SCCA1-MEDIATED BINDING OF HEPATITIS B VIRUS TO HOST CELLS

A Thesis Submitted for the Award of Doctor of Philosophy

By

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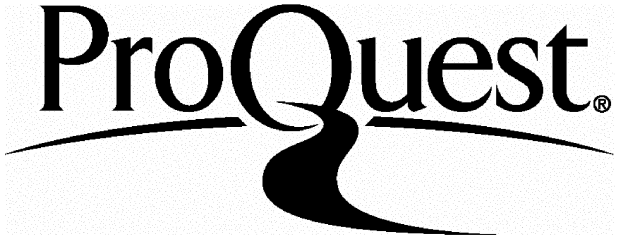

DEDICATION

To EH, with love.

ABSTRACT

The recent report by De Falco *et al.* (J. Biol. Chem., 2001, 276:(39) 36613-23) that SCCA1 may play a role in the infection of host cells by HBV was the basis of the work described in this thesis. The purpose of this study was to confirm the findings described in that paper, and then to investigate the mechanism whereby SCCA1 mediates the binding of HBV to host cells. A cDNA for SCCA1 was cloned, its sequence analysed, and the effect of SCCA1 expression on virus-cell binding assayed in hepatocyte-derived cells. Virus internalisation and replication in SCCA1-transfected cells were also investigated. Recombinant SCCA1 was produced in a bacterial expression system and used in competitive binding experiments with HBV. The possibility that the protease inhibitory activity of SCCA1 was important in the mediation of enhanced virus-cell binding was of interest, especially considering previous reports that treatment of HBV with the V8 protease enhances infectivity. Protease inhibition is mediated by the reactive site loop (RSL) of a serpin. Site-directed mutagenesis was utilised to introduce mutations into the RSL thereby abrogating the ability of the serpin to inhibit proteases. Infectivity assays using the RSL mutants showed that the RSL is not required for enhanced virus-cell binding. The known hepatic clearance of serpin-enzyme complexes was intriguing, and led to investigations into the possibility that HBV subverts this clearance system in order to gain access to hepatocytes. Therefore, studies were performed also to investigate the effect of SCCA1 expression on the receptor proposed to mediate hepatic clearance of serpins, the LDL receptor related protein (LRP). Real-time PCR showed that SCCA1 expression in hepatocyte-derived cells (Huh7) resulted in up-regulation of LRP. However, competitive binding assays which were performed with RAP (receptor associated protein), a well-characterised ligand of the LRP, showed that SCCA1-mediated virus-cell binding does not result from enhanced expression of LRP in SCCA1-transfected cells.

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ABBREVIATIONS

A2M	alpha 2 macroglobulin
A2M*	methylamine activated alpha 2 macroglobulin
ALT	alanine aminotransferase
AMC	7-amino-4-methoxy coumarin
bp	base pairs
cccDNA	covalently closed circular DNA
cDNA	complementary DNA
ddH_2O	double distilled water
DEPC	diethyl pyrocarbonate
DHBV	duck hepatitis B virus
DMEM	Dulbecco's Minimal Essential Medium
DMEM10	Dulbecco's Minimal Essential Medium containing 10% serum
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	dithiothreitol
ER	endoplasmic reticulum
g	grams
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
HBcAg	HBV core antigen
HBsAg	HBV surface antigen
HBV-BP	HBV-binding protein
HBV	hepatitis B virus
HBx	HBV X protein
HCC	hepatocellular carcinoma
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilobases
LB	Luria-Bertani medium

LDL	low density lipoprotein
LHBs	large HBV envelope protein
LRP	LDL receptor related protein
nm	nanometre
M	molar
Mab	monoclonal antibody
ml	millilitre
MHBs	medium HBV envelope protein
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
pgRNA	pregenomic RNA
pHSA	polymersied human serum albumin
RAP	receptor associated protein
rcDNA	relaxed circular DNA
RSL	reactive site loop
SCC	squamous cell carcinoma
SCCA1	squmous cell carcinoma antigen 1
SEC	serpin-enzyme complex
serpin	serine protease inhibitor
SHBs	small HBV envelope protein
ssDNA	single stranded DNA
TE	Tris EDTA buffer
TEMED	tetramethylethylenediamine
TBS	Tris buffered saline
TBST	Tris buffered saline containing 0.02% tween 20
µl	microlitre
µM	micromolar
WHV	woodchuck hepatitis virus

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AMINO ACID ABBREVIATIONS

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lysine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

CHAPTER I

GENERAL INTRODUCTON

1.1 Introduction

Hepatitis, or jaundice, has been observed for centuries. Outbreaks of hepatitis were reported during military campaigns from the 17th to the early 20th centuries, when the disease came to be known as "campaign jaundice" (Zuckerman, 1979). Outbreaks of hepatitis also were common among civilians and in 1912, Cockayne named such epidemics of jaundice "infective hepatitis". By the 1940s, a second form of hepatitis ("serum hepatitis") had been identified and, in 1947, MacCallum (1947) introduced the terms "hepatitis A" for infectious hepatitis and "hepatitis B" for serum hepatitis. The development of the field of viral hepatitis can be traced from the distinction made by Saul Krugman of the two types of hepatitis, type A being transmitted via the faecal-oral route and type B being parentally transmitted (Krugman *et al.*, 1967). Hepatitis B virus was the first of the hepatitis-causing viruses for which protein and genome were identified. The serendipitous discovery (whilst he was looking for polymorphic serum proteins) by Baruch S. Blumberg of "Australia antigen" in 1963 was the first step towards evidence that type B hepatitis was of viral aetiology (Alter and Blumberg, 1966; Blumberg *et al.*, 1967). In 1970, Dane used immunoelectron microscopy to identify the virus-like particles, now known to be HBV, which acquired his name (Dane *et al.*, 1970). These particles were found to contain an endogenous polymerase activity within the core (Kaplan *et al.*, 1973), which was further indicative of the viral nature of the "Dane particles", a fact that was confirmed finally by the identification of the genome of the hepatitis B virus (Robinson *et al.*, 1974).

1.2 Clinical significance of the virus and epidemiology

Despite the 25 years since these first observations were made and the development of an effective vaccine which has been included in the Expanded Program of Immunisation in many countries, the public health significance of hepatitis B virus (HBV) remains extremely high, especially in Africa and Asia. It is estimated that more than 2 billion people today have been infected by the virus (De Meyer *et al.*, 1997) and that HBV infection causes a mortality of 1 – 2 million per year. As many as 387 million people are chronic carriers of hepatitis B surface antigen and of these a

quarter will go on to develop hepatocellular carcinoma (HCC) (Arbuthnot and Kew, 2001). The rapid course of the malignancy and the poor prognosis of HCC patients results in an annual mortality rate from HCC that is virtually the same as its annual incidence (Evans and London, 1998).

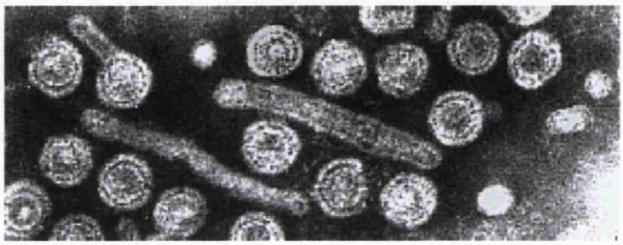
1.3 The structure of hepatitis B virus

1.3.1 Virus morphology

Within the blood of infected persons, there are three particles exhibiting different morphologies. The 42 nm Dane particle is the "true" (i.e. infectious) viral particle. The virus is a spherical double-shelled structure – the outer protein shell is comprised of HBs (S, M and L) protein, with the inner shell, referred to as the core particle or capsid, being comprised of HBc protein. The core encloses the partially double-stranded DNA genome to which the viral polymerase is covalently attached at tyrosine 63 through a phosphodiester bridge to the 5' end of the L(-) strand (Weber *et al.*, 1994). There are also a number of other host proteins which associate with the virus-encoded structural proteins. These include a cellular protein kinase C within the core particles (Gerlich *et al.*, 1982), which is responsible for phosphorylation of serine residues within the carboxyterminal portion of the core protein (Kann *et al.*, 1993).

A heat shock protein, hsc70, associates with the internal preS1 domain of duck HBV (DHBV) and a number of other proteins are thought to associate more loosely with the envelope of the virus, including polymerised human serum albumin (pHSA) and anti-HBs antibodies (Kann and Gerlich, 2000). There are also two types of subviral particles, a spherical 17-22 nm particle comprised of HBs protein (these particles are extremely abundant, reaching concentrations of 10^{11} per ml in serum) and filamentous particles of approximately 20 nm in diameter and of variable length (also comprised of HBs but less abundant in serum) (Dane *et al.*, 1970). Neither subviral particle contains DNA and they are therefore not infectious.

A)



B)

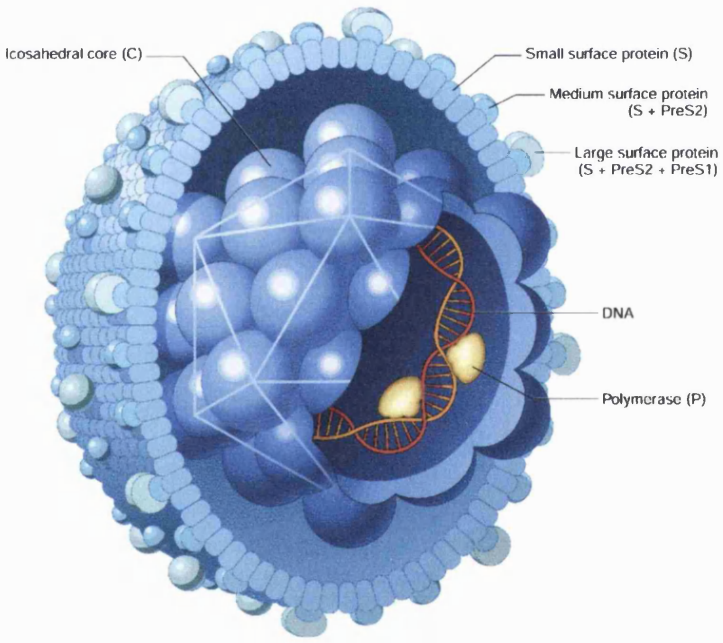


Figure 1.1 (A) Electron micrograph showing the Dane particle and both the filamentous and spherical subviral particles (Linda Stannard, University of Cape Town, South Africa); (B) Schematic showing the structure of the hepatitis B virion (Visible Genetics Inc.).

1.3.2 Genome structure

The genome of hepatitis B virus is a circular, partially double-stranded DNA molecule (Robinson *et al.*, 1974) with a single stranded region of variable length. The longer strand, the minus or L(-) DNA strand, which is complementary to the mRNAs, is approximately 3.2 kb in length. Conventionally, numbering of the bases within the genome starts at the *EcoRI* cleavage site within the genome (or at the corresponding position in those variants which do not encode that restriction site) (Kann and Gerlich, 2000). The shorter plus or S(+) strand is approximately 50-70% of the length of the L(-) strand, with the position of the 5'-ends of both strands being fixed and the position of the 3'-end of the S(+) strand being variable. The partially single-stranded nature of the genome results in a relaxed circular conformation (rcDNA). The circular conformation of the viral genome is maintained by the presence of 224 bp 5' cohesive termini, which also contain an 11 bp direct repeat (DR1 and DR2, 5' TTCACCTCTGT 3') which is required for the initiation of viral DNA synthesis (Molnar-Kimber *et al.*, 1984).

