1 minute, waste medium discarded and the cells resuspended in 200 µl STET buffer (8% sucrose (w/v), 50 mM EDTA, 50 mM Tris-HCl (pH8.0), 0.5% Triton X-100 and 1 mg/ml lysozyme). Cells were boiled for 2 minutes and transferred to ice for 10 minutes. Cell debris was pelleted by centrifugation at 13,000 rpm for 10 minutes in a microcentrifuge, discarded and nucleic acids in solution precipitated with ethanol on ice for 10 minutes. Final nucleic acid pellets were resuspended in 40 µl TE buffer containing 50 µg/ml RNaseA (Sigma). Plasmid content was identified by restriction nuclease digestion using 1 to 2 µl nucleic acid per digestion.

2.8.9 Large scale CsCl preparation of plasmid DNA
A single colony from the bacterial culture was isolated and inoculated into 10 ml 2TY broth containing an appropriate antibiotic to maintain selection, incubated at 37°C overnight with aeration and used to inoculate a 500 ml 2TY broth culture containing the appropriate antibiotic. Cells were grown up at 37°C with aeration to an absorbance at 600 nm of 0.7 to 0.9. 850 µl cells were added to 150 µl glycerol and stored at -80°C as glycerol stocks.
Cells were pelleted by centrifugation at 5,000 rpm, 4°C for 10 minutes in a Sorvall RC5B centrifuge GS-3 rotor (DuPont). The cell pellet was resuspended on ice in 2.5 ml ST buffer (25% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0)) and transferred to a 50 ml tube. 1 ml of 1 mg/ml lysozyme in ST buffer was added and the cells incubated on ice for a further 10 minutes. 2.5 ml 0.5 M EDTA was added with 10 minute incubation on ice. 2.5 ml Triton lysis buffer (1.5% (v/v) Triton X-100, 50 mM Tris-HCl (pH8.0), 50 mM EDTA) was added and the cells incubated on ice until lysis occurred. Cell debris was pelleted by centifugation at 20,000 rpm, 4°C for 45 minutes in an Sorvall RC5B centrifuge SS-34 rotor. The cleared supernatant was carefully transferred to a 15 ml Falcon tube, 1 g/ml CsCl added with mixing at 37°C to dissolve the salt and centrifuged at 2500 rpm in a Mistral 3000i Centrifuge (MSE) for partial CsCl gradient formation. The lower phase was transferred to 2x 3.9 ml heat-seal tubes (Beckman) containing 30 µl 10 mg/ml ethidium bromide solution. Tubes were sealed and spun in a TLN-100 fixed-angled rotor (Beckman) at 80,000 rpm overnight or 100,000 rpm for 5 hours in an Optima TL ultracentrifuge (Beckman).

Plasmid bands were extracted by tube side puncture using a 20 ga. needle and 2 ml syringe and transferred to a 15 ml tube. Repeated extractions with H2O/CsCl saturated isopropanol removed ethidium bromide, aqueous phases removed to a fresh 15 ml tube at each extraction. Plasmid nucleic acid was diluted with 3 volumes TE buffer and precipitated with 2 volumes 100% ethanol at -20 °C overnight. Nucleic acid was pelleted by centrifugation at 2,500 rpm in a Mistral 3000i centrifuge, pellets washed in 2 ml 70% ethanol and final pellets allowed to air dry. Plasmid nucleic acid was resuspended in TE buffer and the concentration determined spectrophotometrically.

2.8.10 Manual 35S-dATP dsDNA sequencing
dsDNA template was sequenced using the Sequenase version 2.0 DNA sequencing kit (United States Biochemical, Amersham Life Science UK) using appropriate oligonucleotide primers following the manufacturer’s protocol.
Briefly, 9 μg DNA template in 9 μl was denatured at 37°C for 15 minutes with 1 μl 2 M NaOH. 1 μl 10 μM primer was added followed by 3 μl 3 M NaOAc (pH5.2) and 75 μl 100% ethanol and the mix precipitated on dry ice for 10 minutes. Nucleic acid/primer was pelleted by centrifugation at 1300 rpm for 5 minutes and washed with 200 ml 70% ethanol. Pellets were resuspended in 10 μl 1x Sequenase buffer. 2 μl was aliquoted to each of 4 Eppendorf tubes and 2 μl labelling mix added to each with incubation at room temperature for 5 minutes.

<table>
<thead>
<tr>
<th>Labelling mix</th>
<th>H₂O</th>
<th>6.5 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 μ DTT</td>
<td>0.4 μl</td>
</tr>
<tr>
<td></td>
<td>α³⁵S dATP (5μCi)</td>
<td>0.5 μl</td>
</tr>
<tr>
<td></td>
<td>Sequenase enzyme</td>
<td>0.25 μl</td>
</tr>
</tbody>
</table>

To each reaction, 2 μl dGTP termination mix was added, incubated at 37°C for 5 minutes and the reactions halted by the addition of 4 μl formamide stop solution. Mixes were heated to 80°C for 2 minutes, quenched on ice and loaded onto a 20 x 50 cm 6% gradient PAGE gel run at 40 W.

When run, the plates were dismantled and the gel fixed for 10 minutes in gel-fix (10% (v/v) industrial methylated spirits (IMS), 10% (v/v) glacial acetic acid). The gel was transferred to 3MM Whatman filter paper, covered with clingfilm and dried under vacuum at 80°C for 1 hour. The gel was exposed to autoradiography film overnight and developed.

For reading sequences close to the primer, an additional 1 μl Mn buffer from the kit was added to the labelling mix.

For the elimination of sequencing compressions, dITP labelling mix and termination mixtures were substituted for the standard dGTP reactants.

2.8.11 DNA amplification by polymerase chain reaction (PCR)
Regions of DNA spanning two oligodeoxynucleotide primer binding sites were amplified by PCR. DNA polymerases used were Taq (Promega), Taq Extender (Stratagene), cloned Pfu (Stratagene), Vent (New England Biolabs) and KlenTaq (Clontech). Reactants were added with additional Mg²⁺ used in the form MgCl₂ (Taq) or MgSO₄ (Vent, Taq Extender, Pfu).
10x DNA polymerase buffer \( 5 \mu l \)
Mg\(^{2+}\) as required
Primer 1 30 pmols
Primer 2 30 pmols
5 mM each ATP, CTP, GTP, TTP mix (Pharmacia) \( 5 \mu l \)
Template DNA (H\(_2\)O for negative control) 20 ng plasmid DNA
DNA polymerase 2.5 U
H\(_2\)O to final volume 50 \( \mu l \)

The reaction mix described above was overlaid with 50 \( \mu l \) mineral oil and the DNA amplified in a cyclogene thermal cycler (Techne) for 25 to 30 cycles following the general cycle parameters,

Template denaturing at 92°C for 30 seconds
Primer annealing at \((T_m - 5 \text{ or } 10)°C\) for 30 seconds
Primer extension at 72°C for 1 minute per kilobase to be amplified
Final template extension at 72°C for 5 minutes after PCR reaction.

For DNA amplifications over 1 kb in length, a hot start was employed. Reactants minus DNA polymerase were pre-heated to 92°C for 5 minutes. DNA polymerase was then added and the PCR reaction started. Nested PCR reactions were performed using 5 \( \mu l \) first round PCR products as template DNA. PCR products were separated by agarose gel electrophoresis with ethidium bromide staining and visualised under UV light.

See table 2.2 for primer sequences and table 2.3 for DNA polymerase buffers used.

2.9 RNA techniques

2.9.1 General considerations for RNA work
As RNA is very sensitive to contaminant RNases, special considerations must be adhered to when carrying out any work with RNA. A clean area was set aside for all RNA work and was regularly washed with a 3% H\(_2\)O\(_2\) solution. All Gilson pipettes were regularly cleaned with a 3% solution of H\(_2\)O\(_2\) and designated for use with RNA-grade solutions only. Dedicated tips and eppendorf tubes used were soaked overnight in a 0.1% solution of
diethyloxycarbonate (DEPC), the waste water removed and the DEPC inactivated by autoclaving. These tips and eppendorfs were subjected to sterilisation by autoclaving at 121°C, 15 psi for 20 minutes followed by further baking in a dry oven at 200°C overnight.

Gloves were changed frequently to avoid cross contamination by hand. For gel electrophoresis of RNA, a dedicated gel tank and mini-gel cast plus comb was soaked in 3% H$_2$O$_2$ and rinsed with DEPC-treated H$_2$O prior to use.

RNA-grade reagents were made up with DEPC-treated H$_2$O, filter sterilised through a 0.2 μm sterile Acrodisk filter (Gelman Sciences) and stored in sterile, 15 and 50 ml graduated tubes where possible. Any glassware used was soaked in a 3% solution of H$_2$O$_2$ and washed thoroughly in DEPC-treated H$_2$O.

2.9.2 Extraction of total cellular RNA from eukaryotic cells

i) RNAzolB reagent extraction
The cell sample of interest (<50 mg tissue or <1 x 10$^7$ cells) was resuspended in 1 ml cold RNAzolB (Biogenesis Ltd) and incubated on ice for 5 minutes. 100 μl chloroform was added with thorough mixing and the sample incubated on ice for a further 5 minutes before centrifugation at 13,000 rpm for 15 minutes. The aqueous phase was removed carefully to a fresh 1.5 ml Eppendorf and the RNA precipitated with an equal volume of isopropanol on ice for 1 hour. RNA was pelleted by centrifugation at 13,000 rpm, washed in 70% ethanol and air dried. The pellet of RNA was resuspended in 60 μl DEPC-treated H$_2$O and re-precipitated with 6.5 μl 2 M NaCl and 133 μl 100% ethanol. RNA was recovered by centrifugation and the final pellet resuspended in TE buffer. Concentration of RNA was determined spectrophotometrically.

ii) TriReagent extraction
In accordance with the manufacturer’s protocol (Molecular Research Center Inc. USA) cells (5-10 x 10$^6$) or tissue (<100 mg) was resuspended in 1 ml TriReagent and incubated at room temperature for 5 minutes. 200 μl chloroform was added with vortexing for 15 seconds and this left to stand at room temperature for a further 10 minutes. Organic and aqueous phases
were separated at 13,000 rpm for 15 minutes and the aqueous phase transferred carefully to a fresh 1.5 ml eppendorf for RNA precipitation by the addition of 500 µl isopropanol. The final pellet was resuspended in TE buffer and RNA concentration determined spectrophotometrically.

iii) Examination of total cellular RNA quality
2 µg total cellular RNA was separated through a 1% agarose gel in 1x TBE buffer. Intact 18S and 28S rRNA bands were taken as an indication of good quality RNA. 2 µg total cellular RNA was digested with 0.2 N NaOH at 37°C for 30 minutes and run on a 1% TBE agarose gel to observe contaminant DNA species.

2.9.3 Extraction of HCV genomic RNA
i) RNA lysis mix
To 100 µl serum was added 190 µl RNA lysis buffer (1% SDS, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl) containing 1 mg/ml carrier yeast tRNA (Sigma) and 1 mg/ml proteinase K (Boehringer Mannheim). The mix was incubated at 37°C for 30 minutes and an equal volume of phenol added followed by phenol chloroform extraction. RNA was ethanol precipitated from the final aqueous phase, washed in 70% ethanol and the final RNA pellet resuspended in TE buffer. RNA concentration was determined spectrophotometrically.

ii) Extraction by viral pelleting
Using the method described by Chan et al (1992) genomic RNA of HCV was extracted from infected patient sera using the following method.

Virus was pelleted by ultracentrifugation of 100 µl serum plus 1.9 ml 1x RPMI 1680 cell culture medium (Gibco-BRL) at 50,000 rpm, 4°C for 90 minutes in an Optima TL ultracentrifuge (Beckman). Supernatant was removed and virus particles resuspended in 400 µl pre-warmed TNE buffer (55 mM Tris-HCl (pH 8.0), 110 mM NaCl, 1.1 mM EDTA, 0.55% SDS) mix (175 ml TNE buffer, 2mg/ml poly A (Boehringer Mannheim), 1 mg/ml proteinase K (Boehringer Mannheim)), transferred to a fresh 1.5 ml eppendorf and incubated at 37°C for 90 minutes. An equal volume of phenol was added and the RNA obtained by phenol chloroform extraction followed by ethanol precipitation and final resuspension in TE buffer.
Presence of nucleic acid was determined by ethidium bromide staining and if sufficiently bright, the RNA concentration was determined spectrophotometrically.

2.9.4 Detection of RNA by ethidium bromide staining
To observe very small quantities of extracted RNA, a dilution series of tRNA (1 pg/μl to 100 ng/μl) in 10 μg/ml ethidium bromide was prepared. 4 μl each dilution sample was placed onto a dark background plus a blank ethidium bromide only control. 2 μl RNA sample was added to 2 μl ethidium bromide. An estimation of RNA concentration was made by comparing the degree of fluorescence of the RNA sample against the tRNA titration under UV light using a hand held transilluminator.

2.9.5 cDNA synthesis and cloning of viral RNA
i) cDNA synthesis
cDNA was synthesised from viral RNA and cloned into pSPORT vector using the Superscript plasmid system (Gibco-BRL) using the manufacturer's protocol. Viral RNA template was resuspended in 8 μl DEPC-treated H₂O. 1 μg HCV3 NotI oligonucleotide primer was added, annealed by heating to 70°C for 10 minutes followed by chilling on ice and first strand cDNA synthesised by the addition of 4 μl 5x 1st strand buffer, 2 μl 0.1 M DTT, 1 μl 10 mM each ATP, CTP, GTP, TTP and 1 μl DEPC-treated H₂O with heating to 42°C for 2 minutes and the addition of 2 μl Superscript II RT enzyme with incubation at 42°C for 1 hour. RT reaction was stopped by placing on ice.

Second strand DNA synthesis was achieved by the addition of the following with incubation at 16°C for 2 hours.

DEPC-treated H₂O 91 μl
5x 2nd strand buffer 30 μl
10 mM each dNTP mix 3 μl
E.coli DNA ligase 1 μl
E.coli DNA polymerase 4 μl
E.coli RNaseH 1 μl
2 µl T4 DNA polymerase was added with incubation at 16°C for 5 minutes and DNA recovered by phenol chloroform extraction followed by ethanol precipitation using 7.5 M NH₄OAc. The final DNA pellet was resuspended in 25 µl DEPC-treated H₂O.

ii) DNA cloning
Sal I adapters were ligated to synthesised DNA by incubation at 16°C for 16 hours with 1x T4 DNA ligase buffer, 10 µl Sal I adapters and 5 µl T4 DNA ligase. DNA was recovered by phenol chloroform extraction and ethanol precipitation and was then subject to digestion with Not I restriction endonuclease in REact 3 buffer (section 2.8.3) and DNA purified by extraction with phenol chloroform followed by ethanol precipitation. The final DNA pellet was resuspended in 100 µl TEN buffer and column purified through a filtration column equilibrated in TEN buffer. Eluate was collected dropwise, each fraction volume measured and all fractions pooled until total volume <550 µl. Salts were removed through a Sephadex G25 spin column (section 2.7.6) and the DNA concentrated by ethanol precipitation.

iii) Ligation of DNA to pSport
DNA was resuspended in 10 µl TEN buffer and added to 1 µl Not I/Sal I linearised pSport vector and 1 µl T4 DNA ligase in 1x DNA ligase buffer to a final volume 20 µl with incubation at 25°C for 3 hours. The reaction was electroporated into electrocompetent AG1 and DH5α cells, plasmid DNA extracted and analysed for successful clones.

2.9.6 RNase protection assay
i) In vitro synthesis of a radiolabelled RNA probe
Template plasmid DNA was linearised with an appropriate restriction endonuclease and the DNA extracted with phenol chloroform followed by ethanol precipitation. DNA was resuspended in H₂O and concentration determined spectrophotometrically. RNA probe was synthesised by addition of the following components in order at room temperature and incubated at 37°C for 2 hours,
5x Transcription buffer (Promega) 4 μl
0.1 M DTT 2 μl
RNasin (Promega) 20 U
2.5 mM each ATP, GTP, CTP (Promega) 4 μl
1 μM UTP (Promega) 2.4 μl
Linearised template DNA 1 μg
α32P UTP (3000 Ci/mmol) (NEN) 5 μl
SP6 RNA polymerase (Promega) 20 U
DEPC-treated H2O to final volume 20 μl

The reaction was terminated by the addition of 1 μl 20 mM EDTA. 1 μl reaction mix was taken for specific activity determination (total cpm) and the DNA removed by the addition of 1 U RQ1 RNase-free DNase (Promega) with incubation at 37°C for 1 hour. RNA probe was extracted by phenol chloroform followed by ethanol precipitation and resuspension in 20 μl DEPC-treated H2O. The probe was added to an equal volume of RNA denaturing loading buffer, heated to 80°C for 5 minutes and gel purified through an 8 M urea 5% polyacrylamide gel cast in 18 x 20 cm glass plates at 40 W in 1x TBE buffer.

When run the gel was dismantled, wrapped in clingfilm and exposed to autoradiography film in a sealed cassette for 5 minutes. When developed the film was used as a template to locate the RNA probe band. This was excised from the gel with a clean blade, diced finely and incubated in 1 ml RNA gel elution buffer overnight at 37°C. The probe supernatant was transferred to a fresh tube and a 1 μl aliquot taken for specific activity determination (incorporated cpm).

ii) Determination of specific activity
Average counts per minute (cpm) were calculated for the 1 μl aliquots taken (total, T and incorporated, i cpm). Each aliquot was added to 3 ml scintillation fluid and counted in a MINAXI 4000 series scintillation counter (United Technologies, Packard). Specific activity of RNA probes was determined using the formulae,

\[
\% \text{ incorporation} = \frac{\text{incorporated cpm}}{\text{total cpm}} \times 100
\]  

(1)
Total cpm incorporated = i cpm x dilution factor x reaction volume \( (2) \)

\[
n\text{mol} \, \alpha^{32}\text{P}-\text{UTP} \text{ incorporated} = \frac{\mu\text{Ci} \, \alpha^{32}\text{P}-\text{UTP}}{\text{concentration (\mu Ci/mmol)}} \quad (3)
\]

\[
n\text{mol cold UTP} = \mu\text{l cold UTP} \times 100 \, \mu\text{M UTP} \times \frac{10^3 \, \text{nmol}}{1 \, \mu\text{mol}} 	imes \frac{1 \mu\text{l}}{10^6 \mu\text{l}} \quad (4)
\]

Total nmol UTP = nmol \( \alpha^{32}\text{P}-\text{UTP} \) + nmol cold UTP \( (5) \)

Maximum RNA yield = Total nmol UTP x 4 rNTPs x 330 ng \( \text{nmol} \)

Total \( \mu\text{g} \) RNA synthesised = \% incorporation x maximum yield RNA \( (7) \)

Specific Activity RNA probe = \( \frac{\text{Total incorporated cpm}}{\text{Total } \mu\text{g RNA synthesised}} \) \( (8) \)

iii) \text{RNase protection assay}

Total cellular RNA was coprecipitated with 1-5 x \( 10^5 \) cpm RNA probe in ethanol and resuspended in 20 \( \mu\text{l} \) RNA hybridisation buffer (0.2 mM NaCl, 40 mM PIPES (pH 4.6), 1 mM EDTA). This was heated to 85°C for 5 minutes and transferred to a waterbath for incubation at 50°C for 16 hours. Reaction mix was diluted with 180 \( \mu\text{l} \) RNase protection assay digestion buffer (10 mM Tris-HCl (pH 7.5), 5 mM EDTA, 200 mM NaOAc) and single stranded molecules of RNA removed by the addition of 1 U RNase ONE exonuclease (Promega) with incubation at room temperature for 1 hour. The reaction was stopped by the addition of 20 \( \mu\text{l} \) stop solution (10% SDS, 1 mg/ml tRNA) and RNA hybrids extracted by phenol chloroform followed by ethanol precipitation with a final resuspension in 10 \( \mu\text{l} \) denaturing RNA loading buffer.

Hybrid products were resolved after heating to 80°C for 5 minutes through a 5% PAGE gel run at 40W with 1x TBE buffer. When run, the gel was dismantled, wrapped in clingfilm with drying under vacuum with heat and exposed to autoradiography film overnight with an intensifying screen at -80°C.
2.9.7 Oligonucleotide protection assay

i) Oligonucleotide radiolabelling

a) Purified oligonucleotide was end labelled by addition of the following in order at 37°C for 1 hour,

- Purified oligonucleotide 1 µg
- γ³²P ATP (10 µCi) 1 µl
- 10x oligonucleotide protection buffer 2 µl
- T4 Kinase (Promega) 5U
- H₂O to final volume 20 µl

1 µl reaction mix was taken for specific activity determination. Probe mix was diluted with H₂O and purified through a G25 spin column (see section 2.7.6).

b) Oligonucleotide was end labelled using the T4 polynucleotide kinase (Gibco-BRL), following the manufacturer's protocol (section 2.8.2). α³²P ATP was substituted for cold ATP. Unincorporated label was removed through a Sephadex G25 spin column and 1 µl final eluate used to determine specific activity of the probe.

ii) Determination of labelled oligonucleotide specific activity

A 1 µl aliquot of labeled oligonucleotide was added to 3 ml scintillation fluid and the average counts per minute (cpm) calculated using a scintillation counter. Specific activity was calculated using the formula,

\[
\text{Specific activity (cpm/pmol) = Average cpm/µl} \times \text{total probe volume} \div 10 \text{ pmol ends}
\]

iii) Oligonucleotide protection assay

Total cellular RNA was coprecipitated with 1 x 10⁵cpm probe in ethanol and the pellet resuspended in 20 µl deionised formamide. 5 µl 5x RNA hybridisation buffer was added, the mix heated to 90°C for 5 minutes and transferred to a waterbath for incubation at appropriate hybridisation temperature for 4 hours. Single stranded molecules of nucleic acid were digested with the addition of appropriate concentration endonuclease (Mung Bean or S1, both purchased from Promega) in 500 µl single stranded nuclease digestion buffer and incubated at 37°C for 1 hour.
Nucleic acids were extracted by phenol chloroform and ethanol precipitated followed by resuspension in 10 μl denaturing RNA loading dye. Hybrids were heated to 80°C and resolved through a thin denaturing 8M urea, 20% polyacrylamide gel run in 1x TBE buffer at 40 W. When run the gel was dismantled and dried down onto 3MM Whatman paper under vacuum. This was exposed to autoradiography film overnight and developed.

2.9.8 *In vitro* synthesis of RNA
Template plasmid DNA was linearised with an appropriate restriction endonuclease, phenol chloroform extracted and precipitated in ethanol. DNA was resuspended in 10 μl DEPC-treated H$_2$O and 1μl taken for nucleic acid quantitation by A$_{260}$nm. An appropriate amount DNA was qualified by agarose gel electrophoresis and if not fully linearised, was further purified by extraction from LMP agarose (section 2.8.5).

RNA was transcribed using the RiboMax T7 RNA synthesis system (Promega). The reaction components were added in the order given at room temperature and incubated at 37°C for 4 hours.

- 5x transcription buffer: 4 μl
- 25 mM each ATP, CTP, GTP, UTP: 1.5 μl each
- Linearised template DNA: 2 μg
- DEPC-treated H$_2$O: to final volume 18 μl
- T7 enzyme mix: 2 μl

Template DNA was removed by the addition of 2 U RQ1 RNase-free DNase (Promega) with incubation at 37 °C for 15 minutes. RNA was recovered by phenol chloroform extraction followed by ethanol precipitation. Final RNA pellet was resuspended in 10 μl DEPC-treated H$_2$O, concentration determined by spectrophotometric absorbance at 260nm and stored at -80°C.
2.9.9 **Single tube reverse transcription coupled PCR**

Cellular or *in vitro* synthesised RNA was used as the template for cDNA synthesis by reverse transcription followed by DNA amplification by PCR. The reverse primer was annealed to the RNA template by heating 3' primer mix to 90°C for 2 minutes and plunging on ice. Reverse transcription was carried out by the addition of 10 μl Avian myeloblastosis virus (AMV) RT mix with incubation at 42°C for 1 hour. As a negative control, H₂O was substituted for AMV-RT.

### 3' primer mix
- **Template**: RNA as required
- **3' primer**: 100 ng
- **10x RT buffer**: 1.00 μl
- **H₂O**: to final volume 10 μl
- Overlay with 50 μl mineral oil

### RT mix
- **H₂O**: 5.25 μl
- **10x RT buffer**: 1.00 μl
- **0.1 M DTT**: 1.00 μl
- **5 mM each ATP, CTP, GTP, TTP mix**: 5.00 μl
- **RNAsin (Promega) 40 U/μl**: 0.25 μl
- **AMV-RT (Promega) 8 U/μl**: 0.50 μl

RT enzyme was inactivated by heating to 90°C for 5 minutes and reactions transferred to ice. To the cDNA was added 30 μl PCR mix and the DNA amplified (section 2.8.11). Positive and negative control PCR reactions were also run.

### PCR Mix
- **10x DNA polymerase buffer**: 3.0 μl
- **5' primer (100 ng/μl)**: 1.0 μl
- **Mg²⁺**: as required
- **DNA polymerase**: 2.5 U
- **H₂O**: to final volume 30 μl

PCR products were separated by agarose gel electrophoresis and visualised under UV light.
2.9.10 Quantitative RT-PCR assay

i) RT-PCR assay

A competitive quantitative assay was set up based on the single tube RT-PCR method. Internal standard control (ISC) RNA was synthesised in vitro from pJF002 plasmid DNA template linearised with Nae I (section 2.9.8), purified and quantified. A series of 5 RT-PCR reactions were run, each containing the same amount of total cellular RNA which was adjusted so that the PCR product signal fell within the range of the ISC RNA used. To these was added a series titration of ISC RNA ranging from $6 \times 10^5$ to $1.5 \times 10^7$ molecules ISC RNA diluted with carrier tRNA. RT buffer and reverse primer were added and the RT-PCR reaction carried out for 25 cycles amplification as described in section 2.9.9 using Taq DNA polymerase in the PCR reaction. A negative RT-PCR reaction was performed on RNA templates substituting H$_2$O for AMV-RT to control for DNA contamination of RNA templates. A positive and negative PCR reaction was also run to control for DNA contamination of PCR reactants. Final PCR products were electrophoresed through a 2% agarose gel in 1x TAE buffer with ethidium bromide staining and visualised under UV light. The PCR product from ISC RNA was 84 bp larger than the product from target mRNA and were well separated from each other on the gel. A photograph was taken and used for product analysis.

ii) RT-PCR product analysis

The gel photograph was digitally scanned into a Power Macintosh computer at 300 dots per inch (dpi) resolution with 256 greyscale using an Aries Relysis scanner. The image was acquired using Adobe Photoshop (Macintosh v2.5) and saved as a standard TIFF graphics file. The image was inverted to facilitate band analysis.

Gel images were analysed using NIH Image software (Macintosh v6.1) using the ‘Gel plotting’ macros system to measure the areas of each target mRNA and ISC RNA PCR product band.

iii) Quantitation of target mRNA

The areas of target and ISC bands were used to calculate the area ratios of target:ISC for each RT-PCR reaction. These ratios were plotted on a double log scale against the known amount of initial ISC RNA in each reaction using Cricket Graph software (Macintosh v1.5.2). Where the ratio of target
to ISC products equals 1 (Loge ratio = 0), the number of molecules of target RNA are the same as the known number of ISC RNA molecules. This gives a value for the expression of target mRNA in molecules per µg total cellular RNA. Values were used for calculating target mRNA when they fell within the range of ISC RNA products.

iv) Worked example
A worked example is described here showing the quantification of PKR mRNA from total cellular RNA extracted from Hut78 cells using the TriReagent RNA extraction procedure (section 2.9.2.ii). 1.5 µg total cellular RNA was used in each RT-PCR reaction with a titration of ISC RNA.