The HBV genome is remarkably compact. It is only slightly larger than the largest open reading frame (ORF) of the virus and utilises a number of strategies to encode four separate ORFs which themselves may encode multiple proteins. The longest ORF, P, encodes the viral polymerase. The S/preS ORF is completely located within ORF P but utilises an alternative reading frame. ORF S/preS allows transcription of three co-carboxyterminal proteins using internal initiation codons. ORF C, which encodes the HBc and HBe proteins, and ORF X, which encodes HBx protein, partially overlap ORF P. Regulatory genetic elements which control the viral transcription, RNA processing and translation of proteins also are situated within coding regions (Kann and Gerlich, 2000).

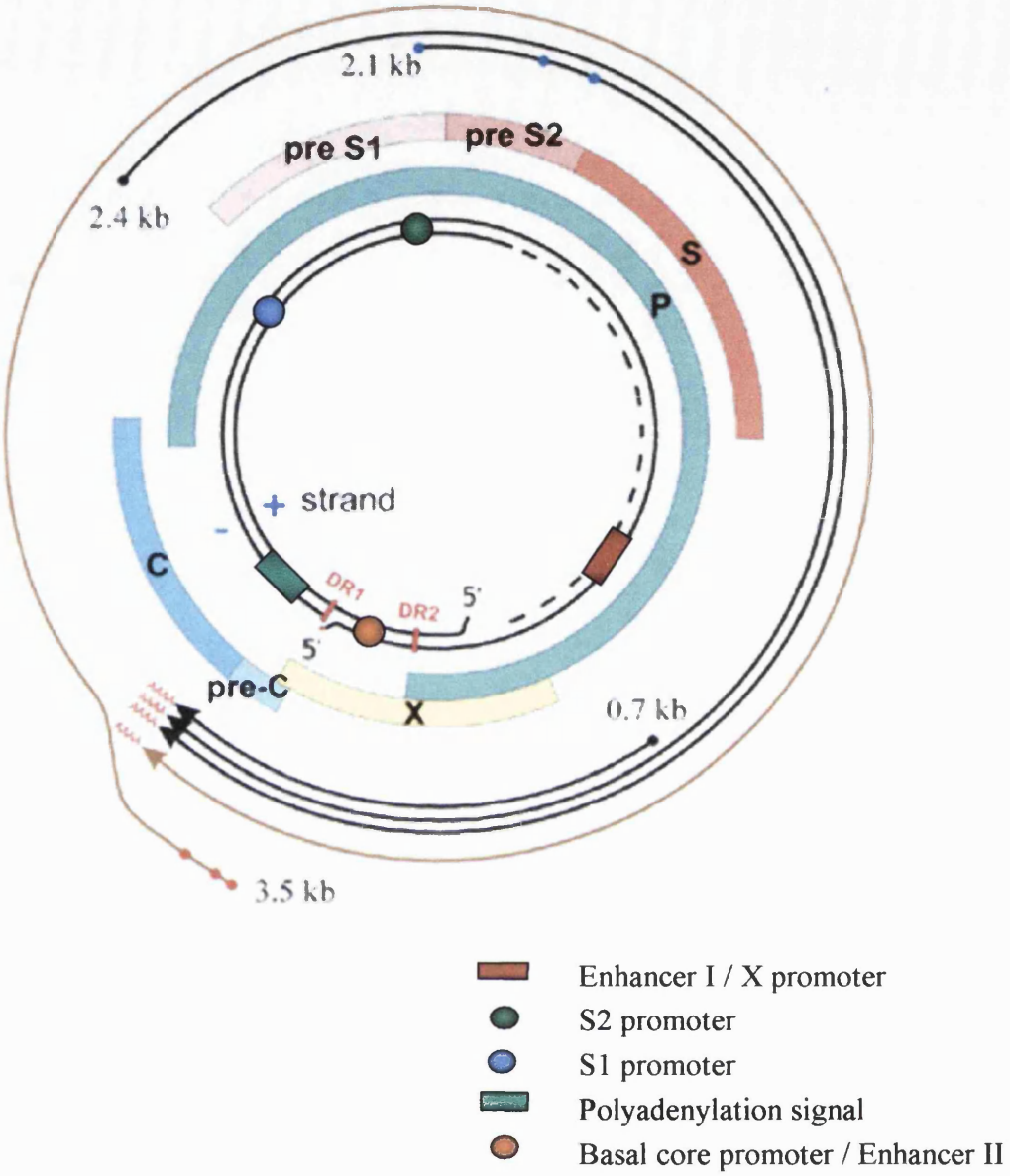


Figure 1.2 Schematic representation of the HBV genome showing four main classes of transcript (arrows), the longest of which corresponds to pregenomic RNA (brown). Variations in 5′ end positions for the 2.1 and 3.5 kb size classes are shown with blue and red dots, respectively. Coloured boxes represent protein-coding regions. DR1 and DR2 are 11 bp repeat sequences with template functions during replication (modified from Kidd-Ljunggren *et al.*, 2002; Moolla *et al.*, 2002).

1.4 Replication and life cycle of HBV

In the 25 years since Dane particles were observed, there has been huge progress in the understanding of the later stages in the viral life cycle. However the earlier stages, namely virus binding and internalisation, remain obscure. This is attributable largely to the lack of a cell culture system in which the virus can be cultivated. The virus life cycle, as with all viruses, can be divided into several stages. These are sometimes arbitrary distinctions and in the case of HBV can be thought to include:

1. attachment of the virus to the host cell
2. penetration of the virus into the cell
3. uncoating of the viral genome and formation of cccDNA
4. transcription of the viral genome
5. translation of viral proteins
6. virus assembly
7. release of viral particles from infected cells

The life cycle for hepatitis B virus is described, using these stages, below.

1.4.1 Attachment of the virus to the host cell

The initial interaction between viruses and their host cell is, in many cases, one of the important determinants of virus tropism. Hepatitis B virus is remarkable in its extremely narrow host and tissue specificity. The human HBV has been shown to be infectious only in humans, chimpanzees, gibbons (and possibly certain macaca) (De Meyer *et al.*, 1997). Furthermore, even within the limited number of host species, the virus is limited almost exclusively to replicating within the hepatocytes of the liver. It is thought that this hepatotropism may be determined, at least in part, by the interaction of HBV with the cellular receptor. This has not been confirmed as the cellular receptor has not yet been identified. The identification of the receptor is hampered by the fact that no known immortalised cell line is susceptible to infection (with a few exceptional reports e.g. Bchini *et al.*, 1990), although a number of hepatocyte-derived lines, e.g. Huh7 and HepG2 cells are permissive for virus

replication after transfection of the viral genome, a process that by-passes the binding and internalisation events.

There has been better progress in identifying the part of the viral surface which interacts with the cellular receptor. Accumulated evidence suggests that the region of the envelope which interacts with host cells is the preS1 domain. This observation was made in 1986 by Neurath *et al.* (1986) who showed that immobilised HBV particles were bound by HepG2 cells and that this binding was inhibited by competition with a peptide corresponding to amino acids 21-47 of preS1 and by antibodies to that peptide. Further experiments were performed by Pontisso *et al* (1989a) showing that recombinant LHBs interacted with immobilised HepG2 membranes and that this interaction was inhibited by a monoclonal antibody (MAb) directed against amino acids 30-36 within the preS1 domain (but not by another MAb directed against an adjacent region of the preS1 domain). Nevertheless, despite this evidence, it has proved impossible to discount the possibility that either preS2 or the small HBs interacts with the cellular receptor. It has been suggested that polymerised human serum albumin plays a role in virus-cell binding and that this role is mediated by the preS2 domain (Pontisso *et al.*, 1989b). Other candidate receptors postulated to interact with the preS2 domain include fibronectin (Budkowska *et al.*, 1995) and the transferrin receptor (De Meyer *et al.*, 1997). It has been suggested also that the SHBs protein is involved in binding to cellular receptors, although it is difficult to reconcile this idea with the extremely high levels of HBsAg in the circulation during infection. Nevertheless, it has been suggested that candidate receptors annexin V (endonexin II) and apolipoprotein H interact with the virus via the small HBs domain (Hertogs *et al.*, 1993; Mehdi *et al.*, 1994). These candidate receptors will be described in greater detail below.

1.4.2 Penetration of the virus into the cell

This process, like the initial interaction with the host cell, is not well understood. Once again, this step is not easily accessible to investigation without a cell culture system in which the virus can be grown. It has been difficult to distinguish viral

binding, whether specific or non-specific, from penetration into the cell. In part, this is because much of the data regarding virus penetration is based on the polymerase chain reaction (PCR) and relies on the selective amplification of the replicative intermediate, cccDNA. Although there have been reports of PCR systems which selectively amplify "across the gap" of the partially double-stranded genome (e.g. Kock *et al.*, 1996a), confirmation of these results requires direct purification of replicative intermediates, a process that is often not possible because of the extremely low levels of viral DNA. During the course of the work described in this thesis, attempts made to utilise selective PCR techniques have proved unreliable.

In general, virus entry into host cells relies on one of two approaches (Marsh and Pelchen-Matthews, 2000):

1) pH independent viruses generally undergo direct fusion: used by some enveloped viruses, utilising fusion peptides containing hydrophobic sequences which insert into the lipid bilayer of the cell membrane, causing fusion of the viral envelope with the cell membrane, and releasing the nucleocapsid into the cytosol (e.g. many retroviruses)
2) receptor-mediated endocytosis (RME), often pH dependent viruses: a more common strategy used by both enveloped and non-enveloped viruses, requires interaction with a specific receptor, which leads to endocytosis into endosomal vesicles. This is followed by acidification of endosomes and/or proteolytic cleavage of viral proteins and movement of the nucleocapsid, or only the viral genome, into the cytosol (e.g. adenoviruses, vesicular stomatitis virus) (Kann and Gerlich, 2000; Marsh and Pelchen-Matthews, 2000)

However, this distinction is not always clear. For most pH-independent viruses, fusion is possible at both neutral and acid pH, so uptake into endocytic vesicles may not impede entry. Also, there are cases of pH-independent viruses which require endocytosis for productive infection e.g. Epstein Barr virus and Human Rhinovirus 14 (HRV14, a major group rhinovirus). In these cases, the trigger for fusion or

penetration of these viruses is not well understood (Marsh and Pelchen-Matthews, 2000). The method of penetration utilised by HBV remains unclear and is often confounded by conflicting reports and by comparisons (which may or may not be valid) between mammalian hepadnaviruses and avihepadnaviruses. Studies using DHBV have shown that the lysosomotropic agents ammonium chloride and chloroquine result in inhibition of virus infection (Offensperger *et al.,* 1991). These agents are relatively lipophilic in their unprotonated forms and are able to penetrate the membranes of cells and vacuoles. In an acidic environment they become protonated and therefore too polar to pass through membranes. Thus, the accumulation of these weak bases in vesicles leads to an increase in the intracellular vesicle pH, due to neutralisation of protons. Therefore, the inhibition of infectivity by such agents suggests that HBV follows the endocytic pathway. These results were contradicted by Rigg and Schaller (1992), who showed that the presence of monensin and ammonium chloride did not affect infection. This finding is compatible with either direct fusion of the virus with the plasma membrane, or with pH-independent fusion after endocytosis. Kock *et al.* (1996b) addressed the same question by examining the effects of ammonium chloride and of energy depletion on virus infection. In this study, ammonium chloride was not shown to inhibit infection. Treatment of cells with sodium azide and 2-deoxy-D-glucose, which block glycolysis and oxidative phosphorylation thereby inhibiting ATP synthesis, did, however, abrogate virus infection. Endocytosis is an ATP-dependent process and therefore these results suggest that endocytosis is required for infectivity, however, subsequent stages do not show low pH-dependence. These data suggest that DHBV shows similarities to Epstein Barr Virus and HRV14, as described above.

An alternative approach to HBV infectivity has involved examining the effect of proteinases on virus binding and internalisation. Lu *et al.* (1996) showed that virion purification and treatment with the streptococcal V8 proteinase resulted in HBV infection of HepG2 cells. The V8 proteinase removes surface-accessible preS domains from the virus envelope and generates new amino termini for the MHBs and LHBs proteins. The authors proposed that proteolysis results in a 16 amino acid

region including a consensus fusion motif which is required for entry. Similar results were obtained using the woodchuck hepatitis virus (WHV) which, after limited proteolysis, was reported to acquire infectivity for HepG2 cells (Lu *et al.*, 2001).

Budkowska *et al.* (1997) reported that activation of HBV envelope proteins by a membrane-type matrix metalloproteinase enabled attachment and entry of HBV into T-lymphocytes (although the presence of replicative intermediates was not shown and the significance of infection of T-lymphocytes *in vivo* is not established). Metalloproteinase treatment of HBV was shown to cleave the N-terminal part of the MHBs protein at the preS2 (136-141) amino acid sequence VRGLYF/L. The authors proposed that proteolysis may trigger a conformational change in the virus envelope which results in exposure of a fusion peptide.

The presence of fusion peptides was originally predicted by Rodriguez-Crespo *et al.* (1994). Sequence analysis suggested the presence of a stretch of 23 hydrophobic amino acids in the amino-terminal region of the SHBs protein which resembles the fusion peptides observed in other viruses. Subsequent studies (Rodriguez-Crespo *et al.*, 1995; Rodriguez-Crespo *et al.*, 1999) showed that a synthetic peptide corresponding to this region had membrane-interacting properties and was able to induce liposome fusion. This finding was confirmed by Berting *et al.* (2000). However, thus far, all studies regarding this putative fusion peptide have been performed using synthetic peptides – confirmation of a role for this fusion motif awaits virus infectivity studies. There is, therefore, little consensus regarding the penetration of HBV (whether human, DHBV or WHV) into cells.

1.4.3 Uncoating of the viral genome and formation of cccDNA

The original observation of intact nucleocapsids in the nuclei of infected hepatocytes (McCaul *et al.*, 1985) led to the assumption that the nucleocapsids were translocated from the cytosol into the nucleus where uncoating occurred and viral DNA replication commenced. It has since been found that the diameter of intact nucleocapids (36 nm) exceeds the diameter of nuclear pores (approximately 25 nm).

It is now thought that the nucleocapsids move through the cytosol to the nuclear membrane, where they are halted and somehow the viral genome is transported into the nucleus (Guidotti *et al.*, 1994). The mechanism whereby this occurs remains unclear, but it appears that the core particles, if they are phosphorylated, mediate the transport of the virus genome to the nucleopore complex (Qiao *et al.*, 1994; Kann and Gerlich, 2000). The presence of viral DNA within the nucleus of duck hepatocytes can be demonstrated as early as 24 hours post-infection (Tuttleman *et al.*, 1986a) so the process occurs rapidly. Once the virus DNA has entered the nucleus the partially single-stranded molecule is converted to a covalently closed circular (ccc) DNA (Tuttleman *et al.*, 1986a; Tuttleman *et al.*, 1986b). The formation of cccDNA is probably dependent on both cellular and viral polymerases. Inhibition of the virus polymerase in woodchuck HBV blocks the formation of cccDNA (Moraleda *et al.*, 1997) suggesting that the viral polymerase plays a role. However, contradictory reports show that viral polymerase activity is not required for the formation of cccDNA, which would indicate that the cellular polymerases perform this role (Kock and Schlicht, 1993). The RNA primer is removed and the HBV DNA is supercoiled and becomes associated with histones, acquiring the architecture of an episomal mini-chromosome from which transcription takes place (Bock *et al.*, 2001). The cccDNA molecule has a fairly short half-life of approximately 2–3 days. Viral persistence is ensured by the continuous replenishment of the nuclear pool of cccDNA. This pool of cccDNA is not acquired via constant new infections of the hepatocyte by HBV, but is maintained from rcDNA in the cytoplasm, which re-enters the nucleus (Tuttleman *et al.*, 1986b).

1.4.4 Transcription of viral gene products

The products of transcription of the human HBV genome include the pregenomic RNA (pgRNA) which also serves as a mRNA for the production of HBc and HBe, three subgenomic RNAs required for the translation of the three envelope proteins and the mRNA for the HBx protein (Seeger and Mason, 2000). Avian hepadnaviruses differ in that there are only two envelope proteins and X is not encoded by the genome. Regulation of HBV transcription is achieved by four known promoters and

two enhancers (Schaller and Fischer, 1991; Siddiqui, 1991). Promoters are required for the binding of transcription factors and mediate the alignment of the RNA polymerase facilitating accurate commencement of transcription. Promoters often contain a TATA box, which allows binding of TFIID, a transcription factor which allows the RNA polymerase II to initiate transcription approximately 30 base pairs downstream. Promoters lacking a TATA site often have multiple initiation sites. Enhancers function by increasing transcription initiation at a given promoter (or promoters, in cases where a single enhancer affects expression of more than one gene). Enhancers, unlike promoters, are not required to be located directly upstream of the affected gene, nor is the sequence orientation always critical (Kann and Gerlich, 2000).

The four virus promoters regulate expression of the HBc/e gene, the LHBs gene, the M/SHBs genes and the X gene. Only one of these promoters, that of the LHBs gene, has a defined TATA box and therefore only the mRNA for LHBs protein has a defined 5' end. The other three TATA-less promoters allow transcription from multiple initiation sites allowing each of the RNA molecules to encode a set of co-terminal proteins e.g. the two longer precore RNAs encode the HBe protein, whereas the shorter pregenomic RNA encodes the HBc protein. The promoters function differently depending on the cell culture system in which experiments are performed and a battery of transcription factors capable of binding to each promoter has been identified (Schaller and Fischer, 1991).

The virus enhancer elements are referred to as En1 and En2 and are both located upstream of the core promoter (Figure 1.2). En1 has been shown to enhance initiation of transcription of the pregenomic/preC and HBx mRNA by a factor of 10-50 in liver cells, although its efficacy in non-hepatocyte derived cells is considerably less (Schaller and Fischer, 1991; Moolla *et al.*, 2002). Termination of transcription is achieved using a TATAAA polyadenylation signal located slightly downstream of the of the start of the HBc gene. It only becomes active if the initiation site of the RNA is more than 400 bases away, therefore the stop signal is inactive at the first pass during

the transcription of the pregenomic RNA (Cherrington *et al.*, 1992). Transcribed RNAs acquire a 5' methyl cap and a poly A tail at the 3' end in the same manner as other eukaryotic RNAs. However, unlike most eukaryotic systems, splicing does not appear to play a significant role in HBV gene expression, although there are situated within the genome numerous potential splice signals (Kann and Gerlich, 2000) and it has been proposed that the virus encodes a polymerase-surface fusion protein, encoded by a spliced RNA derived from the pregenomic RNA with a deletion of 454 nucleotides, which may be able to substitute for the LHBs protein in virion maturation (Huang *et al.*, 2000).

1.4.5 Translation of viral proteins

1.4.5.1 ORF S/preS1

The utilisation of three separate initiation codons allows this ORF to encode three co-terminal proteins. The envelope proteins therefore exist in three forms, a small (SHBs), medium (MHBs) and large (LHBs) form. The initiation site located furthest downstream encodes the smallest protein product, SHBs, comprised of 226 amino acids and referred to as P24 or (when glycosylated) GP27. The second initiation codon is located 165 bases upstream of the first and is responsible for transcription of the MHBs protein (GP33 and GP36), which consists of the SHBs protein with a 55 amino acid extension encoded by preS2. The third ATG start codon, located approximately 357 bases upstream encodes a further 108-109 amino acid extension to produce the LHBs protein (p39 or gp42) including the preS1 extension. The LHBs therefore includes all three antigenic determinants – S, preS2 and preS1 (Kann and Gerlich, 2000). All three envelope proteins are glycosylated type II transmembrane proteins. They form multimers stabilised by disulphide bridges formed between cysteine residues within the S domain (Seeger and Mason, 2000). The LHBs is further modified by N-terminal myristylation which has been shown to be essential for infectivity, but not for virus assembly (Gripon *et al.*, 1995; De Falco *et al.*, 2001a). SHBs is the most abundant envelope protein in both sub-viral and Dane particles, with MHBs being found at relatively low levels in all particles. LHBs is found primarily in Dane particles and to a lesser extent in filamentous sub-viral

particles. Spherical sub-viral particles contain little or no LHBs. It is the proportion of LHBs that determines the morphology of a particle (Marquardt *et al.*, 1987). The avihepadnaviruses encode only two envelope proteins. The equivalent of preS2 does not exist in avian hepadnaviruses and the envelope is comprised only of proteins equivalent to SHBs and LHBs (Pugh *et al.*, 1987). There is apparently no glycosylation of the proteins although, as with human HBV, myristylation of the LHBs is required for infectivity (MacRae *et al.*, 1991). Comparisons between human HBV and the avihepadnaviruses, particularly with regard to envelope proteins, may therefore be of limited value.

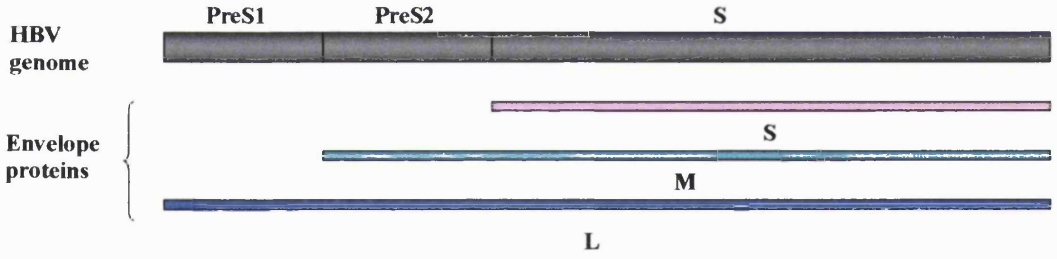


Figure 1.3. Schematic illustrating the envelope proteins of hepatitis B virus, S, M and L, encoded by the preS1/preS2/S gene of the HBV genome.

1.4.5.2 ORF C

ORF C encodes HBcAg, a structural protein which forms the capsid/core particle of HBV. The open reading frame has 212 or 214 codons, but capsid protein translation is initiated at a second initiation codon located 29 codons downstream of the first start codon. The region upstream is referred to as preC and is included within the second protein product of this ORF, HBe, the precursor of a secretory form of the HBc protein. The HBc protein is produced and phosphorylated in the cytosol of infected cells (Roossinck and Siddiqui, 1987) where it is required absolutely for packaging its own mRNA and the viral polymerase after the formation of the RNA-polymerase complex. Thereafter, it assembles into core particles which also contain a cellular protein kinase C. Following DNA synthesis, the assembled core particles acquire envelopes containing the three envelope proteins from the endoplasmic reticulum. The HBc protein is also thought to play a regulatory role in aspects of HBV replication (Guidotti *et al.*, 1994; Nassal, 1992).

The HBe antigen (HBeAg) is not required for viral infection or replication. Indeed, variants deficient in HBeAg production arise naturally during infection and especially during interferon treatment of HBV infected individuals. The preC sequence encodes a terminal hydrophobic α-helix which acts as a secretory signal (Ou *et al.*, 1986; Standring *et al.*, 1988) allowing the HBeAg to move into the lumen of the endoplasmic reticulum. During this translocation, the 19 amino terminal amino acids are removed by a signal peptidase. HBe proteins are incapable of assembling into capsid particles despite the almost total homology of the cleaved protein with HBc protein. This is because the remaining 10 amino acids of the preC portion prevent assembly by interacting with the HBc sequence (Wasenauer *et al.*, 1992). Some HBeAg is transported to the plasma membrane where there is another cleavage event involving a golgi proteinase and then secretion of HBeAg from the cell (Wang *et al.*, 1991).