**Figure 2.7** PCR products from PKR RNA competitive RT-PCR

RT-PCR products stained with ethidium bromide. M=Øx174 DNA/ Hae III cut marker ladder. PKR mRNA product is seen at 303bp and ISC RNA derived products seen at 382bp. 1.5µg total cellular RNA used as template in each reaction with the following titration of ISC RNA (molecules)

**Table 2.10** PCR product band areas measured from Figure 2.7

<table>
<thead>
<tr>
<th>Molecules ISC RNA (x10^5)</th>
<th>Loge</th>
<th>Area PKR mRNA band</th>
<th>Area ISC RNA band</th>
<th>Ratio PKR mRNA:ISC RNA bands</th>
<th>Loge (ratio PKR area: ISC area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.37</td>
<td>13.739</td>
<td>16246</td>
<td>8879</td>
<td>1.830</td>
<td>+ 0.604</td>
</tr>
<tr>
<td>18.75</td>
<td>14.444</td>
<td>13684</td>
<td>11265</td>
<td>1.215</td>
<td>+ 0.195</td>
</tr>
<tr>
<td>37.50</td>
<td>15.137</td>
<td>11559</td>
<td>14464</td>
<td>0.799</td>
<td>- 0.224</td>
</tr>
<tr>
<td>75.0</td>
<td>15.830</td>
<td>13337</td>
<td>22070</td>
<td>0.604</td>
<td>- 0.504</td>
</tr>
<tr>
<td>150.0</td>
<td>16.523</td>
<td>11550</td>
<td>28854</td>
<td>0.400</td>
<td>- 0.916</td>
</tr>
</tbody>
</table>
At the point of RNA species equivalence, ratio PKR:ISC = 1

\[ y = \log_e(\text{area PKR/area ISC}) = 0 \]

As \( x = \log_e(\text{molecules ISC RNA}) \) when \( y = 0 \),

\[ \text{molecules ISC RNA} = \frac{-7.969}{-0.538} = 14.812 \]

Actual number of PKR mRNA molecules = \( e^{14.812} = 2.71 \times 10^6 \)
Therefore in Hut78 cells, there was found \( 2.71 \times 10^6 \) molecules PKR mRNA in 1.5 \( \mu \)g total cellular RNA that equates to \( 1.81 \times 10^6 \) molecules mRNA/\( \mu \)g total RNA.
2.10 Protein techniques

2.10.1 Total cellular protein extraction from eukaryotic cells
i) SDS lysis extraction
Cells were resuspended in an appropriate volume 2x SDS buffer, boiled for 3 minutes and the insoluble fraction pelleted by centrifugation at 13,000 rpm in a microcentrifuge. Soluble proteins were separated by PAGE.

ii) TriReagent extraction
In a method based on that described by Chomczynski (1993), cells (5-10x $10^6$) or tissue (<100 mg) was resuspended in 1 ml TriReagent (MRC Inc. USA) as described in section 2.9.2.iii. After removal of the RNA phase, the DNA interphase was precipitated by the addition of 300 µl 100% ethanol with centrifugation at 13,000 rpm for 5 minutes. The organic phase was transferred to a fresh 15 ml tube and the proteins precipitated with 1.5 ml isopropanol with incubation at room temperature for at least 15 minutes. Proteins were pelleted by centrifugation at 2500 rpm in an Mistral 3000i (MSE) centrifuge for 10 minutes with removal of the supernatant. The protein pellet was resuspended and washed three times, each in 2 ml 0.3 M guanidine-HCl in 95% ethanol, left at room temperature for 20 minutes and pelleted by centrifugation at 2500 rpm for 5 minutes. A final wash was performed in 2 ml 100% ethanol and after pelleting proteins were allowed to air dry. Proteins were resuspended in an appropriate volume 1x SDS buffer, boiled for 5 minutes and insoluble material spun down by centrifugation at 13,000 rpm. Soluble proteins were quantified spectrophotometrically. Proteins to be analysed by SDS-PAGE were added to an equal volume of 2x SDS protein buffer, boiled for 3 minutes and loaded onto a 12% SDS polyacrylamide gel for separation by electrophoresis. Proteins were visualised by staining or by western blot.

2.10.2 Collagenase dissociation of liver tissue

Krebs-Ringer bicarbonate buffer
475 mg Krebs buffer salt (Sigma) was dissolved in 45 ml DEPC-treated H$_2$O. 63 mg NaHCO$_3$ was added, the pH adjusted to 7.2 with HCl and buffer made up to a final volume 50 ml with DEPC-treated H$_2$O before filter sterilisation through a 0.2 µm sterile Acrodisk filter (Gelman Sciences).
The tissue sample was equilibrated in 1 ml Krebs-Ringer Bicarbonate buffer at 37°C for 5 minutes and the buffer removed. 1 ml Krebs buffer/collagenase mix (5 mM CaCl, 1 mM MgCl, 3% (v/v) bovine serum albumen, 1 mM benzamidine-HCl, 1 mM TLCK, 1 mg/ml collagenase Type IV (Sigma) in Krebs-Ringer bicarbonate buffer) was added and the tissue incubated at 37°C with 5% CO$_2$ until the tissue dissociated, generally >4 hours. Tissue cells were pelleted by centrifugation at 13,000 rpm and the buffer supernatant removed. Cells were then extracted as desired.

2.10.3 **Total cellular protein quantitation**

Total cellular protein was quantified using the method described by Warburg & Christian (1941). This measures the excitement of tyrosine and tryptophan residues in constituent proteins. Total cellular protein samples must be assumed to contain relatively equal numbers of each amino acid residue. A diluted sample of total protein was measured spectrophotometrically at 280nm and 260nm against an appropriate blank sample of buffer. The ratio of 280:260 gives a value for % nucleic acid interference which is taken into account when calculating the protein factor (F) in the formula, $F = 0.594x^3 - 2.693x^2 + 4.391x - 1.493$ ($x = 280/260$)

Using F to calculate protein concentration,

| Protein concentration (mg/ml) = A280nm x F x dilution factor |

2.10.4 **In vitro protein expression**

i) **Protein expression**

A pET16b plasmid with sequence encoding PKR amino acids 1-170 cloned into the Nde I to Bam HI restriction sites was used as an expression vector for the production of this PKR protein (PKR1-170). Protein expression is under the control of a T7 promotor region. Plasmid was electroporated into electrocompetent BL21(DE3) cells and grown up to an OD$_{600nm}$ 0.7 to 0.9 in a 500 ml 2TY broth culture with antibiotic selection for ampicillin and chloramphenicol resistant transformants. Addition of 1 mM isopropylthio-β-D-galactoside (IPTG) induced the DE3 lysogen to produce T7 promotor which then induced the expression plasmid to produce PKR1-170 protein. Cells were induced for 3 hours and pelleted by centrifugation at 5,000 rpm for 10 minutes in a RC5B centrifuge (Sorvall).
ii) **Bacterial cell sonication**

Induced cell pellets were resuspended in 10 ml sonication buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 M urea), frozen and subjected to three freeze-thaws. Cells were sonicated on ice with a pre-chilled sonicator probe for 10 x 15 second pulses. Cell debris was pelleted at 15,000 rpm for 15 minutes and the supernatant transferred to a fresh tube.

iii) **His-tagged protein purification**

PKR1-170 expressed from the pET16b vector contains a 10 residue histidine tag at its N-terminal. This was used to purify PKR1-170 from background bacterial proteins using Talon affinity resin (Clontech). Talon resin, pre-washed in sonication buffer was added to the protein supernatant and incubated at room temperature for 30 minutes with gentle agitation. Resin was centrifuged at 2500 rpm for 10 minutes and the supernatant discarded. Presence of protein in supernatants was determined spectrophotometrically by measuring the absorbance at 280nm. The resin was washed in wash buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.5)) with centrifugation at 2500 rpm between washes until no more protein was detected in the wash buffer.

Resin was given a final wash in wash buffer containing 10 mM imidazole (Sigma) and PKR1-170 eluted from the resin by the addition of 100 mM imidazole in wash buffer. Resin was washed several times in 100 mM imidazole wash buffer until no more protein was eluted. Imidazole was removed from the PKR1-170 fractions by dialysis in three changes of wash buffer.

2.10.5 **Purified protein quantitation**

The Bio-Rad protein quantitation assay, based on the Bradford dye-binding technique was used according to the manufactures guidelines. Protein assay reagent was diluted 5x in H$_2$O. To each of 1 ml diluted reagent in a 1cm cuvette was added 30 μl protein sample. PKR 1-170 sample was quantified against serially diluted bovine serum albumin (Sigma) in PKR 1-170 buffer. All samples were incubated at room temperature for 10 minutes and absorbances read at 595nm on a spectrophotometer. A standard curve was created for the BSA protein samples from which the level of PKR1-170 could be calculated.
2.10.6 Protein SDS PAGE
Proteins were separated by electrophoresis through a stacking and then a resolving SDS polyacrylamide gel. To a Mini-Protean II (Bio-Rad) gel assembly was poured 3.5 ml 12% SDS resolving gel mix that was overlaid with H₂O-saturated isobutanol and allowed to set. The solvent was removed, the gel comb inserted and 1 ml 5% SDS stacking gel mix added to fill the gel assembly. 30% stock polyacrylamide Protogel (National Diagnostics) was used for protein gel mixes.

<table>
<thead>
<tr>
<th></th>
<th>5% Stacking gel</th>
<th>12% Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% polyacrylamide</td>
<td>1.00 ml</td>
<td>6.00 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.14 ml</td>
<td>5.00 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl (pH 8.8)</td>
<td>------</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl (pH 6.8)</td>
<td>751 µl</td>
<td>------</td>
</tr>
<tr>
<td>10% SDS</td>
<td>60 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>40 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

For use, the comb was removed and wells washed thoroughly. Protein samples were loaded and run in 1x SDS running buffer (25 mM Tris-HCl, 0.2 M glycine, 0.1% SDS) at a constant 15 mA through the stacking gel and 25 mA through the resolving gel. A standard low molecular weight marker ladder of proteins (Pharmacia) was loaded containing,

- 94 KDa Phosphorylase b
- 67 KDa Albumin
- 43 KDa Ovalbumin
- 30 KDa Carbonic anhydrase
- 20.1 KDa Trypsin inhibitor
- 14.4 KDa α-lactalbumin

Once run, the gel was disassembled and the gel either stained directly or blotted to nitrocellulose membrane.
2.10.7 Protein staining of SDS PAGE gels by coomassie stain
Protein PAGE gels were dismantled and the stacking gel removed. The resolving gel was submersed in Coomassie stain (10% (v/v) IMS, 10% (v/v) glacial acetic acid, 0.2% (w/v) Coomassie brilliant blue) for at least 30 minutes. Stain was removed and the gel submersed in Coomassie de-stain (30% (v/v) methanol, 10% (v/v) glacial acetic acid) with several changes until the background disappeared leaving only protein bands stained blue to the desired intensity. Gels were dried between hydrated sheets of cellophane (BioRad).

2.10.8 Protein transfer to nitrocellulose membrane for western blotting
Hybond C-Super nitrocellulose membrane (Amersham), cut to the gel size, and equivalent pieces of 3MM Whatman filter paper plus the gel were pre-equilibrated in protein transfer buffer (25 mM Tris-HCl, 0.19 M glycine, 20% methanol) for 10 minutes.

i) Semi-dry blotting
Between the cathode base and the anode lid of a semi-dry blotting apparatus was assembled 6 pieces filter paper, protein gel, nitrocellulose membrane and 6 pieces filter paper from the cathode to anode. Proteins were transferred at 80 mA for 1 hour, the apparatus disassembled and proteins detected.

ii) Electroblotting
A sandwich was made of 3 pieces filter paper, gel, membrane and 3 more pieces filter paper. This was transferred to a blotting tank (Cambridge Electrophoresis Ltd.) with the gel on the cathode side. The tank was filled with protein transfer buffer and proteins blotted at 30 V, 4°C for 16 hours.

2.10.9 Protein immunodetection
The membrane was disassembled and gel plus filter paper discarded. The membrane was stained with Ponceau stain (7% (v/v) glacial acetic acid, 0.2% (w/v) Ponceau 3R) with washes in H2O allowing the marker bands to be marked with pencil for later identification. Unbound areas of the membrane were blocked with TMT/SS buffer (10% sheep serum, 3% skimmed milk powder, 0.5% Tween-20, 1x PBS) for 2 hours with rocking.
Buffer was removed and the membrane washed twice in PBS-T (1x PBS plus 0.5% Tween 20).

i) Protein antibody probing
The membrane was incubated in 10 ml mouse monoclonal antibody against human PKR, 71/10 (Ribogene Inc.) (Laurant et al, 1985) at 1 in 10,000 dilution in TNMAT buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% skimmed milk, 0.05% Tween-20) for 1 hour at room temperature with rocking. Antibody was removed, the membrane washed in 1x PBS-T buffer and a secondary anti-mouse Ig antibody added at 1 in 1000 dilution in 10 mls TNMAT buffer and incubated at room temperature for 1 hour with rocking. Secondary antibody was removed, the membrane washed twice in PBS-T for 20 minutes each and twice in PBS for 20 minutes.

ii) Alkaline phosphatase detection
A rat anti-mouse secondary antibody (Dako Ltd.) conjugated to an alkaline phosphatase label was used. After membrane washing, proteins were detected with the addition of alkaline phosphatase colour detection reagents (NBT, X-phos in 1x AP buffer) (Bio-Rad) with incubation at room temperature until protein bands appeared. Reaction was stopped by washing the membrane in H2O.

iii) ECL detection
A goat anti-mouse secondary antibody (Harlan Serolabs) conjugated to horse raddish peroxidase was used. After washing, the membrane was equilibrated in ECL-reagent 2 (Amersham) for 1 minute. An equal volume ECL-reagent 1 was added with incubation for 1 minute. The membrane was blotted from excess reagents, covered with cling film and exposed to blue-sensitive autoradiography film for a timed exposure. Films were developed in a Gevamatic 60 developer (Agfa-Gevaert) and analysed.

iv) α35S labelled antibody detection
A α35S-labelled sheep anti-mouse Ig secondary antibody was used. Blocking buffer substituted foetal bovine serum for sheep serum. After final washes, the membrane was dried and exposed to autoradiography film overnight and developed. Bound antibody and therefore PKR protein bands were also detected by a phosphorImager (Molecular Dynamics Inc. USA) using the ImageQuant software (Molecular Dynamics Inc.).
2.10.10 Quantitative protein assay

i) Protein assay
To a 12% SDS-PAGE gel was loaded a known titration of PKR 1-170 protein spanning a range 5 ng to 45 ng purified protein. Alongside this was loaded and run a known amount of total cellular protein and 4 µl marker ladder. SDS-PAGE gel was western blotted to nitrocellulose membrane as described and detected using an ECL-conjugated secondary antibody.

ii) Protein analysis of ECL detected proteins
Autoradiographs were scanned and analysed using the system described in section 2.9.10 using the NIH Image software (Macintosh v1.61) to measure the average intensities, in pixels, of each standard curve band and a combination of band area and intensity for both the standard curve and cellular-derived full length PKR bands, as described by the program protocol.

iii) Quantitation of target protein
The average band intensity was used to confirm those PKR1-170 bands that were within the linear range of the standard curve. Where the regression coefficient >0.9, analysis of the image was performed.

The analysis program ‘gel plotting’ macros was used to measure the area of each band identified (PKR1-170 at 21kDa and FL-PKR at 68 kDa). The areas of PKR1-170 were plotted against the known amount of protein for each band using Cricket Graph software (Macintosh v1.5.2). From the line of linear regression, given the area of FL-PKR test protein band, the amount of protein in that test band was calculated. This gives a value for the amount of PKR protein (ng) per µg total cellular protein.

iv) Worked example
A worked example is described here showing the quantitation of full length PKR protein species extracted from liver biopsy number 5 using TriReagent, as described in section 2.10.1.ii. 40 µg total cellular protein was tested against a standard curve of PKR1-170 protein with a range 14.9 ng to 59.8 ng. The difference in molecular weights of the two PKR proteins were taken into consideration when quantifying FL-PKR.
The amount PKR1-170 (ng) was converted to number of moles, given that 1ng PKR1-170 = 0.0478 pmoles (MW PKR1-170 = 20920). From this, the number of moles FL-PKR in liver protein could be calculated and converted back to ng quantities protein.

Figure 2.9  Autoradiograph of ECL-detected western blot

Proteins electrophoresed through a 12% SDS-PAGE gel, western blotted using the 71/10 mouse monoclonal antibody against human PKR followed by a secondary goat anti-mouse polyclonal antibody conjugated to a horse raddish peroxidase label. PKR proteins were detected using the ECL reagents (Amersham) with exposure to autoradiography film for 1 minute.

Table 2.11  PKR protein areas measured from figure 2.9

<table>
<thead>
<tr>
<th>ng PKR 1-170</th>
<th>pmols PKR 1-170</th>
<th>Protein band area</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.9</td>
<td>0.712</td>
<td>18481.81</td>
</tr>
<tr>
<td>29.8</td>
<td>1.424</td>
<td>48860.22</td>
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<tr>
<td>44.7</td>
<td>2.137</td>
<td>81950.45</td>
</tr>
<tr>
<td>59.6</td>
<td>2.849</td>
<td>96198.0</td>
</tr>
<tr>
<td>Liver #5 FL-PKR band</td>
<td>--------</td>
<td>78936.35</td>
</tr>
</tbody>
</table>


Figure 2.10  Graph showing PKR1-170 protein area plotted against amount protein.

\[ y = 44542.154x - 13634.173 \quad r = 1.000 \]

From the line of linear regression, the amount FL-PKR protein can be calculated, \( \text{pmoles FL-PKR} = \frac{\text{protein area} - \text{constant}}{\text{line gradient}} \)

For liver #5, \( \text{pmoles FL-PKR} = \frac{78936.35 + 13634.173}{44542.154} = 2.078 \)

amount FL-PKR (g) = moles x MW protein

amount FL-PKR #5 = \( 2.078 \times 10^{-12} \times 68000 = 1.413 \times 10^{-7} \) g = 141.3 ng protein

This is the level of protein from 40 µl total cellular protein.
Therefore, the level of FL-PKR in liver #5 is 3.533 ng/µg total cellular protein
2.9.11 Statistical analyses performed on protein data

Student's paired t-test, F-test (Kanji, 1993) and the Wilcoxon test (SPSS® reference guide, 1990) were used to analyse paired data from the PKR results obtained for HCV infected patients.

\[ \begin{align*}
X_1 &= \text{population of 1st data set} \\
X_2 &= \text{population of 2nd data set} \\
S_1 &= \text{standard deviation of 1st data set} \\
S_2 &= \text{standard deviation of 2nd data set} \\
\bar{X} &= \text{mean of X data set} \\
n &= \text{population number in data set} \\
T^+ &= \text{Sum of ranked positive differences when } X_2 \text{ is subtracted from } X_1 \\
& \quad \text{for each set of paired data}
\end{align*} \]

i) **Student's paired t-test**

Degrees of freedom = (n - 1)

\[ t = \frac{(X_1 - X_2)}{s/n^{1/2}} \]

ii) **F-test**

Degrees of freedom = (n - 1)

\[ F = \frac{(S_2/S_1)^2}{\frac{n(n+1)/24}{\sqrt{[n(n+1)(2n+1)/24]}}} \]

iii) **Wilcoxon test**

\[ Z = \frac{T^+ - [n(n+1)/4]}{\sqrt{[n(n+1)(2n+1)/24]}} \]
Chapter 3

Attempts to construct an \textit{in vitro} replication system for HCV
3.1 Introduction
At the start of this project, there was no method for the propagation of HCV in which molecular analysis of virus replication could be achieved. The chimpanzee was the only animal proven to be infected by HCV, showing all symptoms associated with this virus. As chimpanzees are large and expensive animals to use, this was not a useful model for the majority of work carried out with HCV. Some groups had reported the successful propogation of HCV virus particles in cell culture (section 1.5.2) using HCV infected serum as an initial inoculum. These studies led to the identification of constituent virus proteins and their localisation in infected cells. However, no molecular analysis could be carried out on these virus particles because they contain an RNA genome. This cannot be externally altered in order to analyse the effects of genomic sequence changes on virus protein functions. Molecular cloning of the HCV genome, or regions of the genome, would allow mutational analysis of HCV and its replicative strategy. Expression of virus proteins from a molecular clone could be externally controlled to facilitate analysis of protein expression, localisation, function and the effect of novel therapeutic compounds directed against specific virus proteins.

To obtain a molecular clone of HCV, genomic RNA would first have to be reverse transcribed to cDNA. This would be amplified to dsDNA by the PCR reaction for cloning into a suitable cellular expression vector. Complete molecular clones of other RNA viruses had been developed at the time this project started, including type 14 rhinovirus (Mizutani et al, 1985), poliovirus (Racaniello et al, 1981) and hepatitis A virus (Cohen et al, 1987). Yoo et al (1995) had reported the creation of a full length HCV molecular clone. In vitro transcribed RNA from this was transfected to a Huh7 cell line and the propagation of virus RNA detected though expression of virus proteins was not reported. This indicated there was still a need for the development of a cell culture based system for the expression and analysis of HCV proteins.

The intention of this project was to obtain molecular clones of HCV using the expression vector pBCCX that would express virus like particles (VLPs) in mammalian cell culture (section 3.2). Cloning and expression of the entire HCV genomic sequence might result in production of infectious virus. This would be extremely hazardous as HCV has been classified as a
category III pathogen. For the molecular study of HCV proteins in mammalian cells, production of infectious virus is not necessary. By creating molecular clones of the entire polyprotein coding region that does not include the 5’ and 3’ UTR regions, only virus proteins should be expressed in transfected cell culture. HCV RNA replication would not be possible due to the lack of complete genomic sequence and therefore lack of ability to replicate genomic RNA, ensuring no production of infectious virus particles.

3.2 HCV virus-like protein expression
The vector pBCCX (Haddrick et al, 1996) is a modified version of the plasmid pBC12/CMV/IL2 (Cullen, 1986) in which a short multiple cloning site (Clal to Xhol) replaced the IL-2 region (figure 2.1). Genes inserted into this cloning site should be constitutively expressed in a range of mammalian cells due to the presence of a cytomegalovirus immediate-early (CMV IE) promoter (Boshart et al, 1985; Schmidt et al, 1990). Termination of transcription occurs due to the SV40 polyadenylation (polyA) signal which also facilitates efficient export of transcribed mRNA species from the cell nucleus. As genomic HCV does not encode a polyadenylation sequence the addition of this polyA region is essential for cloned HCV sequence expression. An SV40 origin of replication allows plasmid replication in COS-1 cells while the backbone of pBR322 plasmid allows for pBCCX propagation in bacterial cells. Plasmid transfected into mammalian cells could then express the HCV polyprotein which would be cleaved and processed through a combination of host cell and virus proteins. A diagrammatic representation of the VLP expression system for HCV is shown in figure 3.1.
Construction of HCV polyprotein encoding clones was based on the method successfully employed by Haddrick et al (1996) and Lau (1997) in this laboratory, in which the HCV sequence spanning the entire polyprotein region (354nt to 9400nt) would be amplified from initial virus genomic RNA and then cloned into pBCCX via the restriction sites Hind III and Xho I. Haddrick et al used this system to produce virus-like particles of HIV, from the plasmid pBCCX-CSF, morphologically similar to the wild type virus. Initial experiments would therefore entail extraction of HCV genomic RNA from samples of virus followed by molecular cloning of the polyprotein coding region of HCV.
3.3 HCV genomic RNA extractions
To obtain HCV genomic RNA, samples of HCV infected serum were obtained (a kind gift from Dr L Jarvis, University of Edinburgh, Scotland). HCV from these patients had previously been genotyped as type 3a and samples were known to contain high virus titres (personal communication L Jarvis).

3.3.1 RNA lysis mix extraction
HCV genomic RNA was initially extracted by the RNA lysis method (section 2.9.3) where virus particles are subjected to protein denaturation by SDS and proteinase K so disrupting the virus envelope and capsid structures. Resultant nucleic acid from serum should thus contain virus RNA. Extracted RNA was stored at -80°C in equal aliquots for later use.

The amount of RNA recovered was too low to observe by either agarose gel electrophoresis or by absorbance at 260nm. It was thought that the RT-PCR amplification technique might be sufficiently specific and sensitive enough to detect and amplify regions of any HCV RNA present to an amount observable by agarose gel electrophoresis. Such PCR products could then be cloned to the pBCCX vector. Repeated virus RNA extractions were used in RT-PCR reactions with primers HCV1, HCV2 and HCV3 (table 2.2) though no DNA products were obtained.

At the time this project started, there was no available clone containing type 3a HCV sequence. Therefore, there was no template sequence of HCV for use as a positive control in RT-PCR reactions using primers HCV1, HCV2 and HCV3. It was therefore unclear whether the problems experienced were due to the RNA extraction procedure itself or with the oligonucleotide sequences. In view of this, extracted virus RNA was analysed by nested RT-PCR using oligonucleotides specific for genotype 3a to amplify a short, 253bp section of the 5’UTR region of HCV. This work was carried out in a standardised reaction by L Jarvis, University of Edinburgh. It was confirmed that there was a significant lack of RNA in the sample extracted using the RNA lysis technique thereby suggesting all RNA extractions performed by this method did not yield HCV RNA.

The lack of amplified products may have been due to a number of factors. Both the reverse transcription and PCR reactions may not have been used at their optimum reactions conditions. Equally, some components of these
reactions may have been inactive which would have affected the amplification reaction. The primers HCV1, HCV2 and HCV3 had not been proven in any amplification reaction to anneal with HCV type 3a. A lack of primer to template RNA annealing would not result in any cDNA and therefore no amplified dsDNA PCR products. Contaminant RNases from reagents or even the serum itself may also have played a part in denaturing any RNA present. Repeated freeze-thawing of serum and the RNA extraction method itself are both factors that would influence the presence of virus template RNA. HCV infected serum was stored at -80°C to ensure virus RNA integrity was maintained. Multiple thawing to obtain a fraction of the serum sample might have resulted in virus particle denaturation and thus loss of constituent RNA. The RNA lysis method had been proven effective in extracting HIV RNA from infected cell culture (Haddrick, 1996). However, both the SDS and proteinase k reagents may have been sequestered by the large amount of serum proteins present. This would have resulted in inefficient HCV particles lysis and therefore a lack of virus RNA.

RNA extracted by L Jarvis plus samples of the oligonucleotide primers 209,211 (Garson et al, 1990) and 939,940 (Okamoto et al, 1990) (table 2.2) were obtained from L Jarvis. These were used to confirm that the RT-PCR buffers and enzymes used were of good quality. An RT-PCR reaction using these reactants gave the expected PCR product of 253 bp when observed by agarose gel electrophoresis (figure 3.2). Type 3a HCV RNA extracted by L Jarvis with appropriate primers were subsequently used as positive RT-PCR control reactions.

3.3.2 RNAzolB reagent extraction
As virus RNA may not have been efficiently extracted from serum using the RNA lysis procedure, RNAzolB reagent was used to extract HCV RNA from serum (section 2.9.2). The final pellet of RNA was resuspended in TE buffer. The amount of RNA extracted was too low for quantification by absorbance at 260nm and so was used directly in an RT-PCR reaction with oligonucleotides HCV3 and HCV1. This sensitive technique may amplify any HCV RNA sequence sufficiently for visualisation by agarose gel electrophoresis. No PCR products were observed.
3.3.3 Virus pelleting technique for RNA extraction

HCV virus RNA was extracted from 100μl serum using the virus pelleting method (section 2.9.4). RNA was analysed by RT-PCR using the nested set of oligonucleotides obtained from L Jarvis which resulted in the expected PCR product of 253 bp (figure 3.2). This indicates that both RT-PCR reagents and procedures worked well. That HCV virus was present in the serum sample extracted and that this method of pelleting virus and removal of serum proteins is efficient at extracting HCV RNA from infected serum. The amount of RNA extracted using this method was insufficient for quantitation by absorbance at 260nm or agarose gel electrophoresis. As the sensitive technique of RT-PCR might amplify sufficient amounts of virus RNA for further analysis, samples of nucleic acid extracted from serum were used directly in RT-PCR reactions described further in sections 3.4 and 3.6.

Figure 3.2 Comparison of HCV genomic RNA extraction procedures by nested RT-PCR amplification.