In acute infections, the appearance of HBe in patient serum occurs at approximately the same time as HBs. In many cases there is eventual loss of HBe antigen, which is

associated with a flare-up in immune-mediated hepatitis, elevated ALT levels and hepatocellular necrosis. This is followed by the appearance of anti-HBe antibodies and a concomitant reduction of virus replication and of circulating HBV DNA levels. This seroconversion generally results in a sub-clinical infection with little or no ongoing liver damage. However, loss of HBe antigen in patient serum may also result from the development of a dominant HBV variant which is incapable of producing HBe antigen (a state often due to the presence of a mutation in the pre-core region, normally at nucleotide 1894 or 1896). These patients will continue to replicate HBV at high levels and will show raised ALT levels after apparent "seroconversion". The prognosis for patients infected with such 'pre-core mutant' HBV is rather poorer than for wild type HBV. Nevertheless, it is also possible to find such precore mutants in the serum of asymptomatic HBV carriers (Kann and Gerlich, 2000).

1.4.5.3 ORF P

The longest ORF is the P ORF which consists of four distinguishable domains (Schlicht *et al.*, 1991). The first (amino terminal) domain encodes the *primase* which remains covalently linked to the 5' end of the minus strand of the viral genome. The *primase* is responsible for the priming of minus strand synthesis (Wang and Seeger, 1992; Weber *et al.*, 1994; Zoulim and Seeger, 1994). The second domain functions as a spacer between the first and third domain and appears to have no other function. The third domain encodes the viral polymerase/reverse transcriptase, a protein of approximately 90kDa. The polymerase protein is packaged together with the viral pregenome into the core particles. The polymerase activity is RNA- or DNA-dependent. The carboxyterminal domain encodes an RNase H which is responsible for cleaving the RNA which is associated with the genome as an RNA:DNA hybrid (Schlicht *et al.*, 1991).

1.4.5.4 ORF X

The HBx protein encoded by ORF X is apparently not essential for any stage in the viral life cycle in transfected cells (Blum *et al.*, 1992) but is required for the establishment of infection *in vivo* (Zoulim *et al.*, 1994). The gene is conserved in

similar form in the hepadnaviruses of woodchucks and ground squirrels (although there is no such homologue in the avihepadnaviruses, further suggesting that the protein is not required for replication or particle assembly in avian viruses). The protein (of 17kDa) is encoded by a gene spanning 54 amino acids and can be detected in the serum. Anti-HBx antibodies also may be detected in serum from patients with chronic HBV infection (where there may be a correlation with severity). However the function of the protein remains unknown. It has been postulated to play a role in many processes, including interacting with the p53 tumour suppressor protein, deregulation of cell cycle check points, abrogation of p53-dependent apoptosis and *trans*-activating roles for transcription of both cellular and viral genes (Arbuthnot *et al.*, 2000).

1.4.6 Virus assembly

1.4.6.1 Encapsidation of the pregenomic RNA

Translation of sufficient amounts of core protein leads to initiation of encapsidation of the pregenomic RNA. The nucleocapsid protein, a polymerase molecule, the molecular chaperone hsp90 and protein kinase C assemble to form the core particle. Encapsidation occurs when the polymerase interacts with an RNA stem-loop structure at the 5' end of the pgRNA (Junker-Niepmann *et al.*, 1990; Kidd-Ljunggren *et al.*, 2002). This structure acts as a packaging signal and is referred to as *epsilon* (ε). The packaging signal (ε) comprises a stem, a bulge, a loop and a non-paired U, all of which are necessary for successful encapsidation (Knaus and Nassal, 1993; Pollack and Ganem, 1993; Nassal and Rieger, 1996; Kramvis and Kew, 1998). The hsp90 molecule appears to stabilise a transient conformation of the polymerase that facilitates binding to ε and packaging can be prevented by the use of drugs that are inhibitory to hsp90 (Hu and Seeger, 1996; Hu *et al.*, 1997).

1.4.6.2 DNA maturation within immature core particles

DNA maturation consists of a number of stages, the first of which is reverse transcription which is followed by elongation of the minus strand of the DNA molecule. Reverse transcription generally occurs only within the core particles. The viral polymerase uses the hydroxyl group of its own tyrosine 96 as a protein primer for synthesis of the minus strand DNA molecule (Zoulim and Seeger, 1994). DNA synthesis initiates at the bulge of *epsilon*, the packaging signal described above (Wang and Seeger, 1992). After the first four nucleotides have been synthesised, the polymerase and the nascent nucleotides dissociate from *epsilon* and are relocated to a complementary sequence in DR1 near the 3' end (this occurs despite the fact that the homology between *epsilon* and DR1 is only 3 nucleotides) (Tavis and Ganem, 1993). The exact mechanism of this transfer is not known. Elongation of the minus strand continues as the polymerase moves along the pgRNA. The virus polymerase polyprotein also encodes an RNase H which cleaves the RNA portion of RNA-DNA hybrids to produce RNA oligonucleotides, thereby facilitating synthesis of the nascent DNA strand. However the RNase H is unable to cleave the last 18 nucleotides of RNA. The resulting 18 base fragment dissociates from the 5' terminal DR1 and translocates to DR2 where it functions as a primer for the synthesis of the DNA plus strand (Seeger and Maragos, 1991).

The result of the translocation of the primer for the plus strand DNA is that circularisation occurs, resulting in a relaxed circular (rc) DNA molecule with two modified 5' ends and a shorter plus strand DNA (with up to 50% of the rcDNA single stranded). The newly-made rcDNA located in the cytoplasm therefore acts as a source of HBV genomes for production of enveloped particles and also for re-entry into the infected cell nucleus where the formation of cccDNA leads to maintenance of the nuclear cccDNA pool (Tuttleman *et al.*, 1986a), despite a relatively short half-life of cccDNA molecules of two to three days (Civitico and Locarnini, 1994), ensuring persistent infection.

1.4.6.3 Envelopment of the core particles

Although the assembly of core particles takes place in the cytosol, the HBc protein shows an affinity for intracellular membranes which contain the LHBs protein (Bruss and Ganem, 1991). Virion formation is dependent on maturation of core particles i.e. significant minus-strand DNA synthesis must have occurred before encapsidation is possible (Gerelsaikhan *et al.*, 1996). This may be due to a conformational change in the core protein during the maturation process. It has been suggested also that, in DHBV, changes in phosphorylation of the core protein may play a role (core protein in core particles exhibits a different phosphorylation pattern to that within the cells) (Seeger and Mason, 2000). Binding of mature core particles to the N-terminus of the LHBs protein results in translocation (budding) of the core particles across the endoplasmic reticulum membrane (Ganem, 1991). Although all three envelope proteins are generally incorporated into the membrane, the MHBs protein is dispensable, while the SHBs protein is required in excess. The ratio between the three envelope proteins appears to be critical and virion maturation occurs efficiently only in certain cell lines, e.g. Huh7 and HepG2 cells, both of which are of hepatocyte origin (Kann and Gerlich, 2000).

1.4.7 Release of viral particles from infected cells

Enveloped particles containing all three HBs proteins are transported through the ER into the Golgi complex (Ganem, 1991). During this process, the HBs protein is modified further by glycosylation at an asparagine residue and covalent disulphide bridges appear between the HBs and HBc proteins (Ganem, 1991). Virion release from secretory vesicles does not require any specific signal – secretion apparently occurs using the constitutive secretory pathway. Also, during this transportation from the ER to the Golgi, there is a pH dependent refolding of the preS domain of the LHBs protein from the internal side of the membrane to the surface (Bruss *et al.*, 1994).

1.5 Significance of the lack of a cell culture system

The narrow host range and tissue tropism of the virus suggests a necessity for host and liver-specific regulatory factors for HBV replication. The hepatotropism exhibited by the virus has been attributed, at least in part, to transcriptional regulatory events. The S gene transcript of 2.1 kb can be produced in a wide variety of cell lines derived from various species and diverse tissues (De Meyer *et al.*, 1997). The pregenomic RNA, however, can only be synthesised in well-differentiated hepatocyte-derived cell lines (Sureau *et al.*, 1986). Furthermore, as discussed above, En1 is preferentially functional in hepatocyte-derived cell lines. Liver-specific nuclear factors, such as the members of the nuclear receptor superfamily, nuclear hormone receptors and peroxisome proliferators, have been shown to be required for hepadnavirus replication (Johnson *et al.*, 1995; Raney *et al.*, 1995; Raney *et al.*, 1997; Guidotti *et al.*, 1999; Raney *et al.*, 2001; Tang and McLachlan 2001). However, the extreme host specificity and hepatotropism also may be the result of an extremely specific interaction between the virus and the host cell receptor(s). This possibility has proved difficult to investigate in the absence of a cell culture model for virus replication. The observation that transfection of a rat hepatoma cell line with a greater-than-full-length HBV construct resulted in production of progeny virions (although both *in vivo* and *in vitro* infection experiments using rat hepatocytes resulted in no virus replication) (Shih *et al.*, 1989) suggests the possibility that the species barrier for HBV infection and replication may be effective in the early stages of infection.

The practical difficulties arising from our inability to culture the virus are scientifically and clinically significant. Although there has been huge progress towards an understanding of the basic biology of the virus and in the treatment of infections, that progress is essentially limited by the absence of a cell culture system supporting virus replication.

1.5.1 Animal models and the limits of the DHBV model

Many of the basic virological investigations have taken place in animal models, often utilising the avian hepadnaviruses which differ fundamentally, at a molecular and biological level, from the mammalian hepadnaviruses. Avihepadnaviruses encode only two envelope proteins – there is no equivalent of the preS2 domain. There is also no homologue of the mammalian HBV X gene in avihepadnaviruses. Furthermore, avian hepadnaviruses have been shown to have distinct transcriptional requirements which differ from those of the mammalian hepadnaviruses. Human hepatitis B virus (HBV) replication can occur in non-hepatocyte derived cells when pregenomic RNA synthesis from viral DNA is activated by the expression of the nuclear hormone receptors hepatocyte nuclear factor 4 (HNF4) and the retinoid X receptor alpha (RXRalpha) plus peroxisome proliferator-activated receptor alpha (PPARalpha) heterodimer. Nuclear hormone receptor-dependent HBV replication is inhibited by hepatocyte nuclear factor 3 (HNF3). In contrast, HNF3 and HNF4 support duck hepatitis B virus (DHBV) replication in non-hepatoma cells, whereas the RXRalpha-PPARalpha heterodimer inhibits HNF4-dependent DHBV replication. HNF3 and HNF4 activate DHBV pregenomic RNA synthesis and viral replication synergistically. The conditions that support HBV or DHBV replication in non-hepatoma cells are not able to support woodchuck hepatitis virus replication (Tang and McLachlan, 2002). This makes extrapolation from avian HBV to mammalian viruses, and within the mammalian viruses, difficult.

1.5.2 Use of primary human hepatocytes

Other investigators have made use of primary human hepatocytes which are, for a short time, susceptible to the virus (Seeger and Mason, 2000). Primary hepatocytes are, however, difficult to obtain and of variable quality. Furthermore, the hepatocytes lose susceptibility to the virus within a relatively short period of time. In addition, in many cases where infection of hepatocytes has been reported, the hepatocytes have been maintained in medium containing chemical agents, such as polyethylene glycol or dimethyl sulfoxide, which may modulate the mechanism of entry of the virus into

cells, possibly utilising "unnatural" mechanisms of entry (Gripon *et al.*, 1988; Pugh and Summers, 1989; Gripon *et al.*, 1993).

1.5.3 Transfection of greater-than-full-length constructs

Many virologists have resorted to using an artificial system whereby the binding and internalisation of the virus into the host cell is bypassed. Studies of this type utilise the ability of hepatocyte derived cell lines, such as HepG2 and Huh7 cells, to support the internal replication of the virus i.e. once viral DNA reaches the nucleus, these cell lines are able to provide the necessary "cellular machinery" for HBV gene expression, DNA replication, packaging and production of the progeny virions. Internalisation problems can be avoided through the use of greater-than-full-length HBV genomes cloned into plasmids which may then be transfected into cells and serve as artificial genomes from which virus particles may be produced (Acs *et al.*, 1987). Constructs containing the greater-than-full-length genome are required for production of a full-length terminally redundant pregenome, which would normally be transcribed from the circular genome. Although this system has been used successfully to elucidate much of the biology of HBV, there are also obvious limitations. The development of a more "natural" cell culture system would therefore be extremely valuable in investigating the very early events in the virus replicative cycle and for more practical research such as testing compounds thought to have therapeutic potential.

1.6 Receptor candidates for HBV

There has been a plethora of molecules, shown in Table 1.1, proposed to function as the cellular receptor for HBV. As discussed previously, the viral ligand is generally accepted to be the preS1 domain of the HBV envelope protein. However, this has not precluded the suggestion of candidate receptors proposed to bind to other domains of the envelope proteins.

Table 1.1 Receptor candidates proposed for HBV

Virus binding site	Candidate receptor	Reference
S	Apolipoprotein H	Mehdi *et al.*, 1996
	Annexin V	Hertogs *et al.*, 1993
PreS2	Polymerised Human Serum Albumin	Machida *et al.*, 1983
	Fibronectin	Budkowska *et al.*, 1995
	Transferrin receptor	Franco *et al.*, 1992
PreS1	GAPD	Petit *et al.*, 1992
	IgA receptor	Neurath and Strick, 1990
	Interleukin-6	Neurath *et al.*, 1992a
	Asialoglycoprotein receptor	Triechel *et al.* 1997
	HBV-BP (SCCA1 homologue)	De Falco *et al.*, 2001b

1.6.1 S binding candidate receptors

1.6.1.1 Apolipoprotein H

Mehdi *et al.* (1994) separated the plasma membrane proteins of human liver and used a ligand-blotting technique to identify a 46 kDa protein specifically binding to SHBs protein. This protein was shown to be apolipoprotein H (apoH), also known as beta 2-glycoprotein I. Binding of apoH to SHBs was shown to be saturable and to require only the S protein of HBs. The interaction was shown to be inhibited by excess recombinant SHBs protein, anti-HBs antibodies and antibodies directed against apoH. Apolipoproteins bind to hepatocytes during the normal course of lipid metabolism (Mahley *et al.*, 1984) and the authors proposed that this may provide a route of entry for HBV into hepatocytes. The significance of this interaction has, however, been questioned by Neurath and Strick (1994) who showed that apoH did not interact with delipidated recombinant HBsAg. It was suggested that the reported interaction between HBV and apoH was artefactual and consistent with the known capacity of apoH to bind to lipids.

1.6.1.2 Annexin V

The annexins are a group of at least 12 different proteins which bind to phospholipids in a calcium-dependent manner (Walker *et al.*, 1992). They are associated with the cytoskeleton to varying degrees and certain annexins are able to act as substrates for protein kinases, including protein kinase C. Many functions have been proposed for annexins, both intra- and extracellularly, including a role in calcium-dependent exocytosis, regulation of membrane-cytoskeleton interactions, anticoagulant activity, inhibition of phospholipase A_2 and the formation or regulation of ion channels [reviewed in Walker *et al.*, 1992]. Their precise physiological role has not yet been determined. Annexin V (AxV) is a 35 kDa monomeric protein, which resembles Annexins I, II, III, IV and VIII in that it consists of a unique N-terminal region followed by four repeats of approximately 70 amino acids. The binding of AxV to phospholipids appears to be largely ionic in nature since binding affinity is reduced by increasing ionic strength. Annexin V was proposed as a candidate receptor by Hertogs *et al.* (1993). This report showed that there was a specific interaction between annexin V (formerly referred to as endonexin II) and SHBs, which was inhibited by human liver plasma membrane proteins, recombinant AxV, or anti-AxV antibodies. The rat homologue, which shows 90% homology to the human molecule, was shown to be incapable of binding SHBs. A number of subsequent papers from the same group characterised the role of AxV in HBV infection further. The use of synthetic peptides allowed characterisation of the epitopes within the sHBs molecule proposed to react with human AxV (hAxV), namely amino acids 125-131 and 158-169 (De Meyer *et al.*, 1999a). Gong *et al.* (1999) showed that transfection of a rat hepatoma cell line, FTO 2B, which is refractory to HBV infection, with a construct containing the cDNA for hAxV resulted in infection by HBV. As with apoH, the significance of this interaction remains unknown. The phospholipid-binding properties of hAxV suggest the possibility that the interaction is mediated by the lipids incorporated into the HBV virus particle. This is supported by the finding that phosphatidylserine, a specific ligand of annexin V (utilised in apoptosis assays) was shown to be involved in the interaction of hAxV with HBV (De Meyer *et al.*, 1999b).

Neurath and Strick (1994) suggest that the effect of hAxV on HBV infectivity is an artefact of the phospholipid binding properties of annexin V.

1.6.2 PreS2 binding candidate receptors

1.6.2.1 pHSA

The interaction between polymerised human and chimpanzee serum albumin and HBV was noted in 1983 (Machida *et al.*, 1983). This interaction was found to be specific for the preS2 region. A subsequent study showed by light and scanning electron microscopy that human hepatocytes interact with polymerised human serum albumin (pHSA) (Trevisan *et al.*, 1982). It was also reported that, in the presence of pHSA, recombinant HBsAg particles containing both the large and middle HBs proteins were able to attach to the plasma membranes of human liver cells. Particles containing only the small HBs protein were not able to interact with liver cell membranes even in the presence of pHSA (Pontisso *et al.*, 1989b; De Meyer *et al.*, 1997). It was proposed therefore that the preS2 domain could act, via pHSA, as a site of attachment to human hepatocytes (Pontisso *et al.*, 1989b). However, the physiological significance of these and subsequent findings is not known, particularly as the majority of these studies have involved the use of artificially polymerised HSA (using gluteraldehyde or transglutamase) (De Meyer *et al.*, 1997). Furthermore, the fact that native albumin at physiological concentrations interferes with the interaction between pHSA and HBV (Thomas *et al.*, 1988) and that the amount of pHSA present in human serum is negligible compared with that of unpolymerised serum albumin (De Meyer *et al.*, 1997), makes it difficult to understand how pHSA may play a role in HBV infection *in vivo*.

1.6.2.2 Fibronectin

A second molecule proposed to interact with the preS2 domain of MHBs protein, fibronectin, was identified using an anti-idiotypic antibody (Ab2) raised against the preS2 region-specific MAb F124 (recognising amino acids 120 –126). Ab2 was shown to bind to the extracellular matrix fibronectin of human liver sinusoids. Immunohistochemical studies demonstrated the attachment of viral and recombinant

hepatitis B surface antigen particles (containing the preS2 region) to the fibronectin of human liver sinusoids. The authors suggested that human liver fibronectin may bind HBV *in vivo* via the preS2 region and that the binding of the virus particles to liver sinusoids could facilitate subsequent internalisation by hepatocytes (Budkowska *et al.*, 1995).

1.6.2.3 Transferrin receptor

Franco *et al.* (1992) utilised the fact that activated T lymphocytes expressing class II molecules are able to present complex antigens which bind to the cell surface but which do not require processing. The transferrin receptor was identified as playing a role in the uptake of HBV envelope proteins by T cells. It was proposed therefore, because the transferrin receptor is also expressed by hepatocytes, that similar binding of HBV may occur in hepatocytes. There has, however, been no direct experimental data published supporting this hypothesis.

1.6.3 PreS1 binding domains

Despite the candidate receptors described above, the generally more accepted view remains that the preS1 domain is responsible for attachment of the virus to the cellular receptor. The evidence for this has been described in section 1.4.1 above. Candidate receptors postulated to interact with the preS1 domain are described below. These include GAPD, the immunoglobulin A receptor, IL-6 and the asialoglycoprotein receptor.

1.6.3.1 Liver glyceraldehyde-3-phosphate-dehydrogenase

An immunological approach was used by Petit *et al.* (1992) in which anti-idiotypic antibodies were raised against a monoclonal antibody (F35.25) which recognises the putative receptor binding domain of preS1 (amino acids 21 – 47). A subsequent report showed that the 35 kDa protein recognised by the anti-idiotypic antibody was human liver glyceraldehyde-3-phosphate-dehydrogenase (GAPD) which plays a vital role in glycolysis (Duclos-Vallee *et al.*, 1995).

1.6.3.2 Immunoglobulin A receptor

Neurath and Strick (1990) observed partial homology between the preS1 domain and the human immunoglobulin A (IgA) heavy chain constant region. The authors proposed that the cellular receptor for HBV could be the same molecule utilised as the cellular receptor for IgA. Pontisso *et al.* (1992) showed specific competition between the preS1 sequence and IgA in their binding to human liver plasma membranes. However, this was not supported by Dash *et al.* (1992) who showed no direct role for IgA in the binding of HBV to cells.

1.6.3.3 Interleukin 6

Interleukin 6 (IL-6) has been proposed to contain recognition sites for the preS1 domain and cells transfected with a cDNA for IL-6 were reported to acquire the ability to bind HBV (Neurath *et al.*, 1992a; Neurath *et al.*, 1992b). This interaction occurs between amino acids 21-47 of the preS1 domain and amino acids 35-66 of Il-6. More recently, human IL-6 was reported to facilitate HBV infection in HepG2 cells and in the "trimeric" mouse model (Galun *et al.*, 2000). Contradictory results were obtained by Heinz *et al.* (2001) who assessed the binding of recombinant preS1 to IL-6 and receptor subunits of IL-6 using immunoprecipitation techniques. The authors report that IL-6 and the receptor subunits could not be precipitated with recombinant preS1, but acknowledge a possible role for IL-6 in the elimination of HBV infection.

1.6.3.4 Asialoglycoprotein receptor

Triechel *et al.* (1997) reported that HBV particles interacted with the human asialoglycoprotein receptor (ASGPR) which is expressed in the liver where it mediates the uptake of glycoproteins. A ligand specific for ASGPR, asialofetuin, was reported to inhibit the binding of HBV to HepG2 and Huh7 cells. It was therefore proposed that ASGPR mediates the binding and endocytic uptake of HBV in liver cells.