HCV genomic RNA was extracted from 100 μl serum using the RNA lysis, RNAzolB and virus pelleting procedures. Template RNA was amplified by a nested RT-PCR reaction using AMV-RT and Taq DNA polymerase with primers 209/939 and 211/940. 5 μl each PCR reaction mix was run on a 0.8% agarose gel in 1x TAE buffer. Lanes 1-5 show 1st round, and lanes 6-10 show 2nd round PCR products. Lanes 1,6 = negative PCR reaction, 2,7 = negative RT reaction (virus pelleting extracted RNA), 3,8 = RNA lysis extraction, 4,9 = RNAzolB extraction and 5,10 = Virus pelleting extraction procedures. All negative controls show no system contamination.
3.4 **Subgenomic HCV amplification**

In order to obtain molecular clones of HCV, oligonucleotide primers HCV1, HCV2 and HCV3 were designed against a consensus sequence derived from available published sequences. These included HCV-BK (Genbank #S70787), HCV-Hebei (Genbank #L02836), HCV-JK1G (Genbank #X61596), HCV-L2 (Genbank #U01214) and HCV-N (Genbank #S62220). The expected PCR products for these are given in figure 3.3 where HCV1 and HCV2 amplifies the first half of genomic sequence and HCV1 and HCV3 amplifies the full polyprotein coding sequence. Both 5' and 3' UTR regions of HCV are not encoded within these amplified regions.

**Figure 3.3** Long PCR amplification of subgenomic HCV - Oligonucleotide primer sites plus their expected PCR products.

<table>
<thead>
<tr>
<th>HCV genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' UTR</td>
</tr>
<tr>
<td>HCV1</td>
</tr>
<tr>
<td>HCV2</td>
</tr>
<tr>
<td>HCV3</td>
</tr>
</tbody>
</table>

RT-PCR reactions were performed using HCV virus RNA extracted from serum by the RNA lysis method. HCV2 or HCV3 oligonucleotides were used as the reverse primer for the synthesis of cDNA (section 2.9.9) using AMV-RT. HCV1 oligonucleotide was then used as the forward primer in PCR amplification reactions with Taq DNA polymerase. Initially all three primers were purified by PAGE analysis (section 2.8.1.i) though yields were very low (table 3.1). Primers appeared as discrete bands when visualised under UV light from the PAGE gel and were therefore later purified by lyophilisation (section 2.8.1.ii).

**Table 3.1** Yields of HCV oligonucleotides purified by PAGE analysis

<table>
<thead>
<tr>
<th>Oligonucleotide primer</th>
<th>Amount loaded onto PAGE gel</th>
<th>Amount recovered</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV1</td>
<td>60 µg</td>
<td>5.35 µg</td>
<td>8.92</td>
</tr>
<tr>
<td>HCV2</td>
<td>60 µg</td>
<td>9.10 µg</td>
<td>15.16</td>
</tr>
<tr>
<td>HCV3</td>
<td>60 µg</td>
<td>7.10 µg</td>
<td>11.83</td>
</tr>
</tbody>
</table>
PCR reactions involved an initially extended template denaturing step of 92°C for 1 minute after which the DNA polymerase was added and reactants mixed. This is thought to facilitate the specificity of primer to template annealing reactions (Finckh & Rolfs, 1995). Amplifications generally ran through 30 cycles using a denaturing step at 92°C for 1 minute, a primer annealing step between 57°C and 68°C and a sequence extension step at 72°C between 5 and 10 minutes. The primer annealing temperature was reduced in some reactions to decrease the stringency of primer to template binding as initial amplifications did not yield any products. Extension times were also altered to increase the opportunity for amplified product synthesis.

DNA products were visualised by 0.8% agarose gel electrophoresis. No products were observed from first round PCR reactions. 0.1 volume first round PCR mix was used as template in a second round PCR reaction. It was thought that any trace amounts of first round PCR products might be further amplified to an observable level. In order to increase the specificity and ensure background PCR products were not co-amplified, primer annealing temperatures were increased by 2-3°C.

Despite performing dual rounds of template amplifications and altering reaction conditions (Mg²⁺ ion, dNTP and primer concentration as well as annealing temperature and extension times), no amplified products of HCV were ever obtained. As mentioned in section 3.3 there was no positive control template available at the time this work was carried out in order to determine which stage of the reactions or which reactants were defective.

It later transpired that the RNA extraction procedure used did not yield HCV genomic RNA. This may explain why no amplified products of HCV were observed. It was also suggested that due to recent development in HCV sequence genotyping, oligonucleotide primers should be designed to the specific genotype of virus being used (personal communication L Jarvis). The NZL1 sequence for type 3a virus was subsequently published by Sakamoto et al (1996). Sequences for HCV1, HCV2 and HCV3 were analysed against this using the software program Amplify (v.1.2 Macintosh) and were shown to have poor complementarity. The synthesis of subgenomic cDNA by AMV-RT and therefore subsequent amplification by PCR may also have been impeded by the presence of significant
secondary structures within the genomic RNA. AMV-RT has been reported as lacking processivity for the synthesis of very long regions of cDNA (Gerard et al, 1989; D'Alessio et al, 1990) that may add to the difficulty in obtaining the desired amplified regions of HCV. This last point was the basis for the use of a cDNA synthesis and cloning system described in section 3.5 that utilises the reverse transcriptase Superscript II (Gibco-BRL) and is discussed further in section 3.12.

3.5 **HCV cDNA cloning into pSPORT**

3.5.1 **Subgenomic HCV cDNA cloning**

A modified Moloney Murine Leukemia Virus (M-MLV) without RNaseH activity, Superscript II RT (Gibco-BRL) had been reported to achieve much longer cDNA species in reverse transcription reactions with greater yields of cDNA than other RT enzymes such as AMV-RT (D'Alessio et al, 1990; Rispeter et al, 1997). To amplify and subsequently clone the polyprotein region of HCV, the Superscript plasmid system for cDNA synthesis and plasmid cloning kit was used (Gibco-BRL, section 2.9.5). Virus RNA extracted by the RNA lysis method was used as template for the synthesis of cDNA though the level of RNA extracted was too low to quantify either by OD$_{260}$nm or ethidium staining. First and second strands of cDNA were synthesised and the resultant product ligated into a pSPORT plasmid vector pre-cut with Not I/Sal I. Buffer salts were removed from the ligation reactions by Sephadex G25 column purification (section 2.7.6). cDNA products were then used to transform electrocompetent AG1 and DH5α cells by electroporation that were inoculated onto 2TY agar plate medium with ampicillin selection (section 2.8.7) with overnight incubation at 37°C. As a negative control, H$_2$O was used to transform bacterial cells. When inoculated onto ampicillin medium, no growth occurred confirming that the bacterial cells did not contain any contaminant plasmid DNA. As a positive control, pUC18 plasmid DNA was used to transform bacterial cells. Over 300 bacterial colonies were observed when 1% of the cell volume was inoculated onto ampicillin medium, confirming the transformation reactions were successful.

From the transformations of bacterial cells with cDNA-ligated pSPORT, only 6 bacterial colonies grew up on plate media. Plasmid DNA was extracted by the Triton-lysis miniprep protocol (section 2.8.8) and
linearised by digestion with EcoRI endonuclease. Linearised and undigested samples of plasmid DNA were visualised by electrophoresis through an 0.8% agarose gel. No HCV insert DNA was observed as all plasmid species migrated to either the expected size for pSPORT or to a smaller, deleted form of the plasmid that can be seen in figure 3.4.

Figure 3.4  pSPORT vector deletions

0.05 volume miniprep DNA from cDNA-ligated pSPORT transformed DH5α cells was electrophoresed on a 0.8% agarose gel with ethidium bromide staining. Lanes 21 to 33 and 37 to 40 are different transformed colony preparations showing religated species of pSPORT only. Lanes 34 to 36 show undigested DNA preparations where pSPORT plasmid DNA has religated but is smaller than its expected size indicating a deletion event has occurred. Marker lane = λHind III DNA ladder.

Transformation of the cDNA synthesis product into DH5α cells was repeated 6 times. No DNA inserts were observed but deleted species of pSPORT still occurred.

3.5.2 Incorporation of ³²P dCTP in cDNA synthesis
As no molecular clones of HCV were observed, it was decided to analyse each step of the cDNA synthesis system. This would help in determining the amount of cDNA products synthesised and identify whether any were being lost at each stage. For this a ³²P-dCTP label was incorporated into the first strand synthesis of cDNA according to the protocol given. All steps were carried out according to recommended safety procedures for handling radioactive material.

All subsequent steps were analysed with a Geiger counter, observing where labelled products occurred. At the column chromatography step, fractions were collected and Cherenkov counts measured in a scintillation counter against a blank of TE buffer for each fraction up to a cumulative volume of 550 µl. These were recorded and counts per minute (cpm) measured for each fraction (table 3.2 and figure 3.5).
Table 3.2 Cherenkov counts obtained for each HCV subgenomic $^{32}$P cDNA fraction collected from the Superscript plasmid system.

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Cumulative volume (µL)</th>
<th>Cherenkov counts (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>1</td>
<td>96</td>
<td>117</td>
</tr>
<tr>
<td>2</td>
<td>197</td>
<td>113</td>
</tr>
<tr>
<td>3</td>
<td>219</td>
<td>1481</td>
</tr>
<tr>
<td>4</td>
<td>244</td>
<td>903</td>
</tr>
<tr>
<td>5</td>
<td>269</td>
<td>2087</td>
</tr>
<tr>
<td>6</td>
<td>294</td>
<td>694</td>
</tr>
<tr>
<td>7</td>
<td>320</td>
<td>79</td>
</tr>
<tr>
<td>8</td>
<td>347</td>
<td>89</td>
</tr>
<tr>
<td>9</td>
<td>360</td>
<td>36</td>
</tr>
<tr>
<td>10</td>
<td>388</td>
<td>47</td>
</tr>
<tr>
<td>11</td>
<td>418</td>
<td>2148</td>
</tr>
<tr>
<td>12</td>
<td>433</td>
<td>1248</td>
</tr>
<tr>
<td>13</td>
<td>461</td>
<td>8496</td>
</tr>
<tr>
<td>14</td>
<td>483</td>
<td>155</td>
</tr>
<tr>
<td>15</td>
<td>495</td>
<td>794</td>
</tr>
<tr>
<td>16</td>
<td>525</td>
<td>625</td>
</tr>
<tr>
<td>17</td>
<td>555</td>
<td>not done</td>
</tr>
</tbody>
</table>

Figure 3.5 Column fractionation of labelled HCV subgenomic cDNA species taken from table 3.1.
These results show the majority of label occurred in the lower molecular weight (MW) fractions (fraction 11-13) which would suggest the majority of cDNA products were much smaller than the expected 9 Kb product encoding the HCV polyprotein. Some higher MW products were obtained (fraction 3-5) suggesting a small amount of longer cDNA products were synthesised. The amount of cDNA products from fractions 3, 4 and 5 correspond to 23% of the total amount labelled cDNA. This is around the expected 20% yield of a 9Kb length cDNA products from intact template RNA (personal communication, Gibco-BRL).

These higher MW cDNA products were ligated to pSPORT and used to transform electrocompetent DH5α cells. Plasmid DNA from transformed bacterial cells was extracted by the Triton-lysis mini prep method and linearised by EcoR I endonuclease digestion. No cDNA clones were observed with some DNA bands occurring as deleted plasmid species (figure 3.4).

3.5.3 pSPORT deletion analysis

As the pSPORT vector was provided pre-cut with Not I/Sal I it should have contained incompatible sticky ends. Religation in the absence of any cDNA product would seem unlikely. However, bacterial cells were transformed by the plasmid that did not seem to contain any species of insert DNA. Small pieces of cDNA that may have been sufficient to reigate the vector should have been removed during the column chromatography stage. Spontaneous religations may have occurred and therefore this cut plasmid warranted further analysis. A linker piece of DNA was obtained to specifically reigate the pSPORT vector from the cDNA synthesis kit. A region of DNA was removed from pBluescript II SK+ by digestion with Not I and Sal I and ligated into the Not I/Sal I sites of pSPORT (figure 3.6). To ensure intact pBluescript SK+ vector DNA did not contaminate the transformation reactions, DNA products of the ligation reaction were ethanol precipitated, resuspended in H₂O and subjected to restriction digestion by XhoI endonuclease. This will linearise intact pBluescript II SK+ vector but not pSPORT.

Ligation products were used to transform electrocompetent DH5α cells that were then inoculated onto 2TY agar medium with ampicillin selection. Plasmid DNA was extracted by the Triton-lysis miniprep method, linearised with Mlu I endonuclease and DNA species visualised
by electrophoresis on an 0.8% agarose gel in 1x TAE buffer. Repeatedly, no deletions of pSPORT were observed. Pre-cut pSPORT vector DNA could therefore be religated, given a suitable linker, giving full length plasmid DNA products upon transformation to bacterial cells. The reason for the plasmid deletions observed was not discovered.

Figure 3.6 Strategy for religation of Not I/Sal I cut pSPORT vector

After these experiments were performed it was proven that no HCV genomic RNA was obtained from infected serum by the RNA lysis method (section 3.3). This resulted in an absence of HCV template RNA in the cDNA synthesis system and therefore a lack of final HCV cDNA products. Additionally the primer HCV3NotI had been designed based on the sequence for the 3' primer HCV3. With the publication of type 3a HCV sequence data (Sakamoto et al, 1996), the primer HCV3NotI was found to have poor complementarity to HCV type 3a used here as template for the synthesis of cDNA. The presence of higher molecular weight cDNA products seen in figure 3.5 indicates some synthesis of cDNA may have occurred. This may have resulted from contaminant species of nucleic acid though incorporation of the $^{32}$P-dCTP label should have been a specific
event. The source and type of these cDNA species was not discovered as no molecular clones of these cDNA products were obtained.

As this cDNA cloning system is not the most sensitive method of cloning nucleic acid, especially if there is a limited amount of template RNA available, it was decided to return to using an RT-PCR strategy for the amplification and construction of HCV molecular clones. This is recognised as a very sensitive technique that should result in amplified regions of the HCV genome given that the RNA extractions used yielded very small amounts of template RNA. It was proven that the virus pelleting method for HCV RNA extraction yielded sufficient virus genomic RNA for detection by RT-PCR of the 5' UTR (section 3.3), though insufficient RNA was extracted for visualisation by agarose gel electrophoresis or quantitation by absorbance at 260nm.

Oligonucleotide primers for further RT-PCR amplifications were designed specifically to the type 3a HCV sequence upon publication of this data (Sakamoto et al, 1996) and used in HCV fragment amplifications described in section 3.6.

3.6 HCV fragment cloning
Previous reports suggested the use of fragment amplification for the construction of long DNA clones where very long PCR amplification is otherwise difficult to achieve (Selby et al, 1993; Suter & Pauli, 1995; Mizuno et al, 1995). In this way, shorter regions of genomic RNA sequence can be amplified by RT-PCR and cloned into a suitable vector. These could then be pieced together through suitable restriction endonuclease sites creating the full length sequence desired. Although previous long RT-PCR attempts (section 3.3) failed to amplify the desired region of HCV sequence possibly due a lack of RNA template, the 9065 bp region to be amplified was considered too long for efficient amplification with minimum sequence mutations. As the main aim of this project was to express VLPs in cell culture, it was deemed sufficient to obtain DNA clones of the structural protein coding region only. A plasmid vector encoding all three structural proteins would express these as a single polypeptide in mammalian cell culture. Host signal peptidases would cleave the three proteins from each other as occurs in a naturally infected cell. Core, E1 and
E2 would localise to cell membranes via. the endoplasmic reticulum to form VLPs within transfected mammalian cells.

This system has since become a proven method for the molecular cloning of long regions of the HCV genome. Kohara et al (1996), Wakita et al (1998) and Aizaki et al (1998) have all reported successful attempts at cloning full length HCV cDNA using infected serum as the source of virus genomic RNA.

Oligonucleotides HCV4 to HCV9 (table 2.2) were designed specifically to the HCV genotype 3a sequence NZL1 (Genbank #D17763, Sakamoto et al, 1996) encompassing the structural protein coding region of HCV (figure 3.8). The initial aim was to amplify the whole structural protein region of HCV using HCV4 to HCV9. This would be cloned in reverse orientation into the Hind III/Xho I endonuclease restriction sites of pBluescript II KS+ for sequence analysis and then into pBCCX for mammalian cell expression. Expressed HCV proteins are likely to be toxic to bacterial cells, especially the structural proteins that localise to cell membranes, which may result in bacterial cell disruption. By cloning HCV protein coding sequences in reverse orientation, leaky expression of these proteins should be avoided, ensuring the survival of transformed cells and thus molecular clones of HCV. This has since been reported by Forns et al (1997) where leaky expression of cloned HCV proteins does occur in transformed E.coli cells, resulting in bacterial cell death and therefore loss of the molecular clone.

The three fragments (1, 2 and 3) would also be amplified separately and inserted into a pBluescript II vector. Once cloned, these three sequences would be sequentially pieced together at the compatible endonuclease restriction sites encoded by the PCR primers. Once the HCV sequence was confirmed, this would be cloned into the expression vector pBCCX for VLP expression in mammalian cells.

All oligonucleotide primers used for the amplification of HCV genomic sequences were found to run as pure single bands upon PAGE analysis. These primers were therefore purified by lyophilisation (section 2.8.1.ii) and used in the combinations shown in figure 3.8. Primers annealing to target sequences were confirmed in test RT-PCR reactions using type 3a HCV RNA obtained from L Jarvis, University of Edinburgh. The 3’
oligonucleotide of each pair was used as the reverse primer in the RT reaction with AMV-RT and the corresponding 5’ oligonucleotide as the forward primer in the PCR reaction with Taq DNA polymerase. Upon visualisation, some PCR products were observed although a significant amount of non-specific primer annealing had resulted in background DNA products (figure 3.7). As the RNA extraction procedural problems had been rectified, these oligonucleotides were then used in RT-PCR reactions using HCV genomic RNA freshly extracted from serum.

**Figure 3.7** RT-PCR products using oligonucleotides HCV4 to HCV9

RT-PCR products using HCV type 3a RNA extracted by L Jarvis (University of Edinburgh) electrophoresed on a 0.8% agarose gel run in 1x TAE buffer stained with ethidium bromide. Lanes 1 and 4 are blank (DNA in lane 4 is overspill from lane 5). Lanes 3, 6, 8 and 10 are negative RT-PCR reactions where H2O was substituted for AMV-RT. Lanes 2/3 shows products using primers HCV5+HCV8. Lanes 5/6 shows products using primers HCV4+HCV6. Lanes 7/8 shows products using primers HCV7+HCV9. Lanes 9/10 show 253bp second round control RT-PCR products using primers 209/939 and 211/940. Marker DNA ladder is pBluescript II SK+ / Sau3AI.

HCV type 3a genomic RNA was extracted from 100μl infected human serum using the virus pelleting procedure (section 2.9.3.ii) and stored as 5 equal aliquots under ethanol at -80°C. From each of three aliquots, the virus RNA was pelleted by centrifugation and resuspended in 5 μl H2O. As controls for each RT-PCR reaction performed, one aliquot was used as template RNA for a negative RT-PCR reaction substituting H2O for AMV-RT. When PCR cycling profiles permitted, a further aliquot was used as a positive control using the primers 209/939 and 211/940 to amplify a 253bp region within the 5'UTR (figure 3.2). A further aliquot of extracted virus RNA was used as template in test RT-PCR reactions.

RT-PCR reactions were carried out to optimise the PCR reaction conditions including cycling temperatures and times. Optimum cycling profiles are shown in table 3.3.
Figure 3.8 HCV fragment PCR primer binding sites plus expected PCR products

<table>
<thead>
<tr>
<th>5'UTR</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>940 → 253 ← 211</td>
<td></td>
</tr>
<tr>
<td>939 → 307 ← 209</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5'UTR</th>
<th>C</th>
<th>E1</th>
<th>E2</th>
<th>p7</th>
<th>NS2</th>
<th>NS3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fragment 1

| HCV4 | 1264 ← HCV6 |
| 940 → 1557 ← Nest6 |

Fragment 2

| HCV5 | 1106 ← HCV8 |
| Nest5 | 1292 ← Nest8 |

Fragment 3

| HCV7 | 830 ← HCV9 |
| Nest7 | 909 ← Nest9 |

Fragment 1-3

| HCV4 | 3157 ← HCV9 |
| 940 → 3450 ← Nest9 |

Table 3.3 PCR cycling profiles for HCV fragment amplifications

<table>
<thead>
<tr>
<th>HCV fragment</th>
<th>Template denaturation</th>
<th>Primer annealing</th>
<th>Primer extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>94°C 36 seconds</td>
<td>50°C 42 seconds</td>
<td>72°C 1.5 minutes</td>
</tr>
<tr>
<td>Fragment 1</td>
<td>92°C 30 seconds</td>
<td>55°C 30 seconds</td>
<td>72°C 1.5 minutes</td>
</tr>
<tr>
<td>Fragment 2</td>
<td>92°C 30 seconds</td>
<td>53°C 30 seconds</td>
<td>72°C 1.0 minutes</td>
</tr>
<tr>
<td>Fragment 3</td>
<td>92°C 30 seconds</td>
<td>53°C 30 seconds</td>
<td>72°C 1.5 minutes</td>
</tr>
<tr>
<td>Fragment 1-3</td>
<td>92°C 30 seconds</td>
<td>55°C 30 seconds</td>
<td>72°C 3.5 minutes</td>
</tr>
</tbody>
</table>
Amplification reactions were carried out using primer sets HCV4 to HCV9 but these yielded very little, if any, DNA products. Control RT-PCR reactions using primers 209/939 and 211/940 proved extractions of HCV RNA from serum were successful though template RNA may have been degraded in test reactions by contaminant RNases. As the positive control RT-PCR used a nested amplification system and in view of similar nested PCR reactions used when detecting regions of the HCV genome (Lin et al, 1992; Reid, 1994; Mellor et al, 1995), it was decided to use this system for the amplification of HCV fragments 1 to 3. This strategy is described further in section 3.8.

Amplifications carried out up to this point had all used Taq DNA polymerase. Taq polymerase is known to lack 3' to 5' exonuclease activity so creating a 3' terminal nucleotide overhang of the final DNA product. This would be useful in direct cloning to a vector containing a single T overhang but in this instance, the HCV fragments were to be specifically cloned using primer encoded restriction sites. Taq polymerase is recognised as a highly processive enzyme suitable for amplifying long regions of DNA though it lacks any proof-reading ability, causing between 1 in 10⁴ (Eckert & Kunkel, 1991) and 8 in 10⁶ (Cline et al, 1996) nucleotide misincorporations per duplication. As amplified sequences of HCV were to be cloned for virus expression, it is important to amplify the true sequence. Various DNA polymerases were therefore tested for their ability to amplify sequences of HCV.

### 3.7 Evaluation of different DNA polymerases

Control PCR reactions were carried out using either reverse transcribed HCV RNA with primers 209/939 and 211/940, or template DNA extracted from an HTLV transformed C91 eukaryotic cell line with relevant primers ATK2 and ATK5, a gift from A Lau, University of Leicester (Lau, 1997).

PCR reactions using Taq DNA polymerase yielded the expected DNA products at 253bp or 1077bp when visualised by agarose gel electrophoresis in 1x TAE buffer. Pfu DNA polymerase with a titration of Mg²⁺ ions, dNTP concentration and addition of supplemental BSA did not result in any PCR products (figure 3.9). With Vent DNA polymerase, using the same DNA template and oligonucleotides, a smear was observed upon
agarose gel electrophoresis. Again an Mg\textsuperscript{2+} titration was employed to obtain optimised reaction conditions as well as altering the ratio of DNA template to primer concentration. However, no discrete PCR product was obtained (figure 3.10). It seems unlikely that control DNA and primers were at fault when Pfu and Vent polymerases were used which would imply that these DNA polymerases were not suitable in the reactions carried out. This has since been proven by Kohara et al (1996) who found both Vent and Pfu to be poor at amplifying regions of HCV sequence.

**Figure 3.9** Comparison of PCR reactions using Taq and Pfu DNA polymerase.

![Image of agarose gel showing bands](image1)

Products from a PCR reaction using HTLV transformed genomic C91 DNA amplified with primers ATK2 and ATK5. 0.1 PCR reaction volume was visualised on a 0.8% agarose gel. Lanes 1 and 3 are positive PCR reactions, lanes 2 and 4 show negative PCR reaction controls where H\textsubscript{2}O was substituted for Taq DNA polymerase (lanes 1, 2) or Pfu DNA polymerase (lanes 3,4). Marker ladder = φx174/HaeIII DNA.

**Figure 3.10** Comparison of PCR reactions using Taq and Vent DNA polymerase.

![Image of agarose gel showing bands](image2)

Products from a nested RT-PCR reaction using HCV genomic RNA extracted from infected serum with primers 209/939 and 211/940. 0.1 PCR reaction volume was visualised on a 2% agarose gel. Lanes 1, 3, 5, 7 are negative control reactions. First round PCR products in lanes 1, 2, 5, 6. Second round PCR products in lanes 3, 4, 7, 8. Lanes 1-4 correspond to amplifications using Taq DNA polymerase, PCR product at 253bp. Lanes 5-8 correspond to amplifications using Vent DNA polymerase. Marker ladder = pBluescript II SK+/Sau3AI DNA.
The DNA polymerase mix KlenTaq (Clontech) was then used in control PCR reactions using both the suppliers control template DNA plus primers and C91 DNA with primers ATK2 and ATK5. Expected PCR products were obtained in both reactions. No Mg²⁺ titration was required.

KlenTaq contains both Taq and Deep Vent DNA polymerases. This combination utilises the processivity of Taq while maintaining authentic sequences due to the proof-reading ability of Deep Vent. Barnes (1994) carried out a series of long range PCR reactions proving the combination of Taq and Deep Vent (or Pfu) DNA polymerases to be far more efficient at amplifying the correct DNA sequence than either enzyme on its own. He also found that PCR yields were greater if a polymerase combination is used. This has been supported by Rispeter et al (1997) in which a combination of Taq and Pwo DNA polymerase was used to amplify regions of the HCV sequence. A summary of PCR reaction results for the DNA polymerases used is shown in table 3.4.

<table>
<thead>
<tr>
<th>DNA polymerase</th>
<th>Properties</th>
<th>PCR reaction outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taq</td>
<td>processivity but no proofreading ability</td>
<td>PCR products of the correct size</td>
</tr>
<tr>
<td>Vent</td>
<td>Proofreading ability</td>
<td>No PCR products</td>
</tr>
<tr>
<td>Pfu</td>
<td>Proofreading ability</td>
<td>No PCR products</td>
</tr>
<tr>
<td>KlenTaq</td>
<td>Processivity plus proofreading ability</td>
<td>PCR products of the correct size</td>
</tr>
</tbody>
</table>

Using KlenTaq DNA polymerase should ensure products from HCV RT-PCR reactions contain authentic HCV sequence for correct protein expression in cell culture. The KlenTaq system also employs an antibody hot start mechanism whereby the initial cycle parameter at 92°C irreversibly denatures a Taq-specific antibody bound to the enzyme. This has been shown to increase the specificity and efficiency of PCR amplifications (Kellog et al, 1994) as well as reducing the physical manipulations required by the traditional hot start systems, so reducing contamination risks. KlenTaq was therefore used in all further PCR reactions in the amplification of HCV fragments.
3.8 Nested PCR amplification of HCV sequences
Lin et al (1992), Reid, (1994) and Mellor et al (1995) had reported the use of nested RT-PCR reactions for the detection of specific regions of HCV genomic RNA. This had been suggested a more stringent procedure than using a single round PCR amplification (personal communication, L Jarvis). Nested PCR uses first round PCR products as template DNA for a second round of amplification using a second set of primers designed to anneal within the initial PCR product (figure 3.8). Primer annealing events would be more specific, aided by an increased annealing temperature during the second PCR round.

Oligonucleotides Nest5 to Nest9 (table 2.2) were designed specifically to type 3a HCV using the NZL1 sequence. In this way, second round PCR products would use primers HCV4 to HCV9 and therefore retain the encoded endonuclease restriction sites for use in later cloning experiments. Nested oligonucleotides were observed as pure bands when visualised by PAGE analysis and therefore were purified by lyophilisation.

Nested RT-PCR reactions were carried out to amplify HCV fragments 1 and 2 but did not result in the expected products. Repeatedly, HCV fragment 3 PCR products were successfully amplified (figure 3.11) and these were used in cloning experiments described further in section 3.9.

3.9 HCV fragment 3 amplification and cloning

3.9.1 Fragment 3 amplification and purification
HCV genomic RNA was reverse transcribed using AMV-RT with Nest9 as the reverse primer. cDNA was amplified by PCR with KlenTaq DNA polymerase and Nest7 as the forward primer using the PCR reaction profile shown in figure 3.2 with an annealing temperature of 50°C. 5µl of the PCR product was then used as template DNA for a second round PCR reaction using HCV7 and HCV9 as the two primers. PCR products were visualised by electrophoresis through an 0.8% agarose gel in 1x TAE buffer. A discrete band was observed at the expected size (830 bp) as shown in figure 3.11. Mineral oil was removed from the PCR reaction mix and DNA recovered by phenol chloroform extraction followed by ethanol precipitation. A negative control nested RT-PCR reaction substituting H2O for AMV-RT proved there to be no contamination of the reactions.
precipitation. A negative control nested RT-PCR reaction substituting H$_2$O for AMV-RT proved there to be no contamination of the reactions.

**Figure 3.11** HCV fragment 3 amplified by nested RT-PCR.

Second round PCR products of HCV fragment 3 amplified by RT-PCR from type 3a virus using nested primers Nest7/Nest9 and HCV7/HCV9 with KlenTaq DNA polymerase. 0.1 volume PCR mix loaded onto 0.8% agarose gel in 1x TAE buffer. Lane 1 = test reaction, lane 2 = negative RT-PCR. Marker lane = pBluescript II SK+/Sau3AI. All DNA product sizes in bp.

3.9.2 Restriction digestion of cloning vector plus fragment 3 DNA
The total amount of fragment 3 PCR product was visually estimated from the band intensity when 0.1 PCR reaction volume was electrophoresed on the agarose gel. The band seen in figure 3.11 was estimated to contain 200 ng DNA in 5 µl PCR mix. Therefore 45 µl volume left contained 1.8 µg fragment 3 DNA. An equal amount (1.8 µg) of pBluescript II SK+ and fragment 3 DNA were both digested with 20 U Kpn I endonuclease (section 2.8.3). The enzyme was heat inactivated and DNA recovered by ethanol precipitation followed by digestion with 20 U Xho I. This second enzyme was heat inactivated and digested DNA products recovered from a 1% low melting point agarose gel (section 2.8.5.ii). Purified DNA species were resuspended in 10 µl H$_2$O and a 1 µl aliquot run on a 1% agarose gel in 1x TAE buffer. The amount of DNA present in each sample was estimated visually and the total amount DNA left in 9 µl calculated.

3.9.3 Fragment 3 DNA cloning
Ligation reactions between PCR product and double-digested vector DNA (c) were carried out as described in section 2.8.6. Controls for the levels of undigested vector (a) and single-cut vector that could simply be religated without insert DNA (b) were performed using 30 fmol vector DNA without or with T4 kinase respectively. 0.1 volume ligation reaction was used to transform electrocompetent DH5α cells (section 2.8.7.ii) and inoculated onto 2TY agar medium with ampicillin selection at 37°C.
overnight. Positive and negative transformation controls were also carried out. The resulting colony forming units observed from these transformations are summarised in table 3.5.

Table 3.5 Proportion of bacterial cells transformed by fragment 3 ligation reaction products.

<table>
<thead>
<tr>
<th>% volume transformed cells inoculated onto 2TY agar</th>
<th>Control ligation (a)</th>
<th>Control ligation (b)</th>
<th>Test ligation (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>89%</td>
<td>6 cfu</td>
<td>confluent</td>
<td>confluent</td>
</tr>
<tr>
<td>10%</td>
<td>0 cfu</td>
<td>&lt;200 cfu</td>
<td>&lt;300 cfu</td>
</tr>
</tbody>
</table>

A high level of religated vector without insert was observed in these transformation reactions that may have been due to incomplete endonuclease digestion of the vector. Bacterial cells transformed with vector plus insert ligation mix resulted in higher cfu than vector only though many would have resulted from any religated vector present. To check the fragment 3 bacterial transformants, 24 colonies were taken and plasmid DNA extracted by the Triton-boil mini prep procedure (section 2.8.8). Plasmid DNA was linearised by digestion with Xho I or Nde I and products visualised by agarose gel electrophoresis. Only vector DNA was observed, as all DNA species ran to 2.96 Kb.

The lack of successful ligations between vector and insert species of DNA and high proportion of control vector only transformants may have been due to incomplete endonuclease digestions resulting in compatible DNA ends. Dephosphorylation of digested vector DNA would probably have helped reduce the background level of vector religation though this was not done at the time. Cloning the fragment 3 PCR product and large DNA maxipreps of the clone grown up in bacterial cells would have gained sufficient DNA for multiple sequencing reactions in order to check this PCR product for HCV sequence. As attempts at fragment 3 cloning were unsuccessful, it was decided to directly sequence the PCR products to confirm the success of the PCR reaction itself. Each manual sequencing reaction requires around 9 µg template DNA. This would be more difficult to obtain for PCR products as multiple reactions are required from a limited amount of template RNA.
3.10 Direct sequencing of fragment 3 PCR products

Four PCR reactions were carried out using DNA from a first round RT-PCR reaction proven to amplify the expected species DNA. PCR products were extracted from the PCR mix by removal of the mineral oil followed by phenol chloroform extraction and ethanol precipitation. DNA was sequenced manually (section 2.8.10) using the PCR primers HCV7 and HCV9 as sequencing primers. No sequenced products were obtained upon PAGE analysis. There was not enough PCR product to repeat this and further attempts to amplify fragment 3 did not result in any discrete DNA product.

The failure to successfully sequence fragment 3 DNA could have resulted from the following,

i) There was insufficient DNA product to be sequenced.
ii) The DNA template was not sufficiently clean for a sequencing reaction.
iii) The primers were not sufficiently clean for use in a sequencing reaction.
iv) Primers may not have annealed to the DNA though they were the same primers used in the PCR reaction.
v) The amplified sequence was not that of HCV.

The reason for the sequencing difficulties was not resolved but instead, efforts were directed to amplify and clone the HCV structural protein sequence (fragment 1-3) spanned by the primers HCV4 and HCV9.

3.11 HCV fragment 1-3 amplification and cloning

3.11.1 Fragment 1-3 amplification and purification

Previous attempts to amplify HCV sequences from template RNA had been largely unsuccessful, fragment 3 being the exception. Initial problems with the extraction of HCV RNA from infected serum had been rectified and the use of RT and PCR reactions verified through the use of primers proven to anneal to type 3a HCV sequence. Fragment 3 amplifications had been proven to work, though sequence identity was not proven. KlenTaq DNA polymerase was proven to work for the amplification of HCV regions where Vent and Pfu did not. Therefore a system was in place for the amplification of the structural protein region of HCV.
As there was a limited amount of template RNA available, a system was required that maximised the production of cDNA from initial RNA in order to achieve maximum template for PCR amplifications. To this end, it was decided to use random hexameric oligonucleotide primers in the initial reverse transcription step. These consist of a range of oligonucleotides 6 nt in length and of random sequence. Complementary sequences on template RNA would occur more frequently than the sequences corresponding to the reverse primer Nest9, when fragment 1-3 was to be amplified by nested RT-PCR. This would result in an increased number of cDNA transcripts, some of which would have the potential to be amplified with primers 940 and Nest9. The use of random hexamers would therefore increase the chances of obtaining PCR products of HCV fragment 1-3.

Type 3a HCV RNA was reverse transcribed with AMV-RT using random hexamer primers and amplified using primers 940 and Nest9. Positive and negative controls for this RT-PCR reaction were also run. 0.1 volume product was amplified through a second round PCR using the primers HCV4 and HCV9 with Taq DNA polymerase. Final PCR products were visualised under UV light from a 0.8% agarose gel. Despite repeated attempts to amplify fragment 1-3 of HCV sequence, no amplified products were obtained corresponding to the 3157 bp band expected.

The KlenTaq DNA polymerase mix was then used in a similar reaction as reports had indicated processivity and DNA yields with KlenTaq are significantly higher than for amplification reactions using Taq only (section 3.7). Second round nested PCR reactions resulted in a smear of products. This was used as template for a third round of amplification using the primers HCV4 and HCV9 which resulted in either a ladder of DNA species (one of which occurred at the expected 3157 bp) or a distinct band of the correct size amongst a faint background smear (figure 3.12 and 3.13). Background DNA species probably resulted from non-specific products repeatedly amplified or as a result of specific HCV4 and HCV9 annealing to regions of the HCV sequence other than those expected. Second or third round PCR products were recovered from the reaction mix by removal of the mineral oil layer followed by phenol chloroform extraction and ethanol precipitation.
From third round PCR products, the band of interest corresponding to fragment 1-3 was gel purified for further cloning analysis. Any sequence mutations due to excessive amplification would be corrected according to the published sequence by site-directed mutagenesis once cloned DNA was stable in the vector.

Figure 3.12  PCR products from amplification of HCV fragment 1-3.

HCV fragment 1-3 nested RT-PCR products amplified using KlenTaq DNA polymerase mix. 0.1 volume PCR mix electrophoresed on a 0.8% agarose gel run in 1x TAE buffer. Lanes 1, 3 and 5 show negative RT-PCR controls where H2O was substituted for AMV-RT proving no contamination of reactions occurred. Lanes 1, 2 show first round PCR products using primers 940/Nest9. Lanes 3, 4 show second round PCR products using primers HCV4/HCV9. Lanes 5, 6 show third round PCR products using primers HCV4/HCV9. Marker ladder = λ HindIII.

Figure 3.13  Discrete band corresponding to HCV fragment 1-3 amplified by nested RT-PCR.

2nd round PCR products of HCV fragment 1-3 amplified by RT-PCR from type 3a virus using nested primers 940/Nest9 and HCV4/HCV9 with KlenTaq DNA polymerase. 0.1 volume PCR mix loaded onto 0.8% agarose gel in 1x TAE buffer. Lane 1=negative RT-PCR, lane 2=positive RT-PCR reactions. Marker lane = λ HindIII.
3.11.2 Restriction digestion of fragment 1-3 and vector DNA
pBluescript II KS+ was used as the initial cloning vector as the HindIII and XhoI restriction sites (encoded within the fragment 1-3 DNA) are conveniently situated for reverse orientation cloning of the HCV sequence. Hind III and Xho I were both used with REact 2 buffer.

The amount of fragment 1-3 PCR product was estimated visually by the band intensity of 0.1 volume reaction mix separated by electrophoresis. The total amount of PCR product was calculated for 45 µl reaction mix left. 2 µg each of vector and insert DNA were digested with 10 U Hind III at 37°C for 1 hour. Additional buffer mix was added together with a further 10 U Hind III to ensure complete digestion. DNA was recovered by phenol chloroform extraction followed by ethanol precipitation. 10 U Xho I was added with an appropriate volume buffer mix allowing DNA digestion for 1 hour. Again, an additional 10 U Xho I was added with appropriate buffer and final digested DNA products electrophoresed on a 1% LMP agarose gel for extraction of plasmid DNA plus the PCR product band running to 3157 bp. Final DNA pellets were resuspended in 10 µl H₂O and 1 µl of this run on a 0.8% agarose gel to confirm the initial extraction procedure and to estimate the amount of DNA in each sample.

3.11.3 Fragment 1-3 cloning
A ligation reaction was carried out between 30 fmoles vector DNA and 90 fmoles fragment 1-3 insert DNA as described in section 2.8.6. Controls for background religation reactions were carried out (section 3.8.3). 0.1 volume ligation reaction mix was used to transform electrocompetent DH5α cells followed by inoculation onto 2TY agar medium with ampicillin selection. Resulting colony forming units for these reactions are summarised in table 3.6.

Table 3.6 Proportion of bacterial cells transformed by fragment 1-3 ligation reaction products.

<table>
<thead>
<tr>
<th>% volume transformed cells inoculated onto 2TY agar</th>
<th>Control ligation (a)</th>
<th>Control ligation (b)</th>
<th>Test ligation (c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3 cfu</td>
<td>13 cfu</td>
<td>51 cfu</td>
</tr>
<tr>
<td>10</td>
<td>0 cfu</td>
<td>234 cfu</td>
<td>116 cfu</td>
</tr>
<tr>
<td>10</td>
<td>0 cfu</td>
<td>3 cfu</td>
<td>10 cfu</td>
</tr>
<tr>
<td>10</td>
<td>0 cfu</td>
<td>2 cfu</td>
<td>8 cfu</td>
</tr>
<tr>
<td>10</td>
<td>0 cfu</td>
<td>5 cfu</td>
<td>30 cfu</td>
</tr>
</tbody>
</table>
All fragment 1-3 ligated product colonies were subcultured in 2TY medium with ampicillin selection maintained. Vector DNA was extracted by the Triton-boil miniprep technique and resultant DNA digested with Hind III and Xho I to detect any successful DNA inserts. Digested products were visualised under UV light from an 0.8% agarose gel (figure 3.14). This double digestion would separate vector and insert DNA sufficiently separated for visual identification.

Figure 3.14 Fragment 1-3/pBluescript II KS+ DNA from transformed bacterial cells.

Hind III/Xho I double digested vector DNA extracted from transformed bacterial cell colonies 1 to 19 out of 54. Colony type 6 and 14 show the double bands expected. The lower band corresponds to pBluescript II KS+ DNA. The higher band corresponds to an insert species of DNA of 3150 bp expected for HCV fragment 1-3 PCR product. All other colony types contain vector DNA only. Marker ladder used was λHindIII DNA.

Of 54 colonies analysed, 5 resulted in a visible species of insert DNA around 3150 bp. As a further check, these were subjected to full digestion mapping using the endonucleases Xho I, Hind III, Kpn I and EcoR I. Upon visualisation from an 0.8% agarose gel, 3 looked to be the correct size for fragment 1-3 DNA. These 3 were grown up in 500 ml 2TY broth with ampicillin selection maintained. Vector DNA was extracted by the Triton-lysis maxiprep method (section 2.8.9) followed by full digestion mapping to ensure insert DNA had been maintained. Insert DNA was then sequenced manually (section 2.8.10).

Sequencing reactions using primers HCV4 and HCV9 did not yield any DNA products. Oligonucleotide primers T7 and T3 were also used in order to sequence across the cloning site and would therefore span the vector/insert junction. Initial sequencing of the vector would ensure primer annealing and sequence identification against the published DNA sequence for pBluescript II KS+. Sequencing reactions with T7 and T3 were
successful, initial compressions occurring were removed using additional Mn buffer and the diTP termination mixes. Insert sequences were analysed against the combined nonredundant sequence databases in the Basic Local Alignment Search Tool (BLAST) search engine located at the National Centre for Biotechnology Information (located at the site http://www.ncbi.nlm.nih.gov/BLAST/). All three cloned sequences matched regions of *Saccharomyces cerevisiae* and not HCV. The source of *S. cerevisiae* may have originated from the yeast tRNA carrier used in the initial RNA extractions or possibly as a result of bench-top contamination.

HCV fragment 1-3 amplification and cloning reactions were carried out 5 times, resulting in 11 molecular clones that appeared to contain the correct size of insert DNA. Upon sequencing across vector insertion sites, all clones turned out to contain sequences from *S. cerevisiae*.

The lack of HCV primer binding in the sequencing reactions may suggest that no HCV sequences were cloned though amplified products were obtained using these primers. A possible explanation may be that at some point, contaminant *S. cerevisiae* DNA was ligated to vector DNA instead of PCR products. Despite repeated attempts, no successful clones of RT-PCR amplified fragment 1-3 HCV sequence were obtained.

3.12 Discussion

At the time this project was started, one group had reported the creation, by RT-PCR cloning, of a full length genomic HCV clone propagated in Huh7 cells (Yoo *et al.*, 1995). HCV genomic RNA was transcribed *in vitro* and transfected into cell culture. However, both 5' and 3' termini of genomic HCV were not fully present which, later reports suggest, are necessary for virus replication. Yoo *et al.*, (1995) reported a very low level of HCV replication detected by the presence of both positive and negative strands of HCV genomic RNA, the negative replication intermediate strand proving *in vitro* replication of HCV. No report was made of HCV protein expression that would have confirmed virus replication.

Initial problems in obtaining HCV genomic RNA from infected serum were eventually resolved by the use of a virus pelleting procedure. The lack of an available genomic or part-genomic HCV clone hampered the confirmation of oligonucleotide specificity for the HCV genotype used.
Some time into the project, published data suggested the presence of six different HCV genotypes plus subtypes (section 1.4). Once the genotype for type 3a virus was published, it was possible to design specific oligonucleotide primers HCV4 to HCV9 and Nest5 to Nest9 that proved correct for the amplification of regions of HCV. Until this information was made available, primers had been designed to a best-fit consensus sequence which resulted in areas of mismatch between primer and type 3a HCV template.

There was also an initial problem in the lack of a positive control for any HCV RT-PCR reactions which was later resolved as both HCV type 3a RNA and primers 209/939 and 211/940 were obtained.

Amplification of HCV fragments often resulted in very low yields and smaller background DNA species from which the correct sized fragment had to be gel purified, decreasing the yield further. This may be a result of using the reverse transcriptase enzyme from Avian Myoblastosis virus which contains not only a reverse transcriptase but also some RNaseH activity. Gerard et al (1989) and D’Alessio et al (1990) carried out extensive analysis of RT enzymes that contained RNaseH activity, comparing these with an RNaseH deficient strain of Moloney murine leukaemia virus (M-MLV) reverse transcriptase. RNaseH was shown to significantly increase the degradation between primer and template RNA so reducing the number of primer/RNA hybrids available for cDNA synthesis. AMV-RT was also shown to pause at regions of secondary structure. At these points, RNaseH has an increased opportunity for cleavage of synthesised cDNA reducing the chances of reinitiation of cDNA synthesis at the pause point. This creates a population of smaller cDNA species that are amplified by PCR resulting in the background ladders and smears seen in the HCV amplifications. D’Alessio et al (1990) directly compared the activity of AMV-RT against the RNaseH negative species of M-MLV-RT (Superscript II RT, Gibco-BRL) proving the maximum length cDNA synthesised by AMV-RT was 2600nt with a lower yield than the 5600 nt cDNA synthesised by Superscript II RT.

Gerard et al (1989) went on to prove the increased activity of Superscript II RT at higher temperatures than could be used by AMV-RT. He suggested that at higher temperatures template RNA secondary structure is reduced so reducing the reverse transcriptase pause events, allowing longer cDNA
molecules to be synthesised more efficiently. This fact has subsequently been recognised by various groups that have successfully amplified and cloned long stretches of HCV genomic RNA using RNaseH deficient reverse transcriptase enzymes at an increased incubation temperature of 45°C to 48°C. The amplification of HCV fragments in this project involved the use of AMV-RT at 42°C. This could have been made more efficient by using an RNaseH deficient RT enzyme at an increased, optimised incubation temperature.

Despite the difficulties initially encountered, amplified products from template HCV RNA of the correct size were obtained and purified from background species. Very few clones with inserts of the correct size were obtained, none contained HCV sequence. Background plasmid religation events could have been reduced by the dephosphorylation of digested ends. A low level of successful transformants was also found by Yanagi's group (Yanagi et al., 1997) in their efforts to amplify and clone the entire HCV genome. They found an increased yield was obtained when transformed bacterial cells were incubated at 30°C for 20 hours instead of the usual 16 hours at 37°C. Forcing bacterial cells to grow at a slower rate reduces the rate of transformed plasmid replication and therefore the chance of leaky expression of HCV proteins encoded by the insert DNA. Aizaki et al. (1998) similarly ensured a higher bacterial transformant yield by using a low copy number plasmid, pBR322 in a recombinant deficient strain of E.coli, DH10B. Such practices may have increased the yield of transformed bacterial cells in this project though the successful amplification of HCV sequence was not conclusively determined.

Though Yoo et al. (1995) did not conclusively prove virus replication in mammalian cell culture, other groups have since reported the cloning of the HCV open reading frame and the entire genome, including the full 5' and 3' UTR regions. In vitro transcribed HCV RNA was transfected to cell culture or in vivo into chimpanzees, at all stages proving virus replication occurs with the production of virus proteins and progeny virus.

In 1995, Mizuno et al reported the cloning of full length type 1b HCV and successful expression of all virus proteins when cotransfected with recombinant vaccinia virus in HeLa G cells. Unfortunately virus expression did not seem to persist as cell morphology altered due to the presence of vaccinia virus. Honda et al (1996) also reported the expression
of HCV proteins in vitro from the molecular clone pMN2-1G encoding almost the complete HCV type 1b sequence but lacking the 3'X tail reported by Tanaka et al (1995). In vitro transcribed virus RNA was proven to express core, E1 and E2 proteins when translated in a rabbit reticulolysate system. Huh-T7 cells transfected in vitro with the same clone were also proven to express core, NS3, NS4 and NS5 virus proteins. Though Huh-T7 cells had been cotransfected with recombinant vaccinia virus to increase T7 polymerase expression, there was no report of either long term virus protein expression or altered cell morphology due to vaccinia virus, as reported by Mizuno et al (1995). From Honda’s report, the molecular clone pMN2-1G was successful in expressing the majority of HCV proteins from though long term propagation and detection of virus like particles was not reported.

This was followed by the work of Kolykhalov et al (1997) and Yanagi et al (1997 & 1998) who reported the successful creation of infectious full length HCV clones. Kolykhalov et al proved that in vitro transcribed RNA from their clone, when transfected in vivo into naive chimpanzees, caused persistent hepatitis showing all symptoms associated with hepatitis C virus infection. Virus extracted back from chimpanzee plasma was found to contain HCV sequences identical to the original clone. In a similar fashion, Yanagi et al cloned the full length sequence to HCV genotype 1a (1997) and 1b (1998). Sequences were confirmed against an appropriate consensus sequence and in vitro transcribed RNA from both clones transfected in vivo into naive chimpanzees caused a hepatitis disease. These reports have so far not described any cell culture propagation which must be an important advancement for further study of HCV as chimpanzees offer limited scope for any molecular analysis of the virus.

These infectious full length genomic clones of HCV have greatly advanced the potential for analysis of HCV, especially if they are proven to infect and propagate in mammalian cell culture. However, these clones remain potentially infectious for handling in the laboratory, a hazardous prospect. Of greater advantage would be a clone encoding the HCV open reading frame that expressed all virus proteins so producing virus like particles in cell culture, as would be obtained from infectious clones, without the inherent risk of infection. Such a clone would be invaluable for molecular analysis of specific sequence mutations, identification of the replicative
cycle and the analysis of anti viral compounds directed against specific virus proteins.

Such a molecular clone was constructed by Rispeter et al (1997) who amplified and cloned the entire open reading frame of HCV in two long sections, piecing these together sequentially. *In vitro* transcribed RNA was then translated using a cell free rabbit reticulolysate system resulting in the expression of 7 HCV proteins corresponding to C, E1/E2, NS2, NS3, NS4a/b, NS5a and NS5b. If further analysis proved these proteins to be fully functional and the plasmid, or a suitable derivative was expressed in mammalian cell culture then the goal of a non-infectious HCV clone producing VLPs in cell culture would be achieved.

Aizaki et al (1998) and Moradpour et al (1998) have now achieved this through the construction of full length HCV clones by fragment nested RT-PCR of virus extracted from HCV infected plasma. Expression of the HCV open reading frame was proven by Aizaki et al (1998) when the clone was transfected to both insect and mammalian cells. All 9 HCV proteins were detected with NS3 and NS5b proven to be fully functional. The tetracyclin-regulated transgene of full length HCV sequence reported by Moradpour et al (1998) was proven to be fully transcribed, HCV structural and non-structural proteins expressed and found localised to the cellular endoplasmic reticulum. The development by Moradpour et al (1998) of a continuous human cell line expressing HCV proteins under external controls has undoubtedly opened the door for more extensive analysis of HCV and its constituent proteins than has been achieved so far.

Due to financial and time constraints and in view of recent advances with the production of infectious clones of HCV, it was decided to pursue another line of investigation concerning the pathogenesis of HCV. This involved analysis of the immune response elements to HCV infection, namely 2′5′oligoadenylate synthetase and the dsRNA-induced protein kinase (PKR) in response to interferon therapy undergone by chronically infected HCV patients.

Though the development of an HCV VLP system was unsuccessful, the techniques employed in this investigation were developed for use in later RNA extraction and cloning procedures for the development of a quantitative PKR mRNA assay described in the next chapter.
Chapter 4

Development of a quantitative assay for PKR mRNA
4.1 Introduction
The aim of these experiments was to develop an assay in which the level of PKR and 2’5’oligoadenylate synthetase (2’5’oligoA) mRNA expression could be quantified in human liver from patients chronically infected with hepatitis C virus. The development of an assay to measure the level of protein expression was also studied and is the subject of chapter 5.

Quantified levels of PKR and 2’5’oligoA mRNA would then be correlated with levels of protein expression and compared with patient data (including their biochemical, histological and virus status data). If there proved to be a correlation between pre-treatment levels of PKR and 2’5’oligoA and patient response to treatment, then these two immune effectors could be used as predictive factors in identifying patients who would respond to treatment from those who would not.

At the time these experiments were carried out, 2’5’oligoA and PKR mRNA and protein expression had only been measured in terms of their enzyme activities (Okuno et al, 1991; Okuda et al, 1994; Grander et al, 1996; Pawlotsky et al, 1996). No direct assays to quantify the specific amount of mRNA and protein had been reported, especially in connection with hepatitis C virus infections.

4.2 Patient samples
PKR and 2’5’oligoA are both activated by dsRNA in virus infected cells, once induced by type I interferons (section 1.7). Interferon is given as a therapy for chronic HCV infection with the aim that additional exogenous interferon would boost the patient’s own antiviral immune system.

Zignego et al (1992), Cribier et al (1995) and Radkowski et al (1998) had reported the presence of HCV in peripheral blood mononuclear cells (PBMCs) though replication of HCV in PBMCs remains a controversial subject. Though obtaining samples of patient PBMCs and extraction of their cellular RNA and protein would not be too difficult, for these experiments it was decided to analyse expression of PKR and 2’5’oligoA in liver biopsy samples from HCV infected patients. The liver has been identified as the target organ of HCV with the virus thought to replicate mainly in hepatocytes and therefore this would give an accurate level of PKR and 2’5’oligoA acting at the site of infection.
Liver biopsies intended for this study were taken from HCV infected patients at the same time that biopsies were performed for histological analysis. 24 hours previously, blood was taken from these patients for virus RT-PCR status and biochemical (alanine aminotransferase (ALT) level) analyses. Thus the level of PKR or 2′5′oligoA could be directly compared against other patient factors measured from blood samples taken at the same time. As the presence of virus (and therefore double stranded RNA replication intermediates) activates PKR and 2′5′oligoA, quantification of these two interferon effectors, in light of patient response to therapy, may provide information concerning the effectiveness of the patient’s antiviral system and thus the effectiveness of any interferon therapy given.

Grander et al (1996) reported the analysis of 2′5′oligoA activity in liver cells taken from chronic HCV infected patients. However, cells from liver biopsy samples were kept in culture for at least 24 hours, some of these samples exogenously induced with IFN and would therefore not show accurate analysis of 2′5′oligoA expression within the patient’s body.

HCV is assumed to infect hepatocytes within the patient’s liver. Perfusion of the liver is generally considered the best method to acquire hepatocytes (Berry et al 1991) but this technique cannot be performed on humans. Acquisition of biopsy samples was therefore the method by which liver tissue was obtained for analysis of constituent 2′5′oligoA and PKR. Culturing hepatocytes from liver biopsies can often result in up to 90% loss of cells (personal communication A. Keogh, University of Birmingham). Cell morphology is also known to change within the first 5 days of culture which results in changes to the expression of cellular proteins (Berry et al 1991). The time taken to separate and culture these liver cells would possibly result in changes to the level of PKR and 2′5′oligoA mRNA and protein. As the level of expression of these two effectors at the time of sampling was the aim of these experiments, it was decided to extract total cellular RNA and protein directly from the liver biopsy sample.
Liver biopsies were taken from consenting patients by trained doctors using a biopsy needle (typically an 18 gauge x 1.5 cm Temno Biopsy Needle, Bauer Medical International). Biopsies taken at the Queen’s Medical Centre, Nottingham had been stored at -80°C and were donated by Dr. W. Irving as part of the Trent HCV Study Group. Biopsies taken at the Leicester Royal Infirmary were halved, each snap frozen in liquid nitrogen in cryofreezer vials (Costar corporation, Cambridge, MA). One half of each biopsy was used for this study.