1.7 Carboxypeptidase D – a candidate receptor for the duck hepatitis B virus

Unlike human HBV, DHBV is fairly amenable to investigations of the early events in the replicative cycle. This is due to the relative ease with which primary duck hepatocytes can be obtained, maintained and used for experimental procedures. This has allowed the identification of a convincing candidate receptor for the DHBV, namely carboxypeptidase D (CPD-D). The gp180/p170 protein was originally identified as a putative receptor by observations of binding to the preS region of the DHBV large envelope protein (Kuroki *et al.*, 1994; Tong *et al.*, 1995). Kuroki *et al.* (1994) showed that precipitation of DHBV pre-incubated with primary duck hepatocyte (PDH) cell extracts showed binding to a glycosylated protein of 180 kDa (gp180). Binding was specific to the preS region and was blocked by the presence of neutralising antibodies. Gp180 was shown to be expressed on the cell surface, although it was estimated that only 5 – 10% of the cellular gp180 was localised on cell membranes. Furthermore, the species specificity of binding activity was shown to mirror the known host range of DHBV (Kuroki *et al.*, 1994). However, gp180 was detected in numerous non-hepatic tissues, although many such tissues are not susceptible to DHBV infection. Tong *et al.* (1995) confirmed binding of DHBV preS to a glycoprotein of 170kDa (p170) independently and showed that the interaction was inhibited competitively by incubation with DHBV particles. Protein microsequencing showed similarities between p170/gp180 and mammalian carboxypeptidases H, N and M (Kuroki *et al.*, 1995; Tong *et al.*, 1995). DNA sequence analysis of a cDNA encoding gp180 indicated that gp180 is a novel member of the basic CPD gene family, although gp180 is considerably larger (approximately three times as large as CPD-H) and consists of a "head-to-tail array of carboxypeptidase homology domains". Gp180 was subsequently characterised as the prototype of a new class of membrane-bound carboxypeptidases, designated CPD-D (Song and Fricker, 1995). Homologue proteins from humans and rats have been cloned and characterised, confirming that CPD-D is a type 1 transmembrane protein which consists of a large luminal/extracellular portion, a transmembrane domain and a C-terminal cytoplasmic tail of about 60 amino acids (Breiner *et al.*, 1998). Basic carboxypeptidases are enzymes that remove basic amino acids (lysine or arginine)

from the COOH terminus of polypeptide chains (Skidgel, 1988). The biological function of gp180 in the uninfected host remains unknown (Kuroki *et al.*, 1995). It has been proposed that the carboxypeptidase activity of gp180 could play a role in viral uptake (Li *et al.*, 1999). However, the use of a carboxypeptidase D inhibitor, 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid, was not shown to interfere with DHBV infection (Breiner *et al.*, 1998). The luminal/extracellular portion of CDP-D consists of 3 carboxypeptidase B-like domains (called A, B and C) – in domains A and B all amino acids essential for the enzymatic activity of CPDs characterised so far have been conserved, however those of the C domain are not conserved. It has therefore been suggested that this domain of CPD-D is catalytically inactive and has a distinct but unknown function (Tan *et al.*, 1997). This is suggested further by the fact that, despite the loss of enzymatic activity, the C domains of all CPDs have conserved primary sequences, implying that they have preserved a function which is not yet known (Urban *et al.*, 1998; Urban *et al.*, 2000). This has been confirmed for duck CPD, whose A and B domains display CPD activity, while the C domain contains the DHBV preS binding site (Eng *et al.*, 1998). Breiner *et al.* (1998) showed that Huh7 cells (normally non-permissive for DHBV infection), transfected with gp180, were capable of binding and selectively internalising DHBV and preS polypeptide (although there was no evidence of viral replication). Furthermore, C-terminally truncated gp180 (lacking the cytosolic domain) arrested bound ligand at the cell surface and was unable to undergo endocytosis. Breiner *et al.* (1998) suggested that secondary host-specific factors (co-receptors) are required, in addition to the primary receptor, for virus entry and replication. This hypothesis is supported by the fact that expression of gp180 in LMH cells (chicken hepatoma), which are capable of generating and secreting infectious particles, did not render these cells susceptible to DHBV infection (Kuroki *et al.*, 1995).

Therefore, additional factors may be required for productive virus replication – it has been suggested that such factors may be responsible for fusion, nuclear translocation of the viral genome, or repair of the viral genome prior to the formation of cccDNA. The presence of co-receptors has been noted in other viruses e.g. adenovirus

(Wickham *et al.*, 1993), echovirus (Powell *et al.*, 1997) and HIV (Doms and Peiper, 1997). Li *et al.* (1996) showed that DHBV preS bound a protein of 120 kDa (p120). The p120 binding site was localised to a triplet of amino acids at 100-102 (Phe-Arg-Arg) which are conserved in all DHBV virus strains sequenced thus far (Li *et al.*, 1996). P120, glycine decarboxylase, is expressed on the surface of cells and is restricted to DHBV infectable tissues namely liver, kidney and pancreas. The authors therefore proposed that p120 is a receptor (or part of a receptor complex) for DHBV and that the virus binds the hepatocytes through an initial interaction with p170/gp180, which is followed by "either a direct conformational change of the preS protein or by proteolytic cleavage at Arg-102 to activate p120 binding". This model is partially based on the fact that residues 101 and 102 are both arginine, therefore the p120-reactive preS protein can be generated by proteolytic cleavage by a trypsin-like proteinase or an endopepsidase specific for dibasic residues. Li *et al.* (1996) argue that the observation that proteinase treatment of HBV particles enhanced infectivity in a human hepatoblastoma cell line (Lu *et al.*, 1996) supports this model. However, the significance of the preS interaction with p120 remains unclear and requires clarification e.g. by experiments to determine whether anti-p120 antibodies inhibit binding of DHBV to primary duck hepatocytes, or whether transfection of p120 cDNA (with or without p170/gp180 cDNA) into non-permissive cells confers susceptibility to DHBV infection. Thus far, all the evidence suggesting a role for CPD-D as a receptor for hepadnaviruses has been obtained using avian hepadnaviruses. Attempts to show that the human homologue of the avian carboxypeptidase protein plays any role in human HBV binding or replication have proved unsuccessful. Transfection of a cDNA for the human homologue of CPD into HepG2 cells has no effect on the binding of HBV to cells (Harrison and Ijaz, personal communication).

1.8 Squamous Cell Carcinoma Antigen 1

1.8.1 Introduction

Squamous cell carcinoma antigen (SCCA) was isolated initially as part of the TA-4 complex from squamous cell carcinoma (SCC) of the uterine cervix and refers to a group of at least 14 proteins that react with antibodies raised against the entire TA-4 complex (Cataltepe *et al.*, 2000a). These proteins were initially divided into two arbitrary groups, the acidic SCCA (pI of less than 6.25) and the neutral SCCA (pI of 6.25 or greater). The neutral isoform of SCCA is expressed in both normal and malignant epithelial cells, however, the acidic isoform is expressed primarily in malignant cells and can be detected in the serum of patients with well-differentiated SCC. For this reason, SCCA has become useful as a clinical marker, with elevated circulating SCCA levels correlating with disease progression and infiltrative capacity of the malignancy (Schneider *et al.*, 1995).

Molecular cloning has indicated that there are at least 2 genes encoding SCCA, mapping to a serpin cluster at 18q21.3. The genes are less than 10 kb apart in a head-to-tail arrangement (Silverman *et al.*, 1998). The two genes have been designated SCCA1 and SCCA2 and have a high degree of homology (92% at the amino acid level), with SCCA1 encoding the neutral SCC antigens and SCCA2 encoding the acidic SCCA (Silverman *et al.*, 1998). Both SCCA1 and SCCA2 are, by sequence identity although not by function, members of the serpin family (serine proteinase inhibitors). The serpins are a well conserved superfamily of over 100 proteins expressed in viruses, plants, vertebrates and invertebrates (Salzet *et al.*, 1999). Serpins are involved in the regulation of a variety of proteinases including those of the coagulation, complement, fibrinolytic and inflammatory pathways (Salzet *et al.*, 1999; Wells *et al.*, 1999).

1.8.2 Mechanism of action of serpins

Inhibitory serpins function via an exposed reactive site loop (RSL, also referred to as the Reactive Centre Loop, RCL) of about 20 amino acids and irreversibly inhibit proteinases through a suicide substrate inhibition mechanism (Figure 1.4). The

enzyme recognises the RSL of the inhibitory serpin and binds to it forming a serpin-enzyme complex (SEC) (Worrall *et al.*, 1999). This leads to cleavage of the serpin RSL between the reactive P_1 and P_1' (numbering according to Schechter and Berger (1967), but, for comparative purposes, is relative to conserved P15Gly), causing a significant and irreversible conformational change with complete insertion of the RSL loop into a β-sheet. The enzyme is inactivated reversibly by the formation of an acyl ester linkage between the active site serine and a serpin side chain (Wells *et al.*, 1999; Worrall *et al.*, 1999; Luke *et al.*, 2000).

Although the SEC is kinetically stable, it is thermodynamically unstable and would eventually break down releasing inactivated (cleaved) serpin and active proteinase and, therefore, the irreversible inhibition of the proteinase requires removal of the SEC from the circulation (Wells *et al.*, 1999). Although little is known about the mechanism of action of SCCA1, there is evidence for the removal of other SECs (e.g. thrombin-antithrombin, α-proteinase inhibitor-trypsin and α_1-antichymotrypsin-cathepsin G) from the circulation via a receptor-mediated clearance mechanism. There have been several receptors proposed to be involved in this clearance, including the low density lipoprotein receptor, vitronectin, hepatocyte-associated proteoglycans, Urokinase-plasminogen Activator Receptor (reviewed in Wells *et al.*, 1999) and the LDL receptor related protein (LRP) (Ashcom *et al.*, 1990; David Lomas, personal communication). There is also evidence that SECs are removed from the circulation largely by liver-enriched or even liver-specific pathways – clearance studies of radiolabelled SECs in experimental animals show localisation to the liver and a specific association with hepatocytes (Wells *et al.*, 1999). These observations are intriguing given the possible relationship between SCCA1 and HBV (described below) and the extreme hepatotropism of the virus.

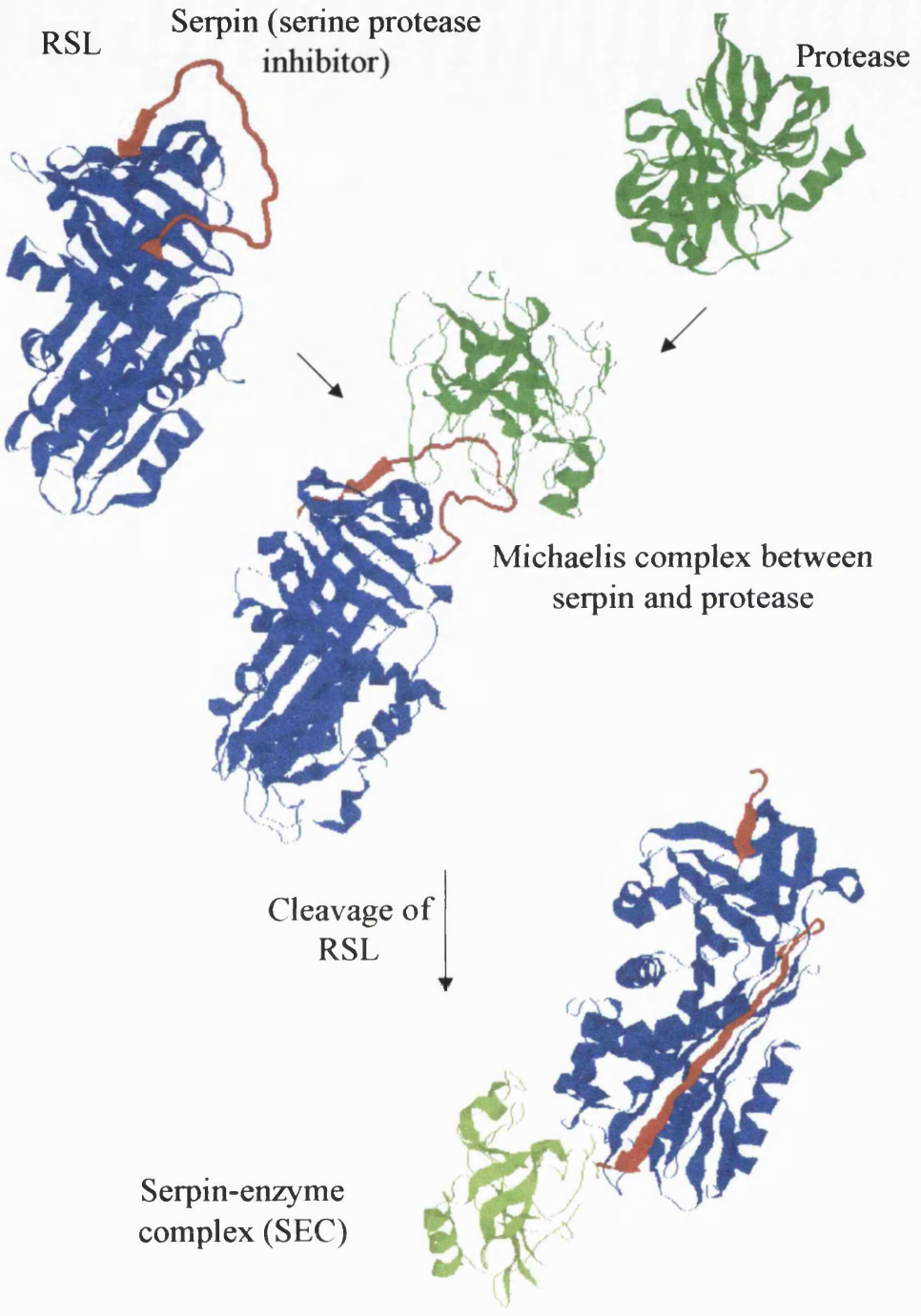


Figure 1.4 Schematic showing mechanism of action of serine proteinase inhibitors. Produced using Genbank Accession Numbers 1HP7 (alpha 1 antitrypsin, uncleaved), 1NES (elastase, engineered), 1EZX (serpin-proteinase complex) and 1K9O (Michaelis serpin-trypsin complex).