A sensitive and accurate quantitative assay was required in which the level of PKR and 2’5’oligoA mRNA could be quantified. Northern analysis of specific mRNAs was considered to lack sensitivity with increased chances of signal loss during RNA to membrane transfer (Davis et al, 1997). An RNase protection assay was therefore chosen as a sensitive mRNA detection technique that can be used quantitatively. Initial experiments were carried out to detect PKR mRNA. Once the assay was proven to work for PKR mRNA it would then be extended to analyse and quantify 2’5’oligoA mRNA from cellular extracts.

4.3 Development of an RNase protection assay for detection of PKR mRNA

The use of an RNase protection assay was investigated for the detection and quantification of PKR mRNA from human cells. Figure 4.1 describes this assay in which an in vitro synthesised RNA probe with an incorporated radiolabel is used to hybridise and detect complementary target mRNA. As a control for this reaction, a constitutively expressed gene transcript, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), would also be assayed. By comparing the band intensities of protected PKR and GAPDH hybrid products, the level of PKR mRNA in the original sample of cellular RNA could be quantified as a proportion of the amount of GAPDH mRNA present. As the level of GAPDH expression would be known, so then the amount of PKR mRNA could be calculated.
Excess radiolabelled antisense probe (F7xl) hybridises to complementary sequences on target mRNA.

Digestion of single stranded RNA by S1 or Mung Bean nuclease leaving hybrids of probe+target mRNA.

Hybrid products separated by denaturing PAGE.

Hybridised products run smaller than the original probe due to loss of the vector derived non-complementary overhang.

A manual sequencing reaction of pBluescript II SK+ is used as the marker ladder as each band is 1nt from the next.
A purified radio labelled negative sense RNA probe was synthesised from the plasmid pNB 92/020. A 510 nt region of PKR spanning the 5' end of the gene had been cloned into the Bam HI/Pst I sites of a pGEM1 vector (work carried out by N. Burley, University of Leicester). pNB 92/020 was linearised with SstI, blunt ended using Klenow DNA polymerase I (section 2.8.4) and gel purified from a 1% LMP agarose gel (section 2.8.5.i). Linearised plasmid DNA was transcribed \textit{in vitro} from the SP6 RNA polymerase site incorporating a $\alpha^{32}$P-dUTP label (section 2.9.6). This resulted in a 578 nt radio labelled antisense RNA probe of which 510nt were complementary to PKR sequence. Figure 4.2 shows the plasmid pNB 92/020 and its use in the synthesis of probe RNA.

\textbf{Figure 4.2} \textit{In vitro} synthesis of probe RNA from pNB 92/020

Transcribed RNA products were visualised by agarose gel electrophoresis (figure 4.3). Smaller RNA species were observed that would have resulted from incomplete transcriptions of the template. This is known to be a problem in transcripts over 300nt in length (Ausubel \textit{et al}, 1994).
In vitro transcribed probe RNA from pNB 92/020 was boiled in denaturing formamide loading buffer and RNA products separated by electrophoresis through a 2% agarose gel in 1x TBE buffer. Marker ladder used was φx174/HaeIII DNA.

Sephadex G25 column purification did not purify the 578 nt probe transcript sufficiently. It was thought that contaminant RNA species present, as they contained complementary sequence to PKR mRNA, might also hybridise to target mRNA resulting in a population of incorrect sized products upon endonuclease digestion. PAGE purification of transcribed probes was therefore used to eliminate all species of RNA other than the probe.

PAGE purified PKR probe was used in RNase protection assays with 1 to 100 µg total RNA extracted from Hut78 or CEM4 cells as described in section 2.9.6. Hybrid products were visualised by PAGE separation and exposure to autoradiography film. Decreases in the amount of original probe were observed though no smaller protected products were seen. A large background smear was present in all lanes suggesting this originated from small transcripts carried over from the in vitro transcription reaction. As the same amount of probe was used in each reaction, the difference between the amount of original and hybridised probe may suggest some probe digestion was occurring.

In view of these difficulties, an oligo protection assay was then tried in which a 5' end labelled synthetic oligonucleotide is used instead of an in vitro transcribed RNA. This would reduce the difficulties encountered in obtaining sufficient amounts of purified probe RNA synthesised in vitro.
4.4 Development of an oligo protection assay for PKR mRNA

This assay is based on the RNase protection assay system and has been successfully reported by Tanay et al (1997) (figure 4.1). Synthesis of an antisense probe to the target sequence is replaced by using a 5' end labelled antisense oligodeoxynucleotide primer with a α32P-dATP radiolabel using T4 polynucleotide kinase (section 2.8.2). Specific activities of end labelled probes would be reduced compared to an incorporated radio labelled probe as only one labelled nucleotide is added per oligonucleotide molecule.

In order to develop this system for the quantification of PKR mRNA, primers to both PKR and an endogenous housekeeping gene transcript were synthesised for use as DNA probes to the target RNA species. Glyceraldehyde 3-phosphate dehydrogenase was chosen as the endogenous control target RNA as it is known to be expressed at constant levels (Edwards & Denhardt, 1985). The band density for any PKR hybrid products would be compared against the band densities of GAPDH hybrid products. As the amount of GAPDH mRNA per μg total cellular RNA is known, the proportion of PKR to GAPDH signal can be calculated and thus the amount of PKR mRNA evaluated.

Oligodeoxynucleotides huPKR and huGAPDH were synthesised (table 2.2) in which the 3' terminus was complementary to a sequence of nucleic acid known to be absent from the laboratory, namely Ebola virus. This ensured no contamination of hybridisation reactions could occur during the assay. In protection assay reactions, the 5' half of the probes (42nt) would hybridise to either PKR or GAPDH leaving the 3' end unhybridised and therefore unprotected from digestion with Mung Bean or S1 endonuclease. Protected labelled probe hybrids would run as smaller bands from the original probe when separated by PAGE analysis.

PAGE analysis of both huPKR and huGAPDH revealed a ladder of premature termination products of each oligonucleotide (figure 4.4) which would be expected during the synthesis of such long sequences. Oligonucleotides were therefore gel purified (section 2.8.1), the largest band taken as full length sequence.
Figure 4.4 Denaturing PAGE analysis of \(^{32}\)P labelled probes huPKR and huGAPDH.

100 \(\mu\)g each oligodeoxynucleotide was loaded onto an 8 M urea/10% polyacrylamide gel. Lane 1 shows huPKR and lane 2 shows huGAPDH.

Low yields of each oligonucleotide were obtained, removal of smaller premature termination products accounting for the major losses. Table 4.1 shows typical yields obtained.

Table 4.1 Typical yields of PAGE purified huPKR and huGAPDH

<table>
<thead>
<tr>
<th>Oligodeoxynucleotide</th>
<th>Amount loaded onto PAGE gel</th>
<th>Amount recovered</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>huPKR</td>
<td>100 (\mu)g</td>
<td>5.4 (\mu)g</td>
<td>5.4 %</td>
</tr>
<tr>
<td>huGAPDH</td>
<td>100 (\mu)g</td>
<td>1.8 (\mu)g</td>
<td>1.8 %</td>
</tr>
</tbody>
</table>

Purified oligodeoxynucleotides were end labelled using either method (a) or (b) as described in section 2.9.7.i, specific activity yields are shown in table 4.2. Method (b) clearly resulted in higher specific activity of probe products.

Table 4.2 Typical specific activities for end labelling huGAPDH by two different methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>pmoles oligo used</th>
<th>Volume probe</th>
<th>Average cpm/(\mu)l</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>36.76</td>
<td>260 (\mu)l</td>
<td>12323 cpm/(\mu)l</td>
<td>8.7 \times 10^4 cpm/pmol</td>
</tr>
<tr>
<td>b</td>
<td>10.0</td>
<td>222 (\mu)l</td>
<td>28524.5 cpm/(\mu)l</td>
<td>6.3 \times 10^5 cpm/pmol</td>
</tr>
</tbody>
</table>
Oligo protection assays were carried out using $5 \times 10^5$ cpm probe, each with 25 μg total RNA from Hut78 cells. Titrations of Mung Bean and S1 endonuclease, hybridisation temperatures and formamide concentrations were used in order to optimise assay conditions as follows,

- 0 units (negative control), 1U, 20U, 50U, 100U, 200U, 300U and 400U Mung Bean and S1 endonuclease titration was used to assess the amount of endonuclease required.

- Hybridisation temperatures of 37°, 50°C, 55°, 57°C, 60°C and 77°C were used.

- Formamide at 20%, 40%, 60% and 80% concentrations used to alter the extent of template mRNA secondary structure melting for efficient RNA-DNA hybridisations.

$5 \times 10^5$ cpm probe was left unhybridised (no RNA in reaction) and digested with 50 U endonuclease as a control to show enzyme digestion was occurring. An additional reaction tube contained neither cellular RNA or endonuclease which showed the original probe for comparisons with hybridised products.

Hybridisation reactions using huPKR and huGAPDH did not result in expected protected products though background ladders were observed. These may have originated from incomplete PAGE purification of the probes. If the amount of endonuclease used was below optimum levels, unhybridised probes may not have been fully digested, leaving this ladder of products.

It transpired that both huPKR and huGAPDH had been synthesised in the reverse orientation and were therefore the same sequence as their target mRNA which would explain the lack of hybrid products. In order to ensure this assay could be optimised correctly, it was decided to concentrate efforts using just the probe for GAPDH as this was thought to be the more abundant transcript in cellular RNA. Once conditions were optimised, detection and analysis of the less abundant PKR mRNA would then be carried out. The oligodeoxynucleotide GAPDH2 was synthesised in the correct orientation containing sequence complementary to GAPDH mRNA. Oligonucleotide synthesis added a 3' dimethoxytrityl label to aid column purification (section 2.8.1.iii). Premature termination products could not be completely eliminated but were sufficiently removed from the full length probe DNA.
Purified GAPDH2 was 5' end labelled with γ²P-dATP and used in oligo protection assay reactions as described above, using a range of hybridisation temperatures and concentrations of S1 nuclease and formamide. Some background product smears were observed. Smearing was reduced by the addition of 0.2 M NaOH (incubated at 37°C for 30 minutes) to S1 nuclease digested products prior to the final ethanol precipitation.

It was possible that RNA-DNA hybrid products were lost at the precipitation stages. This was reported by Davis et al (1997) who analysed sample loss during an RNase protection assay. Up to 86% loss of sample occurred after the second precipitation stage, final losses accumulating to 95% during the assay procedure. This may also have happened in the oligo protection assay used here. Salmon sperm DNA was added as a carrier to aid yields from precipitation steps. No difference was observed between the intensity of product bands precipitated with or without carrier DNA. It was noted however, that final reaction products were very difficult to resuspend in formamide loading buffer. RNA-DNA products may have been lost as RNA is known to stick to the walls of the tubes used. Coating tubes with silicone did not overcome this problem, confirmed by Tanay et al (1997). Conditions for an oligo protection assay using GAPDH2 probe were optimised resulting in the detection of protected hybrid (figure 4.5).

Figure 4.5 shows the products from a successful oligo protection assay in which the amount of labelled GAPDH2 probe has been significantly reduced as a result of hybridisation to complementary sequences from total cellular RNA. The protected DNA probe products can be seen as a faint band at 42nt. From similar reactions, it was deduced that optimum hybridisation conditions used at least 25 μg total cellular RNA for GAPDH detection, hybridisation temperatures of 55°C to 57°C and 40% deionised formamide, followed by single strand nucleic acid digestion with 20 U S1 endonuclease. No carrier DNA was used though digested products were denatured with 0.2 M NaOH.
This oligo protection assay for the detection of GAPDH mRNA was successful. A protected nucleic acid of the correct size was observed. To achieve these results, a minimum of 25 μg total cellular RNA was required. Detection of PKR mRNA, which is assumed to be less abundant than GAPDH, may require substantially more cellular RNA in each assay reaction. On average, 16 μg total cellular RNA was extracted from a half sized liver biopsy sample (section 4.9.5) which is clearly not enough for even one oligo protection reaction, let alone several reactions if repeats were required.

RNase and oligo protection assay systems did not have the degree of sensitivity necessary for detection of PKR mRNA from small amounts of tissue samples. Therefore a more sensitive technique was required. Where protection assays only detect the amount of mRNA present in a sample of cellular RNA, amplification of these transcripts using a reverse transcription coupled PCR reaction (RT-PCR) would allow an increase in detection sensitivity and would therefore require less cellular RNA in the assay.
4.5 Detection of PKR and 2’5’oligoA mRNA by RT-PCR

RT-PCR assays are known for their high sensitivity, Klaassen et al (1998) reporting the detection of a human keratinocyte-specific E48 gene mRNA transcript from just 5 cells. Oligodeoxynucleotide primers were designed against the known sequences of both PKR and 2’5’oligoA. Upon PAGE analysis all primers were observed as pure bands and were therefore purified by lyophilisation from original stocks. Primers PKR1 and PKR2 amplified a 303 bp region within the kinase domain of PKR. Primers 2’5’A1 and 2’5’A2 were designed to amplify a 402 bp region of 2’5’oligoA towards the mRNA 3’ terminus.

A pET16b plasmid (figure 2.3) was available that had the full length sequence for PKR inserted in the Nde I/Bam HI restriction sites (a gift from E. Pollit, University of Leicester). This was used to prove that PKR1 and PKR2 annealed to the correct PKR sequences. 20 ng plasmid DNA was used as template in a PCR reaction (section 2.8.11) using Taq DNA polymerase for 30 cycles. Optimisation of conditions resulted in the following PCR reaction parameters being used for all PKR amplifications.

2 mM MgCl$_2$, 500 µM dNTPs.
Template denaturing at 92°C for 30 seconds
Primer annealing at 57°C for 30 seconds
Primer extension at 72°C for 30 seconds

A single band at 303 bp was obtained from PKR amplifications as expected. 20 ng pET16b/PKR was therefore used as a positive PCR control for subsequent amplification reactions. A negative control using H$_2$O as template was also used. No molecular clone of 2’5’oligoA was readily available for PCR optimisation reactions. Instead, human cellular DNA was obtained from Jurkat, CEM4 and C8166 cell lines (extracted by A.Lau, University of Leicester). PCR reactions were expected to amplify the genes for PKR and 2’5’oligoA. Figure 4.6 shows PCR reaction products for PKR and 2’5’oligoA from Jurkat cellular DNA. Expected 303 bp DNA products were obtained for PKR amplifications, however, 2 bands at around 700 bp and 1500 bp were obtained from 2’5’oligoA amplifications that were larger than the expected 402 bp.
Figure 4.6 PKR and 2'5'oligoA PCR products.

500ng Jurkat cellular DNA used as template in PCR amplification using primers PKR1/PKR2 (lanes 1-5) and 2'5'A1/2'5'A2 (lanes 6-10). Taq DNA polymerase was used with an MgCl\(_2\) titration. Lanes 1 and 6 show negative control reactions (no contamination present). Lanes 2/7 (1 mM MgCl\(_2\)), lanes 3/8 (2 mM MgCl\(_2\)), lanes 4/9 (3 mM MgCl\(_2\)) and lanes 5/10 (4 mM MgCl\(_2\)). Marker ladder used was φx174/HaeIII DNA.

Given that PKR and 2'5'oligoA primers were proven to anneal to template DNA, RT-PCR reactions were carried out using AMV-RT and were optimised for a single tube RT-PCR reaction (section 2.9.9). A negative RT control was used in all reactions substituting H\(_2\)O for AMV-RT. Total cellular RNA was extracted from CEM4 cells using the RNAzol B method (section 2.9.2) in which all RNAses are inhibited and removed by guanidium thiocyanate in the reagent. RNA was quantified by measuring its absorbance at 260nm. Sufficient RNA was extracted to analyse its quality by agarose gel electrophoresis. 2 μg total RNA was boiled in denaturing formamide loading dye and electrophoresed through a 1% agarose gel. The presence of intact 18S and 28S ribosomal RNA bands indicated no RNA degradation had occurred (figure 4.15 shows an example of total human RNA).

Despite repeated attempts, no PCR products were observed for 2'5'oligoA which might suggest levels of the expressed gene were either too low or non-existent for detection by even the sensitive technique of RT-PCR. 2'5'oligoA activities had been analysed from serum, peripheral blood mononuclear cells and liver extracts from HCV infected patients (Okuno et al., 1991; Okuda et al., 1994; Grander et al., 1996; Pawlotsky et al., 1996). However, the level of 2'5'oligoA mRNA or protein expression in human cells had not been reported. The reason why the 402 bp PCR product was not obtained was not found.
At this point, it was decided to concentrate on the detection and quantification of human PKR in patients chronically infected with HCV.

Products at 303 bp were obtained for PKR RT-PCR reactions indicating that PKR is expressed at sufficient levels for detection by amplification. At all stages, positive PCR reactions using the pET16b/PKR plasmid gave a band of the appropriate size at 303 bp. Initially, all negative control reactions showed no contamination of PKR. However, contamination was later observed and despite changing stocks of all reaction components, the source for this contamination was not found.

As the laboratory these reactions were carried out in had been used for the molecular analysis of PKR for many years, it was thought the laboratory itself was a possible source of PKR contamination.

4.6 Eliminating contamination of PKR in RT-PCR reactions

Previous amplification reactions of PKR mRNA by RT-PCR using primers PKR1 and PKR2 had been contaminated with PKR DNA that could not be eliminated. Ultraviolet irradiation of RT-PCR reactants (buffers, MgCl₂, H₂O, dNTPs and mineral oil) did not remove the contamination (thought to originate from the laboratory). To overcome this, all reactions were carried out in a different laboratory that had no contaminant PKR nucleic acid, as was evident in the previous working area. All equipment and reagents used were taken from unopened stocks that had no contact with the previous laboratory. The equipment used for PCR amplifications was located in the original laboratory but at this point, reaction tubes had been sealed. A hot start was not employed as such a short region was to be amplified which also reduced the contamination risk.

All subsequent amplifications of PKR mRNA from total cellular RNA proved to work well with all negative controls remaining negative. The problem of contamination had been solved.

Initial RT-PCR reactions used RNA extracted from Hut78 cells, a T-cell line. CEM4 and Chang cells were also tested for the presence of PKR mRNA to assess whether different human cell types also expressed PKR mRNA, even at low levels. All cell types used showed the presence of PKR message.
4.7 Competitive RT-PCR for PKR by co-amplification with either GAPDH or β-actin mRNA

As the aim of these experiments was to assay the level of expression of PKR mRNA, the next step was to analyse how a quantitative system could be set up. RNase and oligo protection assays previously tried were based on the fact that the level of PKR mRNA signal could be compared to that of a constitutively expressed housekeeping gene, GAPDH. In the same way, the level of RT-PCR amplified PKR mRNA could be compared to and quantified against a known amount of a gene transcript such as GAPDH or β-actin.

Oligodeoxynucleotide primers were available that would amplify a 284 bp region of GAPDH (GAP1 and GAP2) and a 625 bp region of β-actin (B-Act2 and B-Act3) (table 2.2). No positive DNA controls were available for GAPDH or β-actin. RT-PCR reactions for all three mRNA species were carried out using 500 ng total RNA extracted from Hut78 cells. All three sequences were amplified correctly.

To remove tube to tube variation in a quantitative assay, it was necessary to co-amplify PKR with either GAPDH or β-actin. RT-PCR reactions were carried out to optimise co-amplification conditions. PKR and GAPDH were co-amplified but the bands were so close upon separation by agarose gel electrophoresis that identification and therefore quantification would be difficult (figure 4.7). More success was achieved with co-amplification of PKR and β-actin mRNA (figure 4.8). The two PCR products were well separated by electrophoresis allowing easier detection and quantification.
Figure 4.7 Co-amplification of PKR and GAPDH mRNA

0.1 volume RT-PCR products separated by electrophoresis through a 2% agarose gel. 500 ng total cellular RNA was amplified with primers PKR1/PKR2, GAP1/GAP2 or both together. Lanes 2, 4 and 6 show negative RT-PCR reactions. Lane 7 shows a positive PCR control for PKR using 20 ng pET16b/PKR plasmid DNA while lane 8 shows the negative PCR control reaction. Lane 1 shows positive RT-PCR reaction using GAP primers only. Lane 3 shows positive RT-PCR reaction using PKR primers only. Lane 5 shows RT-PCR products for both PKR and GAPDH using both primer sets. Marker ladder used was φx174/HaeIII DNA.

Figure 4.8 Co-amplification of PKR and β-actin mRNA

0.1 volume RT-PCR products separated by electrophoresis through a 2% agarose gel. 500 ng total cellular RNA was amplified with primers PKR1/PKR2, B-Act2/B-Act3 or both together. Lanes 2, 4 and 6 show negative RT-PCR reactions. Lane 7 shows a positive PCR control for PKR using 20 ng pET16b/PKR plasmid DNA while lane 8 shows the negative PCR control reaction. Lane 1 shows positive RT-PCR reaction using β-actin primers only. Lane 3 shows positive RT-PCR reaction using PKR primers only. Lane 5 shows RT-PCR products for both PKR and β-actin using both primer sets. Marker ladder used was φx174/HaeIII DNA.
4.8 Quantitative competitive RT-PCR for PKR mRNA using an in vitro transcribed internal standard control RNA

Though RT-PCR reactions using a co-amplified species of mRNA were successful, the level of PKR mRNA quantified by this method would at best be given in terms of the level of the housekeeping gene message. Edwards & Denhardt (1985) reported that GAPDH was expressed at a constant level in human cells. This indicated GAPDH would be suitable as an endogenous cellular transcript control against which PKR mRNA could be quantified. Later reports by Calvo et al (1997) and Yamagata et al (1998) suggested expression of GAPDH varies between different cell types and therefore, would be too variable as a quantitative assay control.

To overcome this problem, Wang et al (1989) demonstrated the use of a competitive RT-PCR assay using an external transcript. The specific amount of a purified species of mRNA (or DNA) would be known, thus the final amount of target mRNA could be calculated and expressed in terms of number of molecules or copies of mRNA.

An extension of this assay was developed by Becker-Andre and Hahlbrock (1989) resulting in a quantitative competitive RT-PCR assay used here. An in vitro transcribed internal standard control (ISC) species of RNA could be purified and quantified accurately. This RNA is identical in sequence to the target (PKR) mRNA but possesses an additional short sequence inserted between the two primer binding sites such that the final PCR product could be separated by size from the target product (figure 4.11). A direct competition occurs between both ISC and target RNA for all RT-PCR reactants, including primers, which reduces tube to tube variations and variations between primer annealing efficiencies. As long as amplifications to not exceed their exponential phase, direct comparisons can be made between target mRNA and ISC RNA amplified. When the ratio of ISC to target products equals 1, the level of target mRNA can be equated to the amount of ISC RNA amplified. This technique has been used by Clontech in their PCR MIMIC™ assay, available commercially.
4.8.1 Molecular cloning of an ISC plasmid

The plasmid pNB 92/008 (figure 4.10) was given by Dr S Heaphy, University of Leicester. This had the full length sequence for PKR cloned into the Bam HI to Pst I endonuclease restriction sites of a pGEM-1 vector such that in vitro transcription from T7 gave a positive sense strand of PKR RNA.

A convenient unique Afl II restriction site lay between the PKR1 and PKR2 primer binding sites. pNB 92/008 was digested with Afl II, linearised digested ends blunted using Klenow polymerase (section 2.8.4) and the product gel purified (section 2.8.5.i). A 79 bp stuffer fragment of DNA was created by Hae III digestion of pBluescript II SK+. Hae III creates blunt ends of DNA for easier ligation. The specific 79 bp piece was gel purified from all other digested fragments by electroelution (section 2.8.5.ii). Linearised pNB 92/008 and the stuffer fragment were blunt end ligated (section 2.8.6) and used to transform electrocompetent DH5α cells (section 2.8.7). 24 transformed colonies were taken and plasmid DNA extracted by the Triton-boil method (section 2.8.8). Plasmid DNA was digested with Bam HI and analysed by agarose gel electrophoresis.

One of these colony types contained the DNA insert that was confirmed by sequencing manually across the region using PKR1 and PKR2 as sequencing primers. The sequence was correct, confirming the creation of the plasmid pJF001 (figure 4.10). PCR reactions were carried out using pNB 92/008 or pJF001 as template DNA with primers PKR1 and PKR2. PCR products were separated and visualised by agarose gel electrophoresis. Figure 4.9 shows these PCR products, proving pJF001 contains an additional 79 bp sequence that when amplified, results in a band at 382 bp well separated from the original 303 bp product from native PKR sequence.
Figure 4.9 PCR products of pNB92/008 and pJFO01

0.5 volume PCR products visualised by agarose gel electrophoresis. Lane 1 shows positive PCR reaction using pNB 92/008 DNA. Lane 2 shows positive PCR reaction using pJFO01 DNA. Negative reactions (not shown) were negative. Marker ladder used was øx174/Hae III DNA.

Nae II was the only unique restriction site found downstream of the PKR sequence for efficient *in vitro* transcription from the T7 site (figure 4.10). Transcription of pJFO01 would result in an RNA product over 2 Kb which was considered too long. To enable a shorter RNA transcription, the majority of the PKR sequence upstream of the PKR1 primer site was deleted between the Mam I and Bam HI sites (figure 4.10). The Bam HI site was not unique. A partial digestion was therefore used to obtain linearised plasmid cut at the correct site. Linearised plasmid was blunt ended and gel purified. Digestion of linearised pJFO01 with MamI initially proved difficult until it was realised that Mam I was inhibited by the presence of 5' methyladenine. Use of JM110 bacterial cells (table 2.5) solved this and after gel extraction, partially BamHI digested pJFO01 was digested to completion with Mam I. Blunt end ligations were carried out and used to transform JM110 cells (Nae II used to linearise plasmid DNA for *in vitro* transcription is also inhibited by 5' methylcytosine).

Restriction digestion of transformed colony plasmid DNA revealed a molecular clone with the correct size deletion, pJF002 (figure 4.10). This was confirmed by sequencing manually across the deleted region using a T7 primer.
Figure 4.10 Creation of a molecular clone from which ISC RNA could be transcribed *in vitro*

**pNB 92/008**
- PstI 20
- NaeI 4258
- PKR2
- PKR1
- BamHI 1690

4533 bp

79bp insert from *HaeIII* cut *pBluescript II SK+* →

**pJF001**
- PstI 20
- NaeI 4337
- INSERT
- PKR1
- MamI 724
- MamI 766
- T7
- BamHI 1769

4612 bp

1040bp deletion MamI to BamI

**pJF002**
- PstI 20
- NaeI 3297
- PKR2
- PKR1
- MamI 729
- T7

3572 bp
### 4.8.2 Purification of *in vitro* transcribed ISC RNA

pJF002 plasmid DNA was digested with Nae II endonuclease and linearised DNA was gel purified (section 2.8.5.i). ISC RNA was transcribed *in vitro* from the T7 site using the Promega RiboMax system (section 2.9.8).

A number of contamination problems occurred,

- DNase contamination of plasmid DNA stocks.
- Unincorporated nucleotides adversely affecting transcript RNA quantification.
- Plasmid DNA contamination due to inefficient DNase treatment.