1.8.3. Cross-class activity

Despite the high homology between SCCA1 and SCCA2, there are substantial cross-class differences in their inhibition of proteinases: SCCA1 is a potent inhibitor of the cysteine proteinases cathepsins S, K and L (Schick *et al.*, 1998a), whereas SCCA2 (although 92% homologous to SCCA1 at the amino acid level) is an inhibitor of the chymotrypsin-like serine proteinases cathepsin G and human mast cell chymase (Luke *et al.*, 2000). Although serpins generally are limited to inhibiting only proteinases of the serine mechanistic class, there are at least three other serpins which inhibit cysteine proteinases (although these serpins also inhibit serine proteinases in addition to the cysteine proteinases). The cowpox virus serpin *crm*A inhibits ICE (interleukin-1β converting enzyme) (Komiyama *et al.*, 1994) and the human serpin α_1-antichymotrypsin inhibits an novel unclassified PTP (prohormone thiol proteinase) (Hook *et al.*, 1993). SQN-5, a mouse serpin that is similar to SCCA1 and SCCA2, has also been shown to inhibit both chymotrypsin-like serine proteinases and papain-like cysteine proteinases by employing a RSL-dependent inhibitory mechanism (Al Khunaizi *et al.*, 2002).

The RSL of SCCA1 has been well-characterised and has been shown to be essential for cysteine proteinase inhibition (Schick *et al.*, 1998b). Furthermore, replacement of the RSL of SCCA2 (which inhibits serine proteinases) with that of SCCA1 confers cysteine proteinase inhibitory properties on the modified SCCA2, suggesting that the RSL is the only region required for cysteine proteinase inhibition (Luke *et al.*, 2000).

	RSL hinge region							RSL variable region											
	P15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	1'	2'	3'	4'	5'
SCCA1	G	A	E	A	A	A	A	T	A	V	V	A	F	G	S	S	P	T	S	T

Figure 1.5 Amino acid sequence of the reactive site loop of SCCA1, showing the hinge and variable regions. Cleavage occurs between residues P1 and P1' (Schechter and Berger Numbering System (1967) but, for comparative purposes, numbering relative to conserved P15Gly.

Only one other interaction of a serpin with a papain-like cysteine proteinase has been characterised, namely that between antithrombin and papain. Significantly, inhibition of papain by antithrombin occurs in a manner that differs substantially from that of conventional serpins. This interaction showed a poor second-order association rate constant and a high stoichiometry of inhibition. Papain "trapped" by antithrombin is degraded by active, non-complexed papain molecules (Bjork *et al.*, 1998). The authors hypothesised that distortion of the trapped proteinase permitted proteolytic degradation by "free" proteinase molecules. These significant differences in the architecture of inhibition of cysteine proteinases raise questions regarding the relevance of serpin studies, especially involving the RSL, to investigation of SCCA1. Irving *et al.* (2002) have performed studies in which they replaced the RSL of the archetypal serpin, α1-antitrypsin, with that of SCCA1. The modified α1-antitrypsin inhibited papain in the same fashion as SCCA1 and enabled characterisation of the serpin-proteinase complex. It was found that the formation of a serpin-cysteine proteinase complex and the inhibition of that proteinase relied critically on RSL insertion into the body of the molecule (Irving *et al.*, 2002). These results indicate that the body of a serpin molecule is intrinsically capable of supporting cysteine proteinase inhibition and that the complex formed between the serpin and the cysteine proteinase shares analogous architecture with that observed in the inhibition of serine proteinases by serpins (Irving *et al.*, 2002)

1.8.4. SCCA1 as a putative cellular receptor for HBV

SCCA1 was identified initially as an HBV-binding protein using affinity chromatography studies of triton solubilised HepG2 plasma membranes (De Falco *et al.*, 2000). SCCA1 was shown to bind the preS1 [21-47] region of the large envelope protein of HBV specifically, with enhanced binding observed for myristylated preS1. These observations suggested the possibility of a role for SCCA1 as a cellular receptor for HBV. This possibility was further supported by a recent publication (De Falco *et al.*, 2001b) which confirmed, using flow cytometry-based direct binding experiments, the ability of recombinant SCCA1 to bind to preS1 of HBV. Furthermore, transfection of an SCCA1 cDNA clone into HepG2 cells increased the

level of HBV binding to cells two-fold, while CHO cells, which normally do not bind HBV, acquire susceptibility to HBV binding after transfection with a SCCA1 cDNA clone (De Falco *et al.*, 2001b). These results suggest the possibility that SCCA1 plays a role in HBV infection. However, there remain a number of unresolved issues. It has often been proposed that the extreme hepatotropism exhibited by hepatitis B virus may be, at least in part, due to restriction of receptor expression primarily or absolutely to the liver. Therefore, the possibility that SCCA1 plays a role as a receptor or co-receptor for HBV would require that SCCA1 is expressed in the liver. It has been reported that SCCA1 is expressed in a very wide range of tissues, including the skin, lungs, prostate, testis, thymus and tonsils (detectable by RT-PCR and/or immunohistochemistry), but not in the liver (either adult or foetal liver) (Cataltepe *et al.*, 2000b). This report however is not supported by the fact that ourselves and others (De Falco *et al.*, 2001b) have been able to amplify SCCA1 cDNA from HepG2 cells, a hepatocyte derived cell line, by nested RT-PCR (as described in chapter III), although the discrepancy most likely is due to the sensitivity of nested RT-PCR in comparison with immunohistochemistry and single round RT-PCR.

The reported association of HBV infectivity with proteolysis involving e.g. the V8 proteinase (Lu *et al.*, 2001) or metallopoteinases (Budkowska *et al.*, 1997), and the proposed existence of a fusion protein exposed by proteolytic cleavage (Rodriguez-Crespo *et al.*, 1999) raises questions regarding the importance of the proteinase-inhibitory properties of SCCA1 in the mediation of HBV binding to cells. A further possibility is that the hepatotropism of the virus could be defined by a hepatic clearance of SECs rather than liver-specific receptor expression. Another question is whether SCCA1 is merely increasing the ability of transfected cells to bind the virus, or whether the serpin's role affects downstream replicative events too.

1.9. The purpose of this study

The recent report by De Falco *et al.* (2001b) that SCCA1 may play a role in the infection of host cells by HBV was the basis of the work described in this thesis. The purpose of this study was to confirm the findings described in that paper, and then to investigate the mechanism whereby SCCA1 mediates the binding of HBV to host cells. The possibility that the proteinase inhibitory activity of SCCA1 was important in the mediation of virus-cell binding was of interest, especially considering previous reports that treatment of HBV with the V8 proteinase enhances infectivity. The hepatic clearance of serpin-enzyme complexes was intriguing, and led to investigations into the possibility that HBV subverts this clearance system in order to gain access to hepatocytes. The long-term aim of this research is to develop a cell culture system that is permissive for HBV infection, and characterisation of SCCA1-mediated binding of HBV to cells may be useful in this regard.

CHAPTER II

GENERAL MATERIALS AND METHODS

2.1 DNA isolation and analysis

2.1.1 Screening plasmid minipreps

These minipreps were used to obtain DNA of mediocre quality for restriction enzyme analysis only. Five ml Luria-Bertani medium (LB) containing the appropriate selective antibiotic was inoculated with a single colony and incubated overnight at 37°C, 225 rpm. A volume of 1.5 ml of the overnight culture was pelleted at 10,000 *g* for 1 min and the supernatant aspirated. The pellet was resuspended in 100 µl TE buffer (10 mM Tris HCl, pH 7.5, 1 mM EDTA) and 100 µl TE-buffered phenol-chloroform and vortexed vigorously for 20 sec. The tubes were centrifuged at 10,000 *g* for 5 min. The aqueous supernatant was transferred to a fresh microcentrifuge tube containing 25 µl 10 M sodium acetate and 300 µl ethanol (99%), mixed gently and centrifuged at 10,000 *g* for 12 min to pellet the DNA. The pellet was air dried and resuspended in 50 µl double distilled H_20 (ddH_2O) and either used immediately or stored at -20°C.

2.1.2 Qiagen spin minipreps

Qiagen minipreps were used to obtain small amounts of high quality DNA to be used e.g. for subcloning and sequencing. The QIAprep system utilises alkaline lysis of bacterial cells followed by adsorption of the DNA onto silica gel, washing and elution of purified DNA. Bacterial cells from 3 ml of an overnight culture (in LB containing the appropriate selective antibiotic) were pelleted at 10,000 *g* for 2 min. Pelleted bacterial cells were resuspended in 250 µl buffer P1 (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) and 250 µl buffer P2 (20 mM NaOH, 10% SDS) was added. The tube was inverted 4–6 times to mix gently, then 350 µl of buffer N3 (3 M potassium acetate, pH 5.5) was added and the tube immediately inverted a further 4-6 times to avoid localised precipitation. Tubes were centrifuged for 10 min at 10,000 *g*. The clarified supernatant was applied to a QIAprep column inserted into a vacuum manifold (Promega) and a vacuum was applied. The QIAprep column was washed by the addition of 0.5 ml buffer PB, then 0.75 ml buffer PE [The compositions of the buffers PE and PB provided in the kit are not provided by the manufacturer.]. Residual buffer PE was removed by centrifugation at 10,000 *g* for 1

min. The QIAprep column was transferred to a fresh microcentrifuge tube and DNA eluted by the addition of 50 µl ddH_20, incubation at room temperature for 1 min, then centrifugation for 1 min at 10,000 *g*. DNA was either used immediately or stored at -20°C.

2.1.3 Plasmid 'Endofree' Maxi-preps

Plasmid 'endofree' maxi-preps were performed using the Qiagen QiaFilter Endofree Plasmid Maxi Kit. The system is a modified version of the alkaline lysis method, followed by binding of plasmid DNA to an anion-exchange resin. RNA, proteins and low molecular weight impurities are removed by a medium salt wash and plasmid DNA is eluted in a high-salt buffer. The DNA is then pelleted and desalted using isopropanol precipitation. This procedure includes an endotoxin removal stage, critical for effective transfection of DNA into mammalian cells, where the DNA is incubated with an "endotoxin removal" (ER) buffer (the composition of which is not provided by the manufacturer) which prevents LPS from binding to the anion-exchange resin during DNA purification. The detailed procedure was as follows: a single colony was picked from a freshly-streaked plate and used to inoculate a starter culture of 5 ml of LB containing the appropriate selective antibiotic. The starter culture was grown at 37°C, 225 rpm for 8 hours then diluted 1/1000 in fresh selective LB medium and incubated overnight at 37°C, 225 rpm. Cells were harvested by centrifugation at 6,000 *g* for 15 min at 4°C. The bacterial pellet was resuspended in 10 ml buffer P1 (50 mM Tris HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A) then 10 ml buffer P2 (200 mM NaOH, 10% SDS) was added. Tubes were inverted gently 4–6 times and incubated at room temperature for 5 min to allow cell lysis to occur. To the lysate, 10 ml buffer P3 (3 M potassium acetate, pH 5.5) was added for neutralisation. The lysate was filtered using a QiaFilter to remove precipitated proteins, genomic DNA and detergent. The filtrate was treated with ER buffer for 30 min on ice to remove endotoxins from the DNA. The lysate was applied to an anion-exchange column (previously equilibrated with buffer QBT [750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol, 0.15% Triton-X100]) and allowed to enter the resin by gravity flow. The column was washed with 60 ml buffer QC (1.0 M NaCl, 50 mM

MOPS, pH 7.0, 15% isopropanol) and DNA was eluted into an endotoxin-free tube by the addition of 15 ml buffer QN (1.6 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). DNA was precipitated by the addition of 10.5 ml isopropanol, mixed and centrifuged at 15,000 *g* for 30 min at 4 °C. The DNA pellet was washed with 5 ml of endotoxin-free 70% ethanol and centrifuged at 15,000 *g* for 10 min at 4 °C. The pellet was air-dried, then resuspended in TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA). DNA was quantified using spectrophotometry (see below), diluted to a final concentration of 1 μg/μl and stored in aliquots of 50 μl at –20 °C.