DNase contamination was solved by phenol/chloroform extraction and ethanol precipitation of plasmid DNA. Unincorporated nucleotides and short run-off transcripts were removed from the final transcription mix by column filtration. A Nap-10 column (Pharmacia) and Sephadex G-50 spin column (Boehringer-Mannheim) were both tried but in the end a Sephadex G-100 gravity column was assembled (section 2.7.6.ii) and proved the most efficient method. Undigested plasmid DNA proved the most problematical as negative RT-PCR reactions using ISC RNA were positive despite reaction components proving negative for contamination when tested separately. Elution of transcribed RNA from a 1.5% agarose gel using the BioTrap BT100 system (Schleicher & Schuell, Dassel, Germany) did not yield any RNA products. This would have increased the chances of RNase contamination and was therefore not pursued further.
ISC RNA purification was finally achieved using the following method,
- *In vitro* transcribe ISC RNA
- DNase treat plasmid DNA
- Extract ISC RNA with RNAzol B (section 2.9.2.ii)
- Remove small RNAs and single nucleotides through Sephadex G-100 column, collect each fraction separately.
- Analyse each fraction for presence of nucleic acid by ethidium bromide staining (section 2.9.4)
- Analyse each fraction for presence of ISC RNA at 1041nt (figure 4.12)
- Test each fraction for contamination by an RT-PCR reaction.

Column fractions 5 to 10 showed positive for nucleic acid. Figure 4.13 shows that all column fractions containing RNA were positive for ISC RNA and negative for any contaminating DNA. These fractions were pooled and the amount of RNA present was determined spectrophotometrically by its absorbance at 260nm.

**Figure 4.12** Column purification fractions of ISC RNA

3\( \mu \)l each fraction containing nucleic acid was boiled for 2 minutes in 3 \( \mu \)l denaturing formamide loading buffer and electrophoresed through a 1.5% agatrose gel in 1 x TBE buffer. All fractions contained ISC RNA. Marker ladder used was \( \Phi x 174/HaeIII \) DNA.
0.1 volume RT-PCR reactions visualised on 2% agarosed gel in 1x TAE buffer. Negative RT-PCR reactions are shown in lanes 2, 4, 6, 8, 10 and 12. Positive RT-PCR reactions showing the 382bp product are shown in lanes 1, 3, 5, 7, 9 and 11. Positive (lane 14) and negative (lane 13) PCR reactions were carried out using 20 ng pET16b/PKR as template DNA. Marker ladder used was φX174/HaeIII DNA.

4.8.3 Quantitation of ISC RNA
Reading the absorbance at 260nm of purified ISC RNA at a suitable dilution quantified the concentration of RNA. From the molecular weight (MW) of ISC RNA, the number of molecules of RNA could be calculated and thus in each RT-PCR assay, the exact number of ISC molecules added is known.

\[ \text{MW 1041 nt transcript ISC} = 346301 \]
\[ 1 \text{ mole} = 6 \times 10^{23} \text{ molecules} \]
\[ 1 \text{ molecule} = 1.67 \times 10^{-24} \text{ moles} \]
\[ 1 \text{ molecule} = 5.78 \times 10^{-17} \text{ g} \]
Therefore, 1pg ISC RNA = 1.734 x 10^6 molecules

4.8.4 Co-amplification of ISC RNA and PKR mRNA
Initial RT-PCR reactions were carried out using a broad range of ISC RNA series dilutions to assess the level of detection. A constant amount of total RNA from Hut78 cells (500 ng) was added to the RT reaction mix and the two RNA species co-amplified (figure 2.7). The ideal range of ISC RNA in these reactions fell between 1 x10^6 and 1.5 x10^7 molecules. Dilutions of ISC RNA were made using a dilution buffer containing 9 mM DTT, 1 U/µl RNAsin and 1 mg/ml carrier yeast tRNA. Carrier tRNA migrated to the
bottom of the agarose gel upon electrophoresis and so did not impede the detection of the two PCR products as seen in figure 4.14.

**Figure 4.14** Agarose gel electrophoresis of yeast tRNA

![](image)

1 mg yeast tRNA was boiled in denaturing formamide loading buffer and electrophoresed through a 2% agarose gel in 1x TAE buffer. The position of the RNA is well below the 303 bp area that PKR mRNA RT-PCR products would be found. Marker ladder used was øx174/HaeIII DNA.

### 4.8.5 Quantitative competitive RT-PCR assay

Using total RNA from Hut78 cells, an assay to quantify PKR mRNA from human cells was set up. The quality of extracted RNA was analysed by observing intact bands corresponding to 18S and 28S rRNA molecules, found approximately at 1900 nt and 4800 nt respectively (figure 4.15). If rRNA had been degraded it was likely that the mRNA content was also degraded and so of no use in the assay. As an initial test for the presence of PKR mRNA, RT-PCR reactions were carried out using cellular RNA as template only. 250 ng, 500 ng and 1 |ig total RNA was reverse transcribed and amplified. 25 cycles of amplification were used in all RT-PCR reactions to ensure the exponential phase had not been exceeded. PCR products were visualised by agarose gel electrophoresis and the amount of total cellular RNA that gave a positive signal taken as the amount to be used in an assay.

An initial assay was run using a range of ISC RNA molecules. The same amount of cellular RNA was used in each RT-PCR assay. Five reactions, plus a negative RT-PCR, were carried out using 2-fold dilutions of ISC RNA as follows.
1 x 10^6 molecules ISC RNA
2 x 10^6 molecules ISC RNA
4 x 10^6 molecules ISC RNA
8 x 10^6 molecules ISC RNA
1.5 x 10^7 molecules ISC RNA, positive and negative RT-PCR reactions.

Occasionally, when RT-PCR products were visualised a discrete point was observed where PKR products appeared as a strong band in comparison to a lack of visible products in the previous reaction. This indicated that the range of ISC RNA used was too broad, the point at which PKR and ISC RNA's were equal being missed in the reactions carried out. A second assay was then carried out using a narrower range of ISC RNA molecules.

A photograph was taken of the ethidium bromide stained PCR products under UV light. This was scanned into a computer and the image used to measure the relative band intensities of the PKR mRNA and ISC RNA products (section 2.9.9). Comparisons of these products were used to quantify the number of PKR mRNA molecules in the given amount of total cellular RNA used in the assay. A worked example of this is described in section 2.8.10.

4.9 Total cellular RNA extraction from liver tissue
This assay to quantitate PKR mRNA from cell culture was successful. The next step was to use this in quantifying PKR mRNA in total cellular RNA extracted from liver biopsies obtained from HCV infected patients.

Previous RNA extractions had been performed using the reagent RNAzolB. However, this is only useful for obtaining RNA, leaving both DNA and proteins in an inaccessible phase. Liver biopsy samples obtained were very small (18 gauge x 1 cm long). As the aim of this project was to assay both PKR mRNA and protein, a method was required by which both RNA and protein could be extracted and purified from the same sample. Most available methods were designed to extract only one of these components, using reagents that inhibited either RNA or proteins. TriReagent extracts and separates cellular protein, DNA and RNA in three accessible phases, using a combination of guanidium thiocyanate and phenol (Chomczynski, 1993).
4.9.1 Comparison of extraction reagents

A comparison of total RNA extraction methods was carried out between TriReagent and RNAzolB. Cell culture samples were used in initial tests, generally extracting RNA from $5 \times 10^6$ to $5 \times 10^7$ cells each time. Extractions were performed according to the methods described in section 2.9.2. Biopsy sized samples from a freshly sacrificed rat liver were also used to assess the extraction efficiency of TriReagent on tissue samples. Total RNA content was measured by its absorbance at 260nm and total yields calculated. 2 µg each RNA sample was analysed by agarose gel electrophoresis (figure 4.15). 2 µg each RNA sample was also examined for cellular DNA contamination by analysing denatured RNA that had been treated with 0.2M NaOH. Comparisons of total RNA yields were made (table 4.3).

Yields of total RNA using TriReagent were generally lower than those when RNAzolB was used on the same number of cells or amount of tissue. It was also clear that, though yields were lower, TriReagent gave good quality cellular RNA. Cellular proteins extracted using TriReagent were also of good quality (section 5.4.1). This proved that TriReagent was a suitable reagent for the extraction of both RNA and proteins from the same precious liver biopsy sample.

Figure 4.15 Comparison of TriReagent and RNAzolB extraction reagents

Total RNA from $1 \times 10^7$ cells was extracted using TriReagent (lane 1) or RNAzolB (lane 3). 2 µg each RNA sample was analysed by agarose gel electrophoresis. 2 µg each RNA was denatured in 0.2N NaOH for 30 minutes at 37°C and visualised. Lane 2 shows denatured RNA extracted by TriReagent. Lane 4 shows denatured RNA extracted by RNAzolB. Total RNA is intact with 18S and 28S rRNA bands visible. No DNA contamination present seen by the lack of any nucleic acid in lanes 2 and 4.
Table 4.3 Comparison of RNA yields using TriReagent and RNAzolB

<table>
<thead>
<tr>
<th>Cells/tissue</th>
<th>Total RNA yield using TriReagent</th>
<th>Total RNA yield using RNAzolB</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 x10⁶ CEM4 cells</td>
<td>32.6 µg</td>
<td>52.8 µg</td>
</tr>
<tr>
<td>9 x10⁶ Hut78 cells</td>
<td>30.2 µg</td>
<td>48.0 µg</td>
</tr>
<tr>
<td>Rat liver biopsy</td>
<td>31.3 µg</td>
<td>63.9 µg</td>
</tr>
</tbody>
</table>

4.9.2 Total RNA from liver biopsy samples

In total, 62 liver biopsies were obtained from HCV infected patients. 22 of these were obtained from the Leicester Royal Infirmary. Half biopsies (1 cm in length) were snap frozen in liquid nitrogen at the bedside, the maximum time from body to freezing was 2-3 minutes. The other 40 samples were obtained from Dr W. Irving (Queens Medical Centre, Nottingham). These had been taken in the previous 3 years and stored at -80°C to ensure minimum sample degradation.

As all biopsies were infected with HCV, extractions took place in a containment III (CIII) laboratory under strict guidelines. This meant that no sharp implements or glassware could be used due to the potential risk of infection involved. This posed a problem in that liver tissue is not readily dissociated to its constituent cells. TriReagent acts on cell surfaces to lyse the cells, releasing their constituent RNA, DNA and proteins. Initial extractions using samples of rat liver under CIII conditions used the end of a Gilson tip to break up the liver against the tube wall as the only feasible method to increase extraction yields.

Collagenase treatment was investigated as a method to dissociate liver cells prior to extraction. A biopsy sized piece of rat liver was incubated at 37°C under 5% CO₂ with collagenase (section 2.10.2) until the tissue was seen to be dissociated. This took over 4 hours by which time, extracted total RNA had degraded completely, as analysed by agarose gel electrophoresis.

Glass beads (710-1180 µm, Sigma) were known to aid yeast cell disruption. These were added to diced liver samples in TriReagent with vortexing as an additional method of shearing cells from the solid tissue. Glass beads did not pose an infection risk and were provided acid-washed to reduce
potential RNase contamination. Comparisons of total RNA yields with and without glass beads were not made.

4.9.3 Quality of total RNA yields from HCV infected liver tissue

Of the 62 liver HCV infected liver biopsies extracted using TriReagent, only 3 samples contained intact rRNA when analysed by agarose gel electrophoresis. Intact and degraded rRNA from 2 liver samples is shown in figure 4.16.

Figure 4.16 Total RNA quality from HCV infected liver biopsies

Total RNA was extracted from liver samples #3 and #4 using TriReagent. RNA content was determined by absorbance at 260nm. 1.5 μg total RNA was boiled in denaturing formamide loading buffer and electrophoresed through a 1% agarose gel in 1x TBE. Lane 1 shows degraded RNA from liver #3. Lane 2 shows intact rRNA bands from liver #4.

The samples with intact RNA were tested further by RT-PCR analysis using 500 ng total RNA as template. Liver #4 (figure 4.17) was positive for PKR but was also found to contain some DNA contamination. This may have originated from some carry over of the DNA phase during the RNA extraction as all other controls remained negative. No other samples of intact RNA showed any signal for PKR. These samples may have contained too little PKR mRNA to be amplified sufficiently from just 500 ng total RNA.
0.1 volume RT-PCR reactions visualised by agarose gel electrophoresis. Lanes 3 and 5 show negative RT-PCR reaction controls for liver samples #3 and #4 respectively. Lane 1 shows positive RT-PCR reaction control using $1.5 \times 10^9$ molecules ISC RNA as template. Lanes 6 and 7 show positive and negative PCR control reactions respectively, using 20 ng pET16b/PKR DNA as template. Lane 2 shows test RT-PCR reaction for liver #3 RNA. Lane 4 shows test RT-PCR reaction for liver #4 RNA. Marker ladder used was øx174/HaeIII DNA.

4.9.4 Total RNA from non-HCV infected liver biopsies
7 liver biopsy samples were obtained from hepatic patients whose disease were proven not to be caused by any virological or other microbiological agent. 1 sample was obtained from the Leicester Royal Infirmary and was snap frozen on removal from the patient. The other 6 samples were obtained from the Queen’s Medical Centre, Nottingham and had been stored at -80°C. Total RNA was extracted using TriReagent and analysed by agarose gel electrophoresis. All samples from Nottingham contained degraded RNA whilst intact RNA was extracted from the Leicester sample. Upon RT-PCR analysis of the Leicester sample, no PKR signal was obtained indicating that the level of PKR mRNA was too low for detection.

4.9.5 Controls for total RNA liver extractions under ideal conditions
It was later revealed that the liver samples obtained from Nottingham had been left at room temperature for several hours before being stored at -80°C. This would explain the degradation of cellular RNA observed compared to the intact RNA samples from snap frozen liver samples obtained from Leicester. The physical method used for RNA (and protein) extractions was also less than ideal. Yatsuhashi et al (1997) reported the use
of homogenisation to optimise RNA extractions from liver biopsy samples. Homogenisers available at the time were either made of glass or consisted of large apparatus that would be impractical for use in a CIII laboratory. Significant amounts of liver tissue dissociation could also be achieved using a pestle and mortar, keeping the tissue under liquid nitrogen (personal communication, Dr C Stover, University of Leicester). This would be both impractical for such a small sample and dangerous with the potential for tissue loss and therefore contamination of the area with HCV. Chopping the liver sample with a sharp blade into much smaller pieces than were obtained using a pipette tip would have increased the surface area and thus RNA extraction yields though this was not available for use under CIII conditions.

A pellet pestle mixer for tissue homogenisation (Burkard Scientific) was then found that could be used under CIII conditions. This consisted of a plastic disposable pestle that fit snugly into a 1.5 ml Eppendorf tube used for liver biopsy extractions. The pestle could be used by hand to squash the liver tissue against the tube wall so breaking it up to complete dissociation. The pestle could be treated for RNase contamination with DEPC-H₂O. The small and disposable nature of these would make them ideal for processing large number of samples.

Control experiments were performed to compare the yields of total cellular RNA using the different methods of grinding the liver tissue to allow greater action of the TriReagent. A rat was sacrificed and the liver immediately removed. A Temno 18g x 1.5 cm biopsy needle was used to take equal biopsy sized samples. 3 samples were used for each extraction procedure (described in table 4.4) and the average yields of total RNA calculated. The quality of extracted RNA was also analysed by agarose gel electrophoresis.

Table 4.4 Comparison of liver extraction methods

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Total RNA yield (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringe homogeniser</td>
<td>25.76 ± 9.39</td>
</tr>
<tr>
<td>Gilson tip</td>
<td>33.45 ± 6.98</td>
</tr>
<tr>
<td>Pellet pestle</td>
<td>46.93 ± 11.43</td>
</tr>
<tr>
<td>Glass tissue grinder</td>
<td>31.32 ± 5.42</td>
</tr>
</tbody>
</table>
The pellet pestle method of tissue homogenisation gave sufficiently increased yields of total RNA compared to the Gilson tip method. Enough total RNA could be extracted from a half liver biopsy using the pellet pestle to perform an RT-PCR assay for PKR mRNA. Unfortunately, this method was only discovered towards the end of this project and was not used for the extraction of cellular RNA and protein from HCV infected liver biopsies.

The average amount of RNA extracted from a whole liver biopsy under CIII conditions, using a Gilson tip to break up the liver, was around 33 μg. For half biopsies obtained, the amount of RNA would be 16μg. Given that around 7μg would be used to quantify total RNA and determine its quality, there would be insufficient cellular RNA to perform an accurate RT-PCR assay, especially if repeat reactions were required.

A time course of liver samples left at room temperature was used to confirm that the RNA from HCV infected samples obtained from Nottingham had degraded due to a prolonged time at room temperature. Samples were frozen at -80°C after each time point to mimic the conditions used in the Nottingham laboratories. RNA extractions were performed using TriReagent and a pellet pestle. Results can be seen in table 4.5 and figure 4.18. Intact rRNA could be seen in samples up to 6 hours though some RNA degradation was occurring. Sometime between 6 and 12 hours total RNA degradation occurred. It was known that the Nottingham samples had been left out on the bench for more that 12 hours.

Table 4.5 Time course of RNA degradation in liver left at room temperature

<table>
<thead>
<tr>
<th>Time at room temperature</th>
<th>Yield total RNA extracted by TriReagent</th>
<th>rRNA degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hours</td>
<td>46.93 μg</td>
<td>NO</td>
</tr>
<tr>
<td>1 hours</td>
<td>39.36 μg</td>
<td>NO</td>
</tr>
<tr>
<td>3 hours</td>
<td>46.32 μg</td>
<td>NO</td>
</tr>
<tr>
<td>6 hours</td>
<td>49.72 μg</td>
<td>NO</td>
</tr>
<tr>
<td>12 hours</td>
<td>23.68 μg</td>
<td>YES</td>
</tr>
<tr>
<td>23 hours</td>
<td>21.68 μg</td>
<td>YES</td>
</tr>
</tbody>
</table>
4.10 Discussion

Identifying a single (or set of) factors to predict the response of a chronically infected HCV patient to interferon therapy is necessary (section 1.9).

In this study, RNase and oligo protection assays both proved unsuccessful in generating a reliable assay. The level of sensitivity was not sufficient to detect low levels of PKR RNA from the very small amounts of cellular RNA available.

Though PKR mRNA was successfully amplified, 2′5′ oligoadenylate synthetase was not. Reports by Okuda et al (1994), Grander et al (1996) and Pawlotsky et al (1996) all suggested that interferon resistance of HCV is not related to the 2′5′ oligoadenylate synthetase pathway and therefore this interferon effector could not be accurately used as a predictive factor. Efforts were then directed towards analysis of PKR expression levels.

Initial contamination problems of RT-PCR amplification of PKR mRNA were solved, resulting in the development of a quantitative competitive RT-PCR assay using an internal standard control species of \textit{in vitro} transcribed RNA.
Total RNA extractions from HCV infected liver under category III conditions proved difficult with sub-optimal yields of total cellular RNA obtained. The use of the disposable pellet pestle increased yields but was not discovered until the end of this project. Snap frozen liver biopsy samples generally yielded intact cellular RNA whilst samples stored for several hours at room temperature resulted in degraded cellular RNA that was of no use in this assay.

To accurately assay PKR mRNA (and protein) from liver biopsy samples, tissue should ideally be snap frozen in liquid nitrogen at the bedside. This is often difficult as many medical centres do not have this facility. Extraction of RNA and protein with TriReagent in a category III laboratory should be aided by the use of a grinding accessory, namely the pellet pestle. The RNA phase from this extraction should be removed with the greatest care to avoid DNA phase carry over. A whole biopsy sample (18 gauge x 1.5 cm) should provide sufficient total cellular RNA for this RT-PCR assay.

At the time these experiments were carried out, there had not been any reports of the quantification of PKR mRNA in human cells. Basu et al (1997) and Vojdani et al (1997) later reported the quantification of PKR in peripheral blood mononuclear cells by competitive PCR. Basu et al (1997) used a semi-quantitative RT-PCR assay in which expression levels of the housekeeping gene β-actin were used as the standard against which PKR mRNA levels were compared. This was tried in section 4.7. In Basu's report, levels of PKR mRNA cannot be given.

In Vojdani et al's report (1997), a PCR MIMIC system was used to quantify the actual amount of PKR mRNA from a given sample of total cellular RNA. Though the PCR step in this assay was standardised by the use of an internal control species of competitive DNA, the RT step was not. Vojdani et al simply stated that total cellular RNA had been reverse transcribed using primers specific for PKR mRNA. Resultant PKR cDNA was then used in a quantitative PCR assay. Wang et al (1989) reported that, in order to develop a fully competitive assay, the RT step must also be standardised in order to eliminate tube to tube variations that would be exaggerated through PCR amplification.
It seems that a truly competitive RT-PCR assay to quantify PKR mRNA expression in human PBMCs, let alone tissue samples, has not been achieved by other investigators. The assay described in this chapter has been developed to be as accurate as possible with all steps standardised to remove potential points of variation (other than the amounts of ISC RNA used), giving a quantitation of PKR mRNA in terms of the number of molecules present in any given sample of cellular RNA.

PKR protein expression in chronic HCV patients was also examined. The development of a quantitative assay for PKR protein from liver biopsy samples is discussed in the next chapter.
Chapter 5

Development of a quantitative assay for PKR protein
5.1 **Introduction**

This chapter will describe the experiments performed to develop an assay that quantifies PKR protein from HCV-infected liver tissue.

At the time the experiments described in this chapter were carried out, PKR protein had not been accurately quantified in liver tissue from HCV infected patients. Dubois *et al* (1990) and Schwemmle *et al* (1992) had both reported the presence of PKR in the cytoplasm of HeLa cells, Schwemmle *et al* (1992) describing the association of PKR with cellular endoplasmic reticulum. Jeffrey *et al* (1995) reported the presence of functional PKR protein in both the cytoplasm and nucleus of cultured human Daudi and mouse NIH-3T3 cells. PKR expression was quantified from different cellular fractions by western blotting. Protein extracts were electrophoretically transferred to a nitrocellulose membrane and PKR detected with a primary monoclonal antibody followed by a biotinylated secondary antibody. This PKR/antibody complex was detected using strepavidin labelled with a $^{125}$I radiolabel. The amount of PKR protein present was quantified in terms of the number of PKR molecules per cell. However, no quantitation of PKR protein in the livers of HCV infected patients has been reported.

Jeffrey *et al*'s report does not take into account possible protein losses through extraction and assay procedures. Expression of PKR protein in terms of its proportion to total cellular protein would give a more relevant account of PKR levels in the tissues examined, assuming that PKR protein loss during the assay is proportional to the loss of all other cellular proteins.

The aim of these experiments was to develop a similar assay to the one performed by Jeffrey *et al* in which total cellular proteins are extracted from HCV-infected liver tissue. These proteins would then be separated by denaturing polyacrylamide gel electrophoresis (PAGE) and detected by the western blot method using a monoclonal antibody specific to human PKR followed by a labelled secondary antibody. This detection system could then be developed into a quantitative assay for human PKR protein.
5.2 Patient samples

In the time taken to establish a primary cell culture of hepatocytes from a liver biopsy sample, the level of expression of cellular proteins, including PKR, may alter (Berry et al, 1991). The aim of the experiments described in this chapter was to quantify the amount of PKR protein expressed in the liver cells of patients infected with HCV at the same time that other biochemical, virological and histological tests were performed. This would allow accurate correlations of the levels of PKR with factors such as liver histology score, virus genotype and virus titre. Cellular proteins were therefore extracted directly from liver tissue samples to ensure accurate levels of PKR expression would be obtained.

Liver biopsies were obtained from consenting HCV-infected patients, typically using an 18 gauge x 1.5 cm Temno biopsy needle. Samples taken at the Queens Medical Centre, Nottingham had been stored at -80°C, though as described in section 4.9.5, samples had been left at room temperature for several hours prior to storage. Samples taken at the Leicester Royal Infirmary were halved at the bedside and each portion snap frozen in liquid nitrogen within 2-3 minutes of sampling. Tissue samples were stored in liquid nitrogen.

Hut78, Chang, CEM4 and C8166 cell cultures had been proven to express PKR mRNA (section 4.5). These cell lines all showed the presence of PKR protein when detected by western blot (section 5.3) and were therefore used in initial protein assays to optimise the detection and quantitative nature of the assay before patient liver samples were tested.

5.3 Detection of PKR protein

Laurent et al (1985) reported the production of a monoclonal antibody raised in mice, against the human PKR protein. This was termed antibody 71/10 and recognises the first 20 to 40 amino acid region of PKR (personal communication Dr. G. Witherall, Ribogene Inc. USA). This monoclonal antibody was already available in the laboratory and had been proven successful in detecting, by western analysis, both full length PKR and 3' terminal truncated versions of the protein (Pollit, 1997; Gale, 1998). Antibody 71/10 was therefore used in all subsequent experiments to detect full length PKR and a specific truncated PKR protein termed PKR 1-170 which is described further in section 5.6.
The 62055 Dalton molecular weight of human PKR suggests this protein should migrate through an SDS PAGE gel to 62 kDa. In fact, a high degree of phosphorylation increases the apparent size of the protein to 68-72 kDa (Laurent et al, 1984 & 1985; Galabru & Hovanessian, 1987). This was observed when cellular protein samples from both cultured cells and liver tissue were analysed by western blot (figure 5.3). Galabru & Hovanessian (1987) reported the presence of a 48 kDa protein that cross reacted with the PKR specific antibody 71/10. This 48 kDa species was reported to be a product of PKR proteolytic degradation that occurs during protein extraction and analysis processes. PKR degradation and therefore presence of the 48 kDa protein was inhibited by the addition of 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and in view of this, PMSF was added and maintained at 0.2 mM to all protein samples described in this chapter.

A method was required by which full length PKR present in human cells could be separated and identified from any degraded products of PKR. Protein dot blots and antibody capture based methods were not considered sufficiently specific as the antibody 71/10 might bind to non-full length PKR proteins, giving inaccurate values in a quantitative assay. Western blot analysis was therefore chosen as the ideal method. Total cellular proteins extracted from human cells were separated by electrophoresis, initially through a 5% SDS PAGE stacking gel to concentrate the protein sample and then through a 12% SDS PAGE gel (section 2.10.6) which was sufficient to separate the 68 kDa protein from any smaller species of PKR, as well as the 21 kDa truncated PKR 1-170 described in section 5.6. Cellular proteins were electrophoretically transferred to a nitrocellulose membrane (section 2.10.8). Unbound areas of the membrane were blocked using a blocking buffer containing Tween 20 detergent and non-specific proteins in the form of skimmed milk powder and lamb serum.

Western blotting of PKR (section 2.10.9) was carried out by incubation of the membrane with the primary monoclonal antibody 71/10 at an appropriate dilution. 71/10 antibody bound to PKR proteins was detected by a secondary polyclonal antibody specific to mouse Ig that had an attached label for visual detection, further described in section 5.7.
Figure 5.1 Diagram showing PKR detection by western blotting.

Direction of current and protein migration

Current

Protein transfer

5% Stacking polyacrylamide gel

12% Resolving polyacrylamide gel

-current electrode

Resolving gel

Nitrocellulose membrane

+ve electrode

M

Ponceau stain to identify marker ladder

BLOCKING BUFFER TMT/SS

WASH

1° ANTIBODY 71/10

WASH

ANTI-MOUSE 2° ANTIBODY

WASH

DETECT

Marker ladder

Cellular protein

94 KDa
67 KDa
43 KDa
30 KDa
20 KDa
14 KDa

68 kDa
48 kDa
A marker ladder run alongside the cellular protein samples was detected on the blotted nitrocellulose membrane by staining the membrane with Ponceau stain prior to incubation in blocking buffer. As Ponceau stain washes away in blocking buffer, the marker ladder band positions were made permanent with a pencil mark, thereby resultant PKR bands appearing during final detection could be assessed according to the protein's molecular weight. Figure 5.1 shows the western blotting system for detecting specific proteins.

5.3.1 Proving human PKR only was detected by antibody 71/10
To prove that the PKR band at 68 kDa only occurred in human proteins and therefore to prove the primary antibody did not cross react with non-human proteins, western blots were performed using cellular proteins extracted from human cell lines (Hut78) and from pig and rat liver tissue. Extractions were performed using TriReagent (section 2.10.1.ii). Final cellular proteins were resuspended in 1x SDS buffer, boiled in an equal volume of 2x SDS protein buffer and an equal amount of rat and human (or pig and human) protein electrophoresed through a 12% SDS-polyacrylamide gel.

Figure 5.2 shows a coomassie stained gel of rat and Hut78 cellular proteins. There are clearly differences in the pattern of protein bands. When an identical gel was electroblotted to nitrocellulose membrane and western blotted (figure 5.3), a band around 68 kDa was clearly evident in the human cell protein sample corresponding to PKR. The rat protein sample did not contain this band. Cellular proteins from a pig liver did not contain the PKR band. This confirmed that the primary antibody 71/10 used for the detection of human PKR did not cross-react with non-human protein samples, confirming specificity for human PKR.
Figure 5.2 Coomassie stained rat and human cellular proteins

Coomassie stained 12% SDS-PAGE gel showing total cellular proteins extracted from a freshly killed rat liver (lane 1) and from Hut78 human cells (lane 2). Marker ladder was also stained showing the relevant size bands.

Figure 5.3 PKR detected in human cellular proteins only

Western blot detection of full length human PKR using the horseradish peroxidase/ECL system. Total cellular proteins extracted from a freshly killed rat liver (lane 1) and from Hut78 human cells (lane 2) were separated through a 12% SDS-PAGE gel and electroblotted to nitrocellulose membrane. Full length PKR is evident in the human cell sample only.
5.4 Total cellular protein extraction techniques

As mentioned in section 4.2, only very small liver tissue samples were available for the analysis of PKR expression. Therefore both cellular proteins and RNA had to be extracted from this one sample of human liver tissue. The majority of RNA and protein extraction reagents and procedures available were designed for the purification of either RNA or protein but not both. TriReagent (Chomczynski, 1993) acts by extracting and separating out cellular RNA, DNA and protein in a single step procedure using a combination of phenol and guanidine thiocyanate. These cellular constituents are held in separate phases for individual isolation which makes this reagent an ideal method for the simultaneous extraction of total RNA and proteins from the same precious tissue sample.

5.4.1 Comparison of protein extraction reagents

The efficiency of cellular protein extraction from human cells using TriReagent was compared to that using an SDS lysis buffer. Buffer containing the detergent SDS acts to lyse cells, releasing all cellular contents including DNA and RNA and cannot therefore be used as a method for cellular protein purification. However, it was used as a simple and effective method of obtaining cellular proteins for initial analyses. Lysed cell samples were boiled in protein 2x SDS buffer and loaded straight onto an SDS-PAGE gel for electrophoretic separation (section 2.10.6).

To compare cellular protein extraction methods, protein from 8 x10^6 Hut78 cells was extracted using TriReagent (section 2.10.1.ii). The final protein sample was resuspended in 100 μl 1x SDS protein buffer. Additionally, 8 x10^6 Hut78 cells were lysed directly with 100 μl 2x strong SDS buffer (section 2.10.1.i). An equal volume of each sample was loaded onto a 12% SDS-PAGE gel and proteins separated. A 1:2 dilution of the lysed cell sample was also made and the same sample volume also analysed by SDS-PAGE. The gel was stained with coomassie dye followed by destaining (section 2.10.7) (figure 5.4).
Figure 5.4 Coomassie stained cellular proteins extracted either by lysis with SDS buffer or with TriReagent

Total cellular proteins from 8 x 10^6 Hut78 cells were extracted by 2x strong SDS buffer or TriReagent, equal volumes each sample separated by 12% SDS-PAGE and stained with coomassie stain. Lane 1 = SDS lysed cells. Lane 2 = 1:2 dilution of SDS lysed cell sample. Lane 3 = TriReagent extracted protein sample. Lane 4 = insoluble protein pellet from TriReagent extraction.

TriReagent extraction and resuspension of protein in 1x SDS buffer seemed to result in a smaller amount of total cellular proteins present in the sample compared to that seen when cells were lysed directly in 2x strong SDS buffer. Figure 5.4 shows that, though total cellular proteins were extracted by TriReagent and resuspended in 1x SDS buffer, much protein remained in the insoluble protein pellet. It had been reported that cellular proteins extracted using TriReagent are difficult to resuspend. Dialysis of the proteins from the organic phase was recommended but this takes several days which may result in protein degradation due to reactivation of cellular proteases in the extracted sample (personal communication, Dr B. Wilfinger, Molecular Research Center Inc, USA).

TriReagent was therefore proven successful at extracting total cellular proteins from mammalian cells. Together with the successful extraction of cellular RNA from mammalian cells using this method (section 4.9), TriReagent was considered suitable for the extraction and purification of both cellular proteins and RNA from the small, precious samples of tissue available for these experiments.
5.4.2 Liver cell dissociation

For the assays themselves, proteins were extracted directly from liver biopsy samples, as described in section 4.9.1. Glass homogenisers and other sharp implements were unavailable for use with tissues infected with HCV and the tissue sample was too small to be ground with a pestle and mortar. As a result, protein (and RNA) yields were low compared to theoretical yields (table 4.4).

Liver cell dissociation was examined. A control piece of biopsy-sized fresh rat liver was incubated in collagenase until the tissue disintegrated. This took over 4 hours, by which time the RNA had degraded (section 4.9.2). The amount of cellular protein bands, when separated electrophoretically and stained with coomassie, had largely been reduced compared to an undigested sample of liver tissue extracted with TriReagent (figure 5.5). Collagenase treatment of liver tissue was not a suitable method for cell dissociation and was not pursued.

Figure 5.5 Collagenase digestion of rat liver

15 mg fresh rat liver tissue was incubated with collagenase type IV (Sigma) in Krebs Ringer bicarbonate buffer under 5% CO$_2$ at 37°C. After 4 hours tissue was seen to be dissociated. Liver cells were extracted in TriReagent and the RNA and protein phases separated. Protein was resuspended in 150 µl 2xSDS buffer, 0.1 volume loaded onto a 12% SDS polyacrylamide gel and electrophoresed to separate proteins. The gel was stained in Coomassie stain then de-stained until background was removed. Lane 1 = untreated liver extracted with TriReagent. Lane 2 = collagenase treated liver.
5.4.3 Comparison of buffers for resuspension of extracted proteins

Buffers containing different concentrations of SDS were compared for their ability to optimally solubilise extracted protein samples. To reduce any variability in the extraction step, protein from a single aliquot of 5.16 x10^6 Hut78 cells was extracted using TriReagent. The protein phase was aliquoted into 4 equal portions, each one containing cellular protein from the equivalent of 1.29 x10^6 cells. Protein from each aliquot was precipitated, washed and dried (section 2.10.1.ii). Each protein sample was resuspended in 50 μl 1x, 2x, 3x or 4x SDS protein buffer (buffer compositions shown in table 5.1). 15 μl each protein sample was then electrophoresed through a 12% SDS polyacrylamide gel, stained with coomassie stain and then destained to observe the total protein content in the sample (figure 5.6). Less protein content was observed when resuspended in 3x SDS buffer. No difference in the amount of protein was observed for 1x, 2x and 4x SDS buffers.

Table 5.1 SDS buffer contents

<table>
<thead>
<tr>
<th>Constituents</th>
<th>1x buffer</th>
<th>2x buffer</th>
<th>3x buffer</th>
<th>4x buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris pH8.0</td>
<td>25 mM</td>
<td>50 mM</td>
<td>75 mM</td>
<td>100 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>10%</td>
<td>20%</td>
<td>30%</td>
<td>40%</td>
</tr>
<tr>
<td>glycerol</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Figure 5.6 Total cellular protein resuspended in different SDS buffers

Coomassie stain of total cellular proteins extracted from Hut78 cells. An equal amount of cellular protein was resuspended in either 1x SDS (lane 1), 2x SDS (lane 2), 3x SDS (lane 3) or 4x SDS (lane 4) protein buffer. The marker ladder bands are also stained.
Chomczynski (1993) recommended resuspending extracted cellular proteins in a buffer containing 1% SDS. Figure 5.6 shows that the total amount of protein resuspended in 1x SDS buffer was equivalent to that of the 2x and 4x SDS buffers. 1x SDS protein buffer was therefore used in all subsequent experiments.

5.4.4 Quality of cellular protein extractions
A small amount of all extracted protein samples was analysed by SDS-PAGE and stained with coomassie to analyse the quality of the protein sample. Degradation of cellular proteins occurring either in the tissue sample or during extraction processes would result in a lack of discrete bands upon staining. This was occasionally observed though the majority of protein samples yielded the expected discrete protein bands that can be seen in figure 5.2. If protein samples appeared degraded, quantitative analysis of PKR protein content was not pursued.

5.4.5 Quantitation of total cellular protein
The general method for protein quantitation available used the Bio-Rad protein detection reagents, based on the protein binding ability of Coomassie Brilliant Blue G250 (Bradford 1976). This method is affected by the presence of detergents such as SDS which was present in the buffer used to resuspend cellular protein extracts.

To overcome this, total cellular protein samples were quantified using the method described by Warburg & Christian (1942). A dilution of the protein sample was made, generally a 1/25 dilution in H$_2$O, as well as the same dilution of 1x SDS protein buffer for use as a blank. The absorbances at 260nm and 280nm of the diluted protein sample were measured against the blank using 0.5 ml quartz cuvettes. The ratio of A$_{280nm}$/A$_{260nm}$ was used to calculate the protein concentration factor (F) which was then used to calculate the protein concentration in mg/ml, as described in section 2.10.3. This is a standard procedure for the measurement of protein mixtures as it is assumed the total content of tyrosine, tryptophan and phenylalanine residues detected by the wavelengths 280nm (Tyr, Trp) and 260nm (Phe) are proportionally equal throughout the complex of cellular proteins present.
The truncated protein PKR 1-170 used in the protein assay (section 5.10) was quantified using the Bio-Rad protein reagents (section 2.10.5). Resultant protein concentration values (given in ng/µl) were in BSA units as a standard curve of BSA had been used (section 5.6.3). Harlow and Lane (1988) reported that 0.7 absorbance units at 280 nm equalled a 1 mg/ml BSA solution. Total cellular protein concentrations quantified primarily by their absorbance at 280 nm, could be converted to BSA units by taking this 0.7 absorbance unit factor into account. Therefore the protein concentrations of PKR 1-170 and cellular proteins were comparable in terms of their BSA units as a standard measurement.

Once quantified, a known amount of total cellular protein extracted from human cells was separated by SDS-PAGE, transferred to nitrocellulose membrane and the PKR content detected by western blotting.

5.5 Quantitative western detection of PKR protein

In order to quantify PKR protein from human cells by western blotting, a quantitative assay was needed in which the level of PKR in an unknown test sample could be compared to a known protein standard curve, run on the same SDS-PAGE gel. By using a purified source of human PKR as a standard protein, ensuring the 71/10 antibody binding epitope was conserved, the same primary and secondary antibodies could be used to detect both test and standard proteins.

For the standard protein, a cloned PKR protein could be expressed in a bacterial system, purified and quantified. A known series titration of this standard would then be run alongside a specific amount of the test cellular protein sample. A range of standard protein would be used that spanned the detection limits of the assay, from the lowest amount detectable to the largest amount of protein still within the linear range of detection.

After PKR proteins had been detected, resulting in a visible band on a piece of autoradiography film, the intensity of the resultant bands corresponding to human PKR would be a measure of the amount of PKR protein present in the sample loaded. The film would be scanned into a computer and the individual band intensities measured. A standard curve would be made corresponding to the band intensities of the standard protein titration. The intensity of the PKR band appearing in the test sample would also be
measured and compared against the standard curve in order to measure the amount of protein present. Section 2.10.10 shows a worked example of this quantitative western assay in which PKR band intensities are measured in pixels which are then converted back to ng protein according to the standard curve used. The final result is given in terms of ng full length PKR per total µg cellular protein loaded.

5.6 Purified PKR protein as a standard control

In order to create a standard curve against which full length PKR from human cellular protein extracts could be compared, it was necessary to express and purify a single protein that would be detected by the 71/10 monoclonal antibody. This protein would therefore have to be either full length human PKR itself or a truncated derivative that contained the antibody recognition epitope.

Bacterial plasmid clones were available that contained sequences of full length PKR (nucleotides 1-1656) or the first 410 nucleic acid residues of PKR encoding amino acids 1-170 (PKR 1-170) both cloned into the NdeI/Bam HI sites of a pET16b vector (figure 2.3). Proteins would be expressed from the T7 RNA polymerase site directly upstream of the inserted sequence. The BL21(DE3) pLysS strain of E.coli was transformed with these two plasmid clones and used to express both forms of the PKR protein. Leaky expression of PKR, which is thought to be toxic to bacterial cells (Gale, 1998), was inhibited by the presence of the pLysS plasmid which produces the T7 RNA polymerase inhibitor T7 lysozyme. Addition of IPTG induces expression of T7 RNA polymerase from the DE3 lysogen which then induces expression of the cloned PKR protein. Increased levels of T7 RNA polymerase overcome previous inhibition by T7 lysozyme.

At the 5’ terminal of an inserted PKR sequence, the pET16b plasmid contained a coding region of 10 histidine residues (His tag). PKR expressed with this His tag in bacterial cells could be purified by the addition of a metal-chelate affinity resin (Talon resin, Clontech). The His tag would bind to this resin leaving all other bacterial proteins unbound which could simply be removed by washing the resin in buffer. Figure 5.7 shows an outline of this process. Bound PKR protein could then be eluted off the column by the addition of imidazole and used as a purified protein sample.
Figure 5.7 Overview of PKR protein expression and purification

Figure text:

pET16b/PKR

Bam HI

Protein expression

Nde I

T7 promoter

IPTG

His tag

PKR protein + Bacterial proteins

TALON RESIN

Bacterial proteins

TALON RESIN

IMIDAZOLE

TALON RESIN +

PURIFIED PKR PROTEIN

His tag PKR protein + Bacterial proteins
5.6.1 Expression of control PKR proteins

Glycerol stocks of pET16b plasmid DNA, with either full length PKR or PKR 1-170 sequences were used to transform BL21(DE3) pLysS cells by electroporation (section 2.8.7.ii). These were subcultured onto 2TY agar media with additional ampicillin (50 µg/ml) and chloramphenicol (34 µg/ml) to maintain correct plasmid selection. Single colonies were subcultured into 5 ml 2TY broth media (plus ampicillin/chloramphenicol) and incubated overnight at 37°C. 0.5 ml of this was used to inoculate a 10 ml small culture that was incubated at 37°C until the cells reached an OD<sub>600nm</sub> of 0.7. 1 ml cells was removed and lysed for analysis of uninduced proteins. PKR protein expression was then induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours (personal communication Z. Liu-Han, University of Leicester). 1 ml of the induced cultures was removed to a fresh tube, spun down to remove media and lysed directly in 2x SDS protein lysis buffer. Samples were boiled for 5 minutes and 0.25 volume each sample loaded twice onto a 12% SDS PAGE gel. Proteins were separated by electrophoresis after which one half of the gel was stained in coomassie stain followed by destaining until the background colour disappeared (figure 5.8). The second gel half was electroblotted onto nitrocellulose membrane and PKR proteins detected by western blotting using the ECL detection system (section 5.7.3) (figure 5.9).

Full length PKR protein was poorly expressed, in accordance with results found by Gale (1998). This may be due to some toxicity of the full length protein to the bacterial cells. Despite the presence of the additional pLysS plasmid, sufficient full length PKR may have been expressed to inhibit bacterial cellular functions and therefore reduce the amount of PKR expressed under induction. PKR 1-170 was expressed resulting in a large amount of the protein giving very dense bands at around 21 kDa.
Figure 5.8 Coomassie stain of PKR/E.coli proteins

Full length PKR was expressed in BL21(DE3) pLysS bacterial cells. 1 ml uninduced and IPTG-induced cell samples were taken and lysed with 2x strong SDS buffer. Protein extracts were separated by 12% SDS-PAGE and analysed by coomassie stain. Lane 1 = 20 μl IPTG-induced extract sample. Lane 2 = 10 μl IPTG-induced extract sample. Lane 3 = uninduced extract sample. All bacterial proteins are stained.

Figure 5.9 Western blot of PKR proteins expressed from E.coli cells

Full length PKR was expressed in BL21(DE3) pLysS bacterial cells. 1 ml uninduced and IPTG-induced cell samples were taken and lysed with 2x strong SDS buffer. Protein extracts were separated by 12% SDS-PAGE and western blotted using antibodies 71/10 and goat anti-mouse Ig labelled with horseradish peroxidase. Bound antibodies were detected using the ECL reagent system. Lane 1 = 20 μl IPTG-induced protein sample. Lane 2 = 10 μl IPTG-induced protein sample. Lane 3 = uninduced protein sample. A major band appears at 68 KDa, the expected size for full length PKR. Other bacterial proteins are also detected with these antibodies, the reason for this was not found. PKR was observed even in the uninduced protein sample suggesting basal expression of PKR was occurring that may explain poor cell growth observed.
In view of the difficulties in expressing full length PKR, it was decided to express and purify PKR 1-170 for use as a control protein in the quantitative western assay.

5.6.2 Purification of PKR 1-170
PKR 1-170 was expressed in BL21(DE3) bacterial cells (section 2.10.4.i). Cells were collected and lysed to obtain cellular proteins by sonication. PKR 1-170 was purified by binding to the Talon metal affinity resin (Clontech) described in section 2.9.4.iii, followed by elution with 100 mM imidazole. 5 µl each eluted sample was analysed by SDS-PAGE. Figures 5.10 shows the eluted samples containing PKR 1-170 detected by Coomassie stain.

Figure 5.10 Coomassie stained gel showing purified PKR 1-170

5µl each protein sample was boiled in 10 µl 2x SDS protein buffer for 5 minutes and loaded onto a 12% SDS polyacrylamide gel with a 5% stacking gel. Proteins were electrophoresed, the gel assembly dismantled and the resolving gel stained in Coomassie stain followed by several washes in destain until the background was removed. Lane 1 = cell supernatant after sonication prior to Talon resin addition. Lane 2 = cell supernatant after Talon resin added (unbound protein). Lane 3,4 = 4th and 5th wash of Talon resin in wash buffer. Lanes 5 to 9 = 1st to 5th elution fractions from Talon resin. PKR 1-170 is missing from unbound protein sample (lane 2) and returns after elution, proving PKR 1-170 reversibly binds to the Talon resin.
5.6.3 Quantitation of PKR 1-170

Purified PKR 1-170 protein was quantified using the Bio-Rad protein detection reagents. A 5 point standard curve of bovine serum albumin (BSA, 96% pure by weight) was made up in PKR buffer (100 mM Tris (pH 8.5), 50 mM NaCl) ranging from 60 µg/ml to 300 µg/ml. 1 ml Bio-Rad protein assay reagent (1 in 5 dilution in H₂O) was added to 30 µl each BSA sample, 30 µl PKR 1-170 buffer (blank) and 3 x 30 µl samples of PKR 1-170 at 15 second intervals. These were mixed and left to incubate for 15 minutes. The absorbance at 595 nm was measured for each sample at 15 second intervals to ensure each sample had developed for the same amount of time.

A standard curve of OD₅₉₅nm against BSA protein concentration was plotted using the software Cricket Graph (Macintosh v.1.3). From the line of linear regression, the concentration of PKR 1-170 was calculated and the mean value of the three samples tested taken as the given protein concentration.

5.7 Antibody optimisation for detection of PKR proteins

Experiments were carried out to optimise the sensitivity of PKR protein detection using the western blotting system described in figure 5.1. The monoclonal antibody 71/10 was the only antibody available that recognised both full length protein and the truncated PKR 1-170 protein. Different secondary anti-mouse antibodies were used (section 2.10.9), each conjugated to a different detection label.

A series titration of purified PKR 1-170 protein (ranging from 1 ng up to 206 ng) was electrophoresed through a 12% SDS-PAGE gel and electroblotted to nitrocellulose membrane. Proteins were detected using a 1/10,000 dilution primary antibody followed by one of the secondary antibodies described in this section. Figure 5.11 shows how each secondary antibody label acts giving a visualisation of protein detection.
Figure 5.11. Secondary antibody detection methods used

1) **Alkaline phosphatase (AP) label**

- \( X\text{-phos + NBT} \)
- Precipitated blue indole group
- Coloured product on blot membrane

2) **\( ^{35}S \) label**

- \( ^{35}S \)
- \( \beta \)-particle
- Autoradiography film

3) **Horseradish peroxidase (HRP) label**

- Peracid
- Light 428nm
- Oxidised luminol

**KEY:**
- Nitrocellulose membrane
- Primary antibody (mouse anti-human PKR)
- Bound protein
- Secondary antibody with conjugated label
- Detected protein band
5.7.1 Alkaline phosphatase labelled secondary antibody
A rabbit anti-mouse antibody was used that contained a conjugated alkaline phosphatase enzyme label. After the final wash, the membrane was incubated in alkaline phosphatase detection reagents (section 2.10.9.ii) at room temperature until no further detection of coloured bands occurred. The lowest amount of PKR 1-170 detected using alkaline phosphatase was 60 ng.

5.7.2 \(^{35}\text{S}\) labelled secondary antibody
A sheep anti-mouse Ig antibody conjugated to a \(^{35}\text{S}\) radio-label was used to detect human PKR 1-170. This was bought from Amersham Life Science who, despite repeated attempts to label the antibody stock, could only achieve a final product with half the minimum specific activity usually expected (100 μCi/mmol). This was used at a concentration of 1 μCi/ml buffer (section 2.10.9.iv) as directed by the manufacturer’s protocol (personal communication Dr. C. Brown). After the final wash, the membrane was air dried and exposed to blue sensitive autoradiography film over a time course of 14, 28 and 42 hours to observe any increase in detection of PKR 1-170 in the lower end of the range. After 14 hours bands were observed for PKR 1-170 from 55 ng to 206 ng. After 28 and 42 hours, no further bands were observed. This indicated that longer exposures did not enhance detection of the lower range of protein.

The labelled membrane blot was also analysed by phosphor imaging. The membrane was exposed to a phosphor screen overnight which was then scanned using a PhosphorImager (Molecular Dynamics Inc. USA). Areas of storage phosphors (BaFBr:Eu\(^{2+}\)) excited by the \(^{35}\text{S}\) radiation coupled to bound antibody and therefore PKR 1-170 protein bands were detected and measured using ImageQuant software (Molecular Dynamics Inc.). The lowest amount of PKR 1-170 detected using this system was 55 ng. This showed that phosphor imaging of a \(^{35}\text{S}\) labelled blot is no more sensitive than detection using autoradiography film which can itself be scanned into a computer and the visible bands analysed.

If the sheep anti-mouse antibody used here had an optimum specific activity (rather than half the optimum received), the sensitivity of this assay may then have been twice that observed. A lower level of 25 ng PKR 1-170 may have been detected. This indicates that \(^{35}\text{S}\) labelled secondary
antibodies are more sensitive at detecting PKR 1-170 protein by western blotting than an alkaline phosphatase labelled antibody. The resultant image from a $^{35}$S labelled blot is also more stable for long term storage and analysis than the coloured products of an alkaline phosphatase labelled western blot.

5.7.3 Horseradish peroxidase labelled secondary antibody
A western blotting assay was performed using a goat anti-mouse secondary antibody conjugated to a horseradish peroxidase (HRP) enzyme label. Enhanced chemiluminesence (ECL™, Amersham Life Science) reagents were added as described in section 2.10.9.iii, and the blot exposed to blue sensitive autoradiography film for a range of times (generally 5 seconds to 10 minutes) until no further protein bands were observed.

Protein detection using ECL reagents has been reported by Ong et al (1990), Cumming & Wensley (1993) and Venembre et al (1994) as being the most sensitive detection method generally available. From the PKR 1-170 titration experiments, as little as 10 ng protein was detected proving this to be the most sensitive method for the western blot detection of PKR proteins.

Table 5.2 shows the results of these detection systems together with a guide to their ease of use.

<table>
<thead>
<tr>
<th>Antibody label</th>
<th>Time of detection step</th>
<th>Permanent record</th>
<th>Lowest amount PKR 1-170 detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>&gt; 5 minutes</td>
<td>NO</td>
<td>60 ng</td>
</tr>
<tr>
<td>$^{35}$S</td>
<td>&gt; 24 hours</td>
<td>YES</td>
<td>55 ng</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>5 seconds - 10 minutes</td>
<td>YES + multiple exposures</td>
<td>10 ng</td>
</tr>
</tbody>
</table>

Given that ECL was the easiest, quickest and most sensitive detection system used, all further western blot experiments for the detection of PKR from human cells were carried out using these ECL detection reagents.

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5.7.4 Antibody titrations
Venebre et al (1994) described how high background signals were obtained when using too high concentration of secondary antibodies with the ECL detection system. The effect of antibody concentrations on the detection of PKR using the ECL detection system was therefore analysed. Both primary (mouse 71/10) and secondary (goat anti-mouse Ig) antibodies were used in different concentration combinations to detect a series titration of PKR 1-170. The primary antibody was diluted down 1:1000 and 1:10000 whilst the secondary antibody was used at 1:1000, 1:1500 and 1:2000 dilutions. A 1:1000 dilution primary antibody repeatedly failed to detect PKR protein. A 1:10000 dilution was successful at detecting the protein with any diluted secondary antibody, as recommended by the suppliers. The secondary antibody was observed to give the clearest protein detection at a dilution of 1:1000. All subsequent western blot detection of human PKR therefore used the primary antibody at a 1:10000 dilution and the secondary antibody at a 1:1000 dilution.

5.8 Comparison of nitrocellulose membranes
0.4 μm nitrocellulose matrices, either as a pure cast of unsupported nitrocellulose or supported by an inert matrix, were compared for efficiency of transfer of PKR proteins. An identical titration of PKR 1-170 protein was electroblotted to either supported or unsupported nitrocellulose membranes from a 12% SDS-PAGE gel (section 2.10.8). PKR was detected on both membranes by western blotting using HRP labelled secondary antibodies and the ECL detection system. Both membranes were treated in identical conditions. The supported membrane showed the presence of human PKR 1-170 with little background evident (figure 5.12). Figure 5.13 shows detection of human PKR on an unsupported membrane. A large amount of non-specific binding was evident on this unsupported nitrocellulose as antibodies had bound to unblocked areas of the membrane itself and the PKR 1-170 proteins seem to have transferred irregularly, though the identically treated supported membrane showed the transfer and blocking procedures to have been successful.
Titration of PKR 1-170 was electrophoresed through a 12% SDS-PAGE gel. Proteins were electroblotted to a supported nitrocellulose membrane. Protein titration used the following amounts of PKR 1-170: 60 ng (lane 1), 30 ng (lane 2), 12 ng (lane 3), 6 ng (lane 4) 1.2 ng (lane 5).

Titration of PKR 1-170 was electrophoresed through a 12% SDS-PAGE gel. Proteins were electroblotted to an unsupported nitrocellulose membrane. Protein titration used the following amounts of PKR 1-170: 60 ng (lane 1), 30 ng (lane 2), 12 ng (lane 3), 6 ng (lane 4) 1.2 ng (lane 5).

It was evident that, as unsupported nitrocellulose gave a high level of background, supported nitrocellulose was the better type of membrane to use and was therefore used for all subsequent experiments.
5.9 Comparison of protein electrotransfer methods

To compare the efficiencies of different protein electrotransfer methods, electroblotting was compared with semi-dry electrotransfer. Duplicated amounts of PKR 1-170 and cellular proteins extracted from CEM4 cells were separated by electrophoresis through a 12% SDS polyacrylamide gel. One half of the gel was subjected to semi-dry electrotransfer of the proteins to nitrocellulose membrane, as described in section 2.10.8.i. The proteins from the other gel half were transferred to nitrocellulose membrane by electroblotting (section 2.10.8.ii).

PKR from both membranes was detected by western blotting using the ECL detection system. Figure 5.14 shows protein transfer by semi-dry blot while figure 5.15 shows transfer by electroblotting. It was evident that, though quicker, semi-dry blotting did not yield as good quality protein signal than was obtained when electroblotting was employed. It was also noted that the membrane had been significantly singed during the semi-dry electrotransfer. It was therefore decided to transfer proteins from the resolving polyacrylamide gel to a nitrocellulose membrane by electroblotting.

Figure 5.14 Semi-dry blotting of Hut78 cellular proteins

Western blot detection of PKR 1-170 titration and cellular proteins extracted from CEM4 cells. Proteins transferred by semi-dry blot from SDS-PAGE gel to supported nitrocellulose membrane. PKR 1-170 titration used 2.4 μg protein (lane 1), 240 ng (lane 2), 100 ng (lane 3), 24 ng (lane 4) and 10 ng (lane 5). Total cellular protein from 4 x 10^5 CEM4 cells extracted with TriReagent was run in lane 6. Total cellular protein from 4 x 10^5 CEM4 cells lysed with 2x strong SDS protein buffer was run in lane 7.
Western blot detection of PKR 1-170 titration and cellular proteins extracted from CEM4 cells. Proteins transferred by electroblotting from SDS-PAGE gel to supported nitrocellulose membrane. PKR 1-170 titration used 2.4 µg protein (lane 1), 240 ng (lane 2), 100 ng (lane 3), 24 ng (lane 4) and 10 ng (lane 5). Total cellular protein from 4 x 10⁵ CEM4 cells extracted with TriReagent was run in lane 6. Total cellular protein from 4 x 10⁵ CEM4 cells lysed with 2x strong SDS protein buffer was run in lane 7.

5.10 Quantitative assay performed using Hut78 cells
Section 5.5 described the quantitative nature of a western blot assay for PKR extracted from human cells using purified PKR 1-170 as a standard protein to create an internal standard curve. Cellular proteins extracted (with TriReagent) from Hut78 cells were initially used in this assay to assess the procedures used. All assay components were used in identical conditions. Section 2.10.10 describes the assay procedure used to quantify PKR from human cells.

5.10.1 Differences in loading protein samples
Differences between similar loadings of a single sample of cellular protein were analysed to see what effect minor sample loading errors may have on the final assay results. Table 5.3 shows the amount of total cellular protein loaded onto a single 12% SDS-PAGE gel and the resultant amounts of PKR protein.
Table 5.3 shows a maximum variation of 19% occurring, possibly due to pipetting errors or variation in proteins precipitating out of solution between quantitation and sample loading. Some sample losses may also have occurred after quantitation due to variable condensation events during the boiling step (protein samples were boiled in a reducing loading buffer for 5 minutes, section 2.10.1.i).

5.10.2 Assessing gel to gel variation
To analyse gel to gel variations occurring, the same amount of total cellular protein from a single sample of Hut78 cells was loaded onto three different 12% SDS-PAGE gels and analysed for the PKR content. Identical amounts of PKR 1-170 standard proteins were used to create the standard curve for PKR quantitation. Table 5.4 shows the results from this assay.

Table 5.4 shows a maximum gel to gel variation of 9%. Variations may have occurred due to inaccurate sample loadings (as evident in section 5.10.1), different efficiencies of protein electroblotting to nitrocellulose membrane or even slight differences in antibody preparations. Variations of 9% may be within acceptable limits of the nature of the assay and therefore were considered sufficiently low to continue with this assay for the quantitation of PKR from human liver samples.
5.10.3 Differences in the protein extraction procedure

Variations in the PKR content of Hut78 cells cultured under constant conditions were analysed to determine whether the TriReagent extraction procedure has any effect on the proportion of PKR in total protein extracts. On 3 different occasions, protein from Hut78 cells was extracted, quantified and analysed on the same gel. Table 5.5 shows the results from this assay.

Table 5.5 Differences in cellular proteins extracted at different times

<table>
<thead>
<tr>
<th>Extraction number</th>
<th>Amount total cellular protein loaded</th>
<th>ng amount PKR per μg total cellular protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38.70 μg</td>
<td>15.36 ng/μg</td>
</tr>
<tr>
<td>2</td>
<td>36.28 μg</td>
<td>12.56 ng/μg</td>
</tr>
<tr>
<td>3</td>
<td>33.20 μg</td>
<td>14.50 ng/μg</td>
</tr>
</tbody>
</table>

Table 5.5 shows the differences in the PKR content of Hut78 cells that were extracted at different times. The largest difference evident is 18%. Some of these differences may be due to variations in loading techniques, solubility of the protein sample or because the Hut78 cells were under slightly different growth conditions at each sampling, thus reflecting genuine variation in PKR expression.

It was decided to proceed with analysis of PKR from HCV infected human liver samples. The 9% to 19% differences seen when test samples were compared may prove to be acceptable within the levels of protein detection.

5.11 PKR from livers of HCV negative patients

7 liver biopsies from patients previously tested negative for HCV infection were obtained from the Queens Medical Centre, Nottingham (6) and Leicester Royal Infirmary (1). Total cellular protein was extracted using TriReagent under category III laboratory conditions as described. Table 5.7 shows the results from these extractions.
Table 5.6 Results of PKR quantified from non-HCV infected livers

<table>
<thead>
<tr>
<th>Liver biopsy sample number</th>
<th>ng full length PKR per µg total cellular protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>no PKR detected</td>
</tr>
<tr>
<td>B2</td>
<td>no PKR detected</td>
</tr>
<tr>
<td>B3</td>
<td>2.98 2.37</td>
</tr>
<tr>
<td>B4</td>
<td>no PKR detected</td>
</tr>
<tr>
<td>B5</td>
<td>4.77 4.62</td>
</tr>
<tr>
<td>B6</td>
<td>no PKR detected</td>
</tr>
<tr>
<td>B7</td>
<td>no PKR detected</td>
</tr>
</tbody>
</table>

The results shown in table 5.6 indicate that PKR protein was detected in 2 out of the 7 livers tested. Patient B3 had been tested negative for all known microbiological causes of hepatitis and was therefore diagnosed with autoimmune hepatitis. Patient B7 had been admitted to hospital suffering severe hepatitis. All biochemical and microbiological tests ruled out known causes of hepatitis. The levels of PKR seen for patients B3 and B7 may have been due to some unknown micro-organism infecting the liver cells or even some cellular (maybe genetic) process that naturally upregulated PKR expression at the time the liver biopsy samples were taken.

PKR protein was not evident in the cellular extracts of livers from patients B1, 2, 4, 6 and 7. The reasons for the apparent increase in PKR expression in patients B3 and B5 could not be found. It seems from these results that PKR expression in human liver may not be constant, even in the absence of an interferon inducing factor such as a virus infection. This may then indicate that the level of PKR protein in HCV infected patients will not be dependent on their virus infection but may alter due to some other, unknown factor.
5.12 Quantitative western assay for PKR protein from HCV infected liver tissue

Total cellular protein from 62 liver biopsies obtained from HCV infected patients was extracted using TriReagent. Protein concentrations were quantified and the PKR content detected and quantified using the western assay described in section 2.9.10. The majority of liver samples yielded very small amounts of protein. When possible, sample duplicates were analysed though quite a few protein samples were too small to be analysed more than once. This occurred most often with the liver samples obtained from the Leicester Royal Infirmary. Section 4.9.5 discussed the limitations and low yields of cellular RNA extracted from these half biopsy samples. The same limitations would be true for the yields of proteins extracted. At least three results would be required for each sample to prove the significance of the data obtained, however this was not always possible. Table 5.8 and figure 5.16 the PKR results obtained from HCV infected human liver samples. The limit of PKR detection was 0.01 ng/µg. Figures 5.17 to 5.22 show these PKR results plotted against various patient and virus parameters.

All patient and virus data was supplied by the Trent HCV Study Group.

Knodell scores give a measure of the severity of liver cell damage when biopsy specimens were analysed by microscopy. The Knodell score is taken as the sum of four sub-scores (Table 5.7). All samples were assessed by Dr J. Underwood (Queens Medical Centre, Nottingham) and were given on a scale of 0 to 22 (personal communication Dr W. Irving, Queens Medical Centre, Nottingham) where 0 indicates no damage and 22 indicates very high levels of liver damage.

HCV virus titres were all quantified using the Quantiplex™ HCV RNA 2.0 bDNA assay (Chiron Diagnostics Ltd, Essex, UK). Results were given as MEq/ml, or $10^6$ copies/ml, HCV genomic transcripts present per ml of serum.

Table 5.7 Histological factors contributing to a Knodell score

<table>
<thead>
<tr>
<th>Histological feature</th>
<th>Sub-scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periportal/bridging necrosis</td>
<td>0, 1, 3, 4, 5, 6 or 10</td>
</tr>
<tr>
<td>Intralobular necrosis</td>
<td>0, 1, 3 or 4</td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>0, 1, 3 or 4</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0, 1, 3 or 4</td>
</tr>
<tr>
<td>Sample number</td>
<td>ng PKR per µg total protein</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>1</td>
<td>1.82</td>
</tr>
<tr>
<td>2</td>
<td>3.88</td>
</tr>
<tr>
<td>3</td>
<td>1.01</td>
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<td>15</td>
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<tr>
<td>16</td>
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<tr>
<td>17</td>
<td>0.2</td>
</tr>
<tr>
<td>18</td>
<td>3.12</td>
</tr>
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Figure 5.16 Graph showing duplicated PKR protein assay results

Key: + ◊ ○ all denote duplicated assays (1st, 2nd and 3rd respectively) for the same cellular protein sample.
Figure 5.17  Duplicate PKR protein assay results plotted against sex of the patient (1=male, 2=female)
Figure 5.18  Duplicate PKR protein assay results plotted against age of the patient at time of biopsy
Figure 5.19  Duplicate PKR protein assay results plotted against Knodell score of the biopsy specimen
Figure 5.20 Duplicate PKR protein assay results plotted against HCV genotype of virus detected in patients
Figure 5.21 Duplicate PKR protein assay results plotted against patient response to interferon alpha treatment
Figure 5.22 Duplicate PKR protein assay results plotted against HCV virus titre measured from patient serum
Figure 5.16 shows that there is very little relationship between duplicated protein sample assays. Statistical analyses using the paired Student’s t-test and the Wilcoxon test (section 2.10.11) were performed on the duplicated PKR results data to measure the degree of difference present. An F-test (section 2.10.11) was also performed to measure the degree of variation between paired data. The results can be seen in table 5.9 in which the values for the 5% confidence intervals are given for comparison.

<table>
<thead>
<tr>
<th>Paired PKR assay data</th>
<th>Paired t-test</th>
<th>Wilcoxon test</th>
<th>F-test</th>
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<tr>
<td></td>
<td>t</td>
<td>t at 5%</td>
<td>z</td>
</tr>
<tr>
<td>1st vs. 2nd</td>
<td>-5.44</td>
<td>± 1.699</td>
<td>-4.45</td>
</tr>
<tr>
<td>2nd vs. 3rd</td>
<td>-3.45</td>
<td>± 2.015</td>
<td>-1.99</td>
</tr>
<tr>
<td>1st vs. 3rd</td>
<td>-6.31</td>
<td>± 2.015</td>
<td>-2.20</td>
</tr>
</tbody>
</table>

Though the Wilcoxon test compares the similarities of paired data, it is known to be less reliable than the Student’s t-test, especially for very small data sets. This is seen in table 5.9 in which the data set comparing 2nd and 3rd PKR assays only contains 6 paired data entries. The value for z seems to be within the 5% confidence level. However, the more reliable Student’s t-test gives a comparative value well outside the 5% level of confidence.

The overall results suggest that the paired data are outside the 5% level of confidence in these tests which proves that there are significant differences between these duplicated sets of data. Some factor (or factors) of the assay must therefore have a significant effect on the final results. Many of these sample duplicates were run on different gels and so this variation may be due to differences in pipetting and sample loading, efficiencies in gel blotting or differences in the antibody probes, though all parameters were run to the same procedure. There may be a degree of natural variation (that is significant) between samples run on different gels at different times.
PKR 1-170 used in the PKR quantitative western assay ranged from 10 to 45 ng protein. This spans less than half an order of magnitude. Below 10 ng PKR 1-170, protein bands were detected as background due to the background colour of the autoradiography film. Above 45 ng PKR 1-170, protein bands were all detected as black and were therefore above the linear range of detection which measured protein bands on a grey scale from 1 to 256 (black being measured at the 256 level).

Venembre et al (1994) and Amersham International plc. (chicken myosin quantitation described in their ECL western blotting protocols) both used a range of standard protein in quantitative western blotting assays spanning at least one order of magnitude. The range of PKR 1-170 standard protein used here was therefore half that used in assays reported elsewhere. Using such a narrow range of standard protein made differences between test sample duplicates more evident as natural variations between assays were enhanced rather than smoothed out, as would be expected from using a larger range of standard protein.

Variations arising from pipetting errors, differences in protein transfers and variable protein precipitation between samples prior to loading may all have played a role in the variations seen which would also have been exaggerated due to the narrow range of standard protein used.

Due to the variation present between duplicated samples, any further analysis of patient liver PKR expression in light of factors such as patient sex, age, severity of liver damage (Knodell score), virus genotype and virus titre would not be meaningful. The graphs shown in figures 5.17 to 5.22 show the varied nature of this data but unfortunately, no statistical inferences may be reliably made.
5.13 Discussion

Prior to the experiments described in this chapter, little work had been reported of the quantitation of PKR in humans. In 1993, Haines et al analysed the presence of PKR proteins in squamous cell carcinoma cells from the head and necks of patients. Jeffrey et al (1995) analysed the subcellular localisation of PKR protein in a NIH3T3 cell line using a $^{125}$I labelled secondary antibody to detect PKR protein bound to nitrocellulose in a western blotting procedure. A quantitative assay for PKR protein was used, though the expression of the amount of PKR per number of cells extracted did not take into account possible protein losses during the extraction and quantitation procedures.

The purpose of the experiments carried out in this chapter was to analyse the expression of PKR protein in the livers of HCV infected patients in light of patient factors such as age, HCV viral titres and genotypes, alanine aminotransferase levels, histological damage (Knodell score of extent of liver cell fibrosis and necrosis) and response to interferon alpha treatment. In light of the presence of a 48 kDa species of human PKR protein, thought to be a product of PKR proteolysis, that would be detected using the monoclonal antibody 71/10, it was decided to develop a quantitative western blotting assay.

Total cellular proteins extracted from human liver tissue were separated by SDS-PAGE, electroblotted to a supported nitrocellulose membrane and probed with the 71/10 primary monoclonal antibody specific to human PKR and then a secondary antibody specific to the primary antibody that had a conjugated label used for the visual detection of the PKR protein/antibody sandwich.

Experiments carried out to optimise the detection system of this western assay proved that electroblotting of the cellular proteins to a supported nitrocellulose matrix reduced background noise upon detection and also resulted in sharper band images.
Use of differently labelled secondary antibodies resulted in the use of horseradish peroxidase conjugated to a goat anti-mouse polyclonal antibody. This was detected by the addition of enhanced chemiluminescence (ECL) reagents that not only gave a very fast result but had the advantages of being cheaper, more stable and less hazardous than a radioactive label such as $^{35}$S. The use of ECL reagents also meant multiple exposures over a time range could be used to ensure the maximum signal to background ratio was achieved. These findings were in accordance with those of Ong et al (1990) and Venembre et al (1994).

In 1997, Basu et al assayed peripheral blood mononuclear cells (PBMCs) for the presence of PKR protein and mRNA from patients with haematological malignancies. Total cellular proteins were separated by SDS-PAGE and detected by western blotting using the ECL system. PKR was not quantified in this report, merely the proportion of cells containing PKR protein were analysed. Similarly, Vojdani et al (1997) used the ECL detection system to quantify PKR proteins in PBMCs from patients suffering from chronic fatigue syndrome. The latter report described that a quantitative western assay was developed though the nature of any standard to which PKR present was compared, or the relative value of units used to quantify PKR protein was not made.

The nature of the quantitative western assay described in this chapter has been proven by other groups as the best way in which full length PKR can be identified from smaller proteins containing PKR epitopes (products of PKR degradation). ECL is the most sensitive method of protein detection that has other advantages described previously. The assay, by which full length PKR was quantified against a standard purified protein titration, was thought to be the most suitable method for PKR quantification. Values of PKR protein in relation to the amount of total cellular protein present in an extracted sample took into account protein losses through the extraction and assay procedures as the amount PKR lost would be proportional to all other cellular protein losses.
Experimental assays carried out using cellular proteins from cell culture samples indicated inter-gel variations of up to 9%. When PKR was detected and quantified from cellular protein samples extracted from HCV infected liver samples, duplicate assays varied by over 9%. This suggests that, though PKR can be quantified in a single assay, the system used has an inherent variation that alters with each assay performed. This may be due to a too narrow range of the standard protein used though at either end of this range (10 to 45 ng PKR 1-170 protein), the assay becomes non-linear and cannot be used.

The major problem with the assays performed on patient liver tissue was the size of the sample. The small size (half a liver biopsy in many cases) proved a problem in obtaining sufficient cellular protein to be able to carry out many duplicate assays. Larger tissue samples would have resulted in higher yields of cellular proteins, allowing more duplicate assays to be performed, thus giving a much larger set of data that would have proven to be statistically more significant. The size of biopsy samples available were limited due to the invasive nature of the procedure. Ethical guidelines would therefore not permit further samples to be taken.

Venembre et al (1994) reported the lack of success when using an ECL detection western blot assay to develop a quantitative assay for the human α1-antitrypsin protein. The manufacturers of the ECL detection reagents (Amersham International plc.) carried out a quantitative western blot assay to measure chicken myosin from test samples against a standard curve of the same protein. This assay was not, however, repeated and so any gel to gel variation not reported.

Due to the highly variable nature of the quantitative western assay for PKR protein, no firm conclusions could be made from the data obtained for PKR protein expression in HCV infected patients.

To reduce gel to gel variation, much larger SDS-PAGE gels could have been used in order to analyse more samples on the same gel. However this would not be possible, or desirable, in any clinical setting where cellular
protein samples would be analysed for their PKR content over time. In order for an assay of this sort to be of use, it would have to be proven repeatedly accurate so that results from the same and different samples would all be comparable. This cannot be proven true for the assay developed here though future developments in the separation and detection of specific proteins may result in a reliable system for the quantitation of full length human PKR protein in tissue samples such as the livers of HCV infected patients.
Chapter 6

Conclusions
6.1 Conclusions

The lack of an *in vitro* system for the propagation of HCV that would facilitate molecular analysis of virus protein localisation and function has hampered studies of HCV. Such an *in vitro* system would also aid the study of novel anti-viral compounds in the bid to find a vaccine or therapy that is more effective than interferon-alpha.

The work carried out in chapter 3 attempted to develop an *in vitro* system for the expression of virus-like particles in which a molecular clone of HCV, encoding the polyprotein coding region, would express HCV proteins in mammalian cell culture. In order to achieve this, attempts were made to clone regions of the HCV RNA genome. Initial problems in extracting genomic RNA for use in RT-PCR reactions from HCV infected patient serum occurred. These were due to using a sub-optimal extraction method and oligonucleotide primers that were non specific for genotype 3a HCV (type 3a had been proven present in the serum samples). The lack of an available cloned HCV sequence DNA template that could be used as a positive control in PCR reactions hampered the identification of where problems in such amplification reactions were occurring. Also, the amounts of genomic RNA extracted were too small to be spectrophotometrically quantified which meant it was not known how much HCV genomic RNA was being used in any one RT-PCR reaction.

On the advice of Dr L. Jarvis (University of Edinburgh), the method of genomic RNA extraction was rectified by removal of serum proteins prior to virus disruption by SDS and proteinase k. The publication of a sequence for type 3a HCV allowed the design of primers specifically targeted to HCV RNA extracted from infected serum. The availability of control primers directed to the 5'UTR region of type 3a HCV allowed control RT-PCR reactions to be carried out.

Nested RT-PCR reactions were carried out to increase the specificity of amplified products, as used by Lin *et al* (1992), Reid *et al* (1994) and Mellor *et al* (1995). Analysis of different DNA polymerase enzymes proved that KlenTaq (a mix of Taq and Deep Vent) was the best system for the amplification of long HCV sequences.
The initial aim was to create a molecular clone of almost the entire length of the HCV genome. This would entail amplification of about 9.0 kb nucleic acid which did not result in any PCR products. Selby et al (1993), Suter & Pauli (1995) and Mizuno et al (1995) described the use of fragment cloning to amplify a very long sequence. Fragment cloning of the HCV genome was therefore used. To create a molecular clone of HCV that encoded the structural proteins, primers were designed against type 3a HCV that amplified the first half of the genome in three overlapping regions that could be sequentially cloned and pieced together using internally encoded restriction sites. The idea was to clone these fragments in reverse orientation, removing potential problems of leaky expression of HCV proteins that may be toxic to the bacterial cells.

RT-PCR amplifications of the different fragments of HCV encoded by the new set of primers resulted in DNA bands of the correct size. Despite repeated attempts to ligate amplified DNA products into correctly digested vector DNA and use this to transform bacterial cells, no molecular clones containing HCV sequence were obtained. Some clones containing sequences of *Saccharomyces cerevisiae* were found, the source of this contamination was not determined. Insufficient PCR products were obtained to sequence directly and therefore the nature of the PCR products could not be determined.

After these experiments were performed, several groups reported successful attempts at cloning the HCV genome, most notably clones developed by Kolykhalov et al (1997) and Yanagi et al (1997 & 1998). RNA transcribed In vitro from full length genomic clones of HCV was found to cause hepatitis in previously naive chimpanzees, with HCV virus corresponding to the cloned sequence recovered from these chimps. Other groups have also reported the cloning of various proteins of HCV and their expression in cell culture for analysis of these protein structures and functions. To date, however, no report has been made of the propagation of a full length clone of HCV in mammalian cell culture resulting in production of virus like particles of HCV. The aim of the project described in chapter 3 still has relevance in the development of a cell culture based system for the molecular analysis of HCV and its constituent proteins. This would enable a greater understanding of how this virus acts together with a method by which potential anti-viral compounds could initially be tested.
The uses of PKR and 2′5′oligoadenylate synthetase as factors for the prediction of HCV patient response to interferon-alpha therapy was also investigated. Assays to quantitate PKR mRNA and protein in the livers of HCV infected patients prior to interferon-alpha treatment were the subject of chapters 4 and 5. Initial experiments to develop a quantitative assay for 2′5′oligoadenylate synthetase mRNA did not succeed. In view of reports by Okuda et al (1994) and Pawlotsky et al (1996) that this interferon effector cannot be used as a predictive factor of interferon-alpha treatment response by HCV patients, it was decided to concentrate on the analysis of PKR expression in the livers of HCV patients.

Hut78, CEM4 and Chang cell cultures were all proven to express PKR mRNA (by RT-PCR) and protein (by western blotting analysis). Cellular RNA and protein extracts from these mammalian cell lines were used as templates in developing the quantitative assays described. The samples of HCV infected patient liver tissue obtained were very small, generally half a biopsy sized piece of tissue. From each sample, both mRNA and protein had to be extracted for analysis of their PKR content. For this, TriReagent was chosen and proven to be efficient in the extraction and separation of total cellular RNA, DNA and protein in obtainable phases. RNA and proteins extracted in this way were not degraded when fresh cell samples were used.

Initial experiments to develop a quantitative RNase protection assay for PKR mRNA did not succeed though subsequent oligo protection assays did. Unfortunately, a relatively large amount of cellular RNA was required for each test reaction, more than was obtainable from half a liver biopsy. A more sensitive method was needed that would detect PKR mRNA from a much lower amount of cellular RNA, RT-PCR amplifications. Initial experiments became contaminated with PKR DNA, probably originating from the laboratory. This problem was solved by moving laboratories and ensuring all reagents and apparatus used had not come into contact with the previous laboratory.

Co-amplifications of PKR and both GAPDH and β-actin mRNA were successful. However, in light of a report made by Wang et al (1989), to develop a fully quantitative assay for PKR mRNA, it was decided to use an RT-PCR assay where an in vitro transcribed RNA acted as a competitor within each test reaction. A molecular clone was created from the plasmid
pNB 92/008 which contained the sequence of PKR amplified by primers PKR1 and PKR2 but had an additional 79 bp piece of DNA inserted within the two primer binding sites. Amplified PCR products from this competitor RNA were distinguished from PKR mRNA products by size.

A fully competitive assay to quantify PKR mRNA in a known amount of total cellular RNA extracted from human cells was successfully developed. Unfortunately, problems arose when analysing PKR mRNA from the samples of HCV infected liver tissue obtained. Cellular RNA was degraded in samples obtained from the Queens Medical Centre in Nottingham. Those samples obtained from the Leicester Royal Infirmary (these had been snap frozen in liquid nitrogen) generally contained intact cellular RNA but were half a biopsy in size and therefore did not yield sufficient cellular RNA to perform an assay using a range of amounts of control RNA. In conclusion to these experiments, the assay for PKR mRNA was developed as a fully competitive quantitative system but the samples used were either degraded or too small for levels of PKR mRNA to be analysed.

Quantitation of the expression levels of PKR protein was the aim of experiments carried out in chapter 5. Due to the possible presence of a 48 kDa species of PKR, suggested by Galabru & Hovanessian (1987) to be a product of full length PKR degradation, separation of cellular proteins by SDS-PAGE was the only method available that distinguished full length PKR from its products of degradation. Western blotting of human PKR from cell samples was successful. The antibody 71/10 was proven to detect human PKR only and a horseradish peroxidase labelled secondary antibody detected by ECL reagents proven to be the most sensitive method of protein detection.

To create a quantitative western blot assay, a standard curve of an internal control protein was used in each gel blot, against which the amount of full length PKR protein was compared and quantified. The truncated PKR 1-170 was used as the standard control protein and was successfully expressed and purified from a bacterial cell culture system.

Unfortunately, many of the liver samples obtained were very small and yielded too little cellular protein to run many duplicate assays. Those samples that were run 2 or 3 times on different gels showed a significant
degree of variation between duplicates. These results indicated that there was some inherent problem of variation with the assay itself. As a result of this, PKR results obtained could not be analysed against other patient or virus data with any degree of confidence. Larger samples of patient liver tissue could not be obtained due to the physiological and therefore ethical stresses on the patient. Use of the assay to quantitate PKR protein was therefore severely limited.

To date, no other group has reported the quantitation of PKR protein or mRNA in a truly quantitative manner that takes into account losses during extraction procedures and tube to tube variations between RT-PCR reactions. Certainly no one has reported the evaluation of PKR as a factor to predict the response of an HCV infected patient to interferon therapy. Should samples of HCV infected liver that have been stored correctly at -80°C or below since acquisition be made available, provided they are at least the size of a full biopsy, the quantitative competitive RT-PCR and western blot assays could be used by other groups for the quantitation of PKR mRNA and protein for the evaluation of PKR as a predictive factor of interferon-alpha response by HCV patients.

Due to financial and time constraints and the limited availability of biopsy material, further analysis of the quantitative western assay for PKR protein could not be continued. As a major limitation of this assay seemed to be the narrow range of standard protein used, future developments of the ECL detection system and analysis software packages would be expected to facilitate the use of an increased standard protein range, dampening the effect of natural gel to gel variation found during this work. This quantitative western blot assay should then be ideal for use in further experiments where the amount of a given protein is to be determined, provided the user has a monoclonal primary antibody specific to that protein and can obtain purified samples of that protein (or a truncated species containing the antibody-recognition epitope).
6.2 Future work

This work remains important in having developed assays to quantify PKR mRNA and protein for use in future analyses into the role of PKR in HCV infection. Gale et al (1997 & 1998) proposed that the NS5a protein of HCV interferes with the dimerisation domain of PKR. However, there have been no reports about the effect this interaction has on the level of cellular PKR. Questions arise that should be addressed in order to understand the effect PKR has on HCV replication and the effect that HCV has on the host’s immune response. Some questions that may be addressed include,

- Does the level of PKR mRNA expression in HCV infected cells remain unaltered?
- Is the level of PKR protein in HCV infected cells reduced?
- Does the NS5a protein bind irreversibly to PKR protein such that no PKR monomers can be detected in the cell cytoplasm?
- If the NS5a protein sequesters PKR in the cytoplasm, what happens to the nuclear fraction of PKR, is it reduced or does it remain unaltered?

Use of the assays developed here to quantitate PKR mRNA and PKR protein could be used to answer such questions in order to understand the pathogenic mechanisms by which HCV replicates.
References


Barnes WM. (1994) PCR amplification of up to 35-kb DNA with high fidelity and high yield from bacteriophage templates. Proceedings of the National Academy of Science (USA) 91: 2216-2220.


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is not a predictor for sustained response to treatment. *British Journal of Haematology* **86**: 816-819.


Yanagi M, Purcell RH, Emerson SU, Bukh J. (1997) Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proceedings of the National Academy of Sciences (USA)* **94**: 7738-8743.