2.1.4 DNA quantification

DNA (10 μl) was diluted 1/100 in distilled water and analysed spectrophotometrically at wavelengths of 260 nm and 280 nm using a UV 300 spectrometer (Unicam).

The concentration of DNA was determined, where 1 absorbance unit (A_{260}) is equivalent to 50 μg/ml of double-stranded DNA, as follows:

DNA concentration in μg/ml = 50 x Abs_{260} x dilution factor.

Purity of DNA was assessed by obtaining the relative absorbances at 260 nm and 280 nm. Proteins absorb more heavily around 280 nm (primarily due to tryptophan absorbance, with a lesser contribution from tyrosine residues) and will shift the A_{260}/A_{280} ratio lower if present as contaminants in a DNA sample. The A_{260}/A_{280} ratio was normally 1.8 to 2.0 indicating low levels of protein contamination.

2.1.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed in 0.8 – 4% agarose containing 0.5 μg/ml ethidium bromide, using TAE buffer (40 mM Tris HCl, 20 mM sodium acetate, 2.5 mM EDTA, pH 8.0). Samples were mixed with 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water) before loading on the gel. Gels were run at 50-100 V until the dye front had moved to approximately 1 cm of the end of the gel. Gels were visualised and photographed using the Epi Chemi II Darkroom and Labworks Image Acquisition and Analysis software (UVP).

2.1.6 Purification of DNA from LMP agarose

DNA was electrophoresed using 1-2% low melting point (LMP) agarose (Gibco BRL) in 1x TAE buffer. The band of interest was viewed through glass on a transilluminator and excised from the gel (maximum 300 mg of agarose) using a clean scalpel. The gel slice was placed in a microcentrifuge tube and purified using the Wizard® PCR preps DNA purification system (Promega) as recommended by the manufacturer. Briefly, the agarose slice was incubated at 70°C until the agarose had completely melted. Resin (1 ml) was added and the slurry was mixed well for 20 sec. The resin/DNA slurry was applied to a Wizard® minicolumn, using a vacuum manifold. The column was washed under vacuum using 2 ml of 80% isopropanol and dried for 30 seconds. The column was further dried by centrifuging at 10,000 *g* for 2 min to remove residual isopropanol. The column was transferred to a clean microcentrifuge tube and DNA was eluted by the addition of 50µl ddH_2O, incubation at room temperature for 1 min, then centrifugation at 10,000*g* for 1 min. DNA was stored at -20°C until required.

2.1.7 Purification of PCR products

This procedure was used also for the removal of restriction enzymes or DNA fragments of less than 100 bp. Direct purification of PCR products (i.e. those products not requiring LMP agarose gel purification) was performed using the Wizard® PCR preps DNA purification system (Promega) as recommended by the manufacturer. Briefly, 30-300µl of the PCR reaction was added to 100 µl of Direct Purification Buffer and mixed by vortexing. Resin (1 ml) was added and the mixture vortexed briefly three times over a one minute period. The resin/DNA slurry was applied to a Wizard® minicolumn using a vacuum manifold. The column was washed under vacuum using 2 ml of 80% isopropanol and dried for 30 sec. The column was further dried by centrifuging at 10,000 *g* for 2 min to remove residual isopropanol. The column was transferred to a clean microcentrifuge tube and DNA eluted by the addition of 50 µl ddH_2O, incubation at room temperature for 1 min, then centrifugation at 10,000 *g* for 1 min. DNA was stored at -20°C until required.

2.2 Transformation of InvαF' / Top10 cells

A single vial of One Shot™ INVαF'/Top10 chemically competent cells was thawed on ice for each transformation. An aliquot of the ligation mixture (4 µl) was added directly into the competent cells and mixed by tapping gently. Vials were incubated on ice for 30 min, then heat shocked at 42°C for exactly 30 sec and snap cooled on ice for 2 min. Pre-warmed SOC medium (250 µl, see appendix I) was added to each vial and vials were incubated horizontally at 37°C, 225 rpm for 1 hour. The transformation mixture (50 µl to 200 µl) was spread on LB plates containing an appropriate selective antibiotic and incubated overnight at 37°C. Individual colonies were then picked for further characterisation.

2.3 Enzymatic analysis and modification of DNA for cloning purposes

2.3.1 General Restriction Digests

Restriction enzymes (RE) were obtained from New England Biolabs and were supplied with the appropriate buffers. Restriction digests were performed as follows:

Component	Volume	
DNA	x µl (0.5-1 µg)	x µl (2–5 µg)
H_2O	6-x µl	30-x µl
BSA (1 mg/ml)	1 µl	5 µl
RNase A	1 µl	5 µl
RE buffer (provided by manufacturer)	1 µl	5 µl
RE	1 µl	5 µl
Total Volume	**10 µl**	**50 µl**

Reactions were incubated for 2-3 hours at the recommended temperature for individual enzymes. Where more than one enzyme was used simultaneously, the buffer used was that recommended by the manufacturer for double or triple digests.

2.3.2 Ligation

DNA ligations were performed as follows:

Component	Volume
vector backbone DNA	1 µl
insert DNA	7 µl
DNA ligase buffer (10x)	1 µl
DNA ligase (1 U/µl) (New England Biolabs)	1 µl
Total volume	**10 µl**

Ligations were incubated overnight at 16°C and either used for transformation immediately or stored at -20°C until required.

2.3.3 Dephosphorylation

Dephosphorylation was performed as follows:

Component	Volume
DNA (in dH_2O)	x µl
H_20	40-x µl
SAP buffer (10x)	5 µl
Shrimp Alkaline Phosphatase (SAP)	5 µl
Total Volume	**50 µl**

Dephosphorylation was performed at 37°C for 1-2 hours and then the SAP was heat inactivated at 65°C for 15 min.

2.3.4 Blunt -ending

Blunt ending of restriction digested and purified DNA was performed as follows:

Component	Volume	
DNA (in dH_2O)	x µl	x µl
H_2O	15-x µl	75-x µl
dNTP (2 mM)	2 µl	10 µl
T4 DNA polymerase buffer (10x)	2 µl	10 µl
T4 DNA polymerase	1 µl	5 µl
Total Volume	**20 µl**	**100 µl**

Reactions were incubated at 15°C for 30 min before heat inactivation of the T4 DNA polymerase at 75°C for 15 min.

2.4 Southern blotting and detection

2.4.1 Transfer of DNA

After agarose gel electrophoresis, gels were soaked in 500 ml denaturing solution (1.5 M sodium chloride, 0.5 M sodium hydroxide) for 45 min and rinsed in ddH_20. Gels were then soaked in 500 ml neutralising solution (1.5 M sodium chloride, 1 M Tris HCl, pH 7.2) for 30 min and again in 500 ml for a further 15 min. Capillary southern blotting was performed as shown in Figure 2.1 below. Transfer was performed using 10x SCC (Appendix 1).

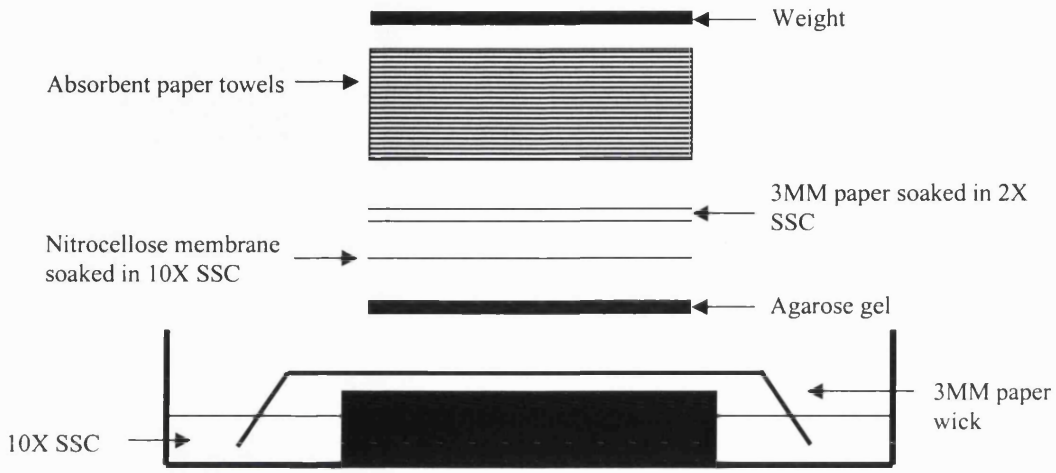


Figure 2.1. Schematic illustrating the apparatus used for Southern blotting.

2.4.2 Random labelling of probes

DNA probes were labelled with ^{32}P dCTP as follows: DNA (1 µl containing 50 ng DNA) was added to 36 µl water and heated to 100°C for 5 min before snap cooling on ice. The labelling mixture was prepared, using the denatured DNA above as follows:

Component	**Volume**
Denatured DNA (in dH_2O)	32 µl
bovine serum albumin (10 mg/ml)	2 µl
oligonucleotide labelling buffer (OLB, see appendix 1)	10 µl
^{32}P dCTP (Amersham)	5 µl
Klenow enzyme (New England Biolabs)	1 µl
Total volume	**50 µl**

The labelling mixture was incubated at room temperature overnight. The mixture was then added to 50 µl TNE (10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5). A spin column of Sephadex G-50 (Amersham Pharmacia) was prepared by blocking a syringe barrel with autoclaved glass wool and loading the syringe with Sephadex G-50 equilibrated in water. The column was equilibrated with TNE buffer by adding 100 µl of the buffer to the column and centrifuging the column at 1,500 rpm for 4 min. The equilibration was performed three times. The diluted labelling mixture was then loaded on the column and centrifuged at 1,500 rpm for 4 min. The unincorporated nucleotides were retained in the column and the DNA eluted into a sterile tube. Scintillation counting was used to estimate incorporation efficiency. The purified labelled DNA was added to 25 µl of 10 mg/ml salmon sperm DNA and heated to 100°C for 5 min before being snap cooled on ice. Probes were used immediately or stored at -20°C until required. The composition of all buffers is described in detail in Appendix 1.

2.4.3 Hybridisation of probes to membranes

Membranes were prehybridised while rotating overnight at 65°C in a hybridisation oven. Prehybridisation was performed using 10 – 15 ml of hybridisation solution (see appendix 1). The prehybridisation solution was removed and replaced with 10 – 15 ml of fresh hybridisation solution containing radiolabelled DNA probes (approximately 25 ng/blot). Hybridisation was performed for 6 to 12 hours at 65°C. The hybridisation solution was removed and the membranes were washed twice with 2x SSC at 65°C and once with 2x SSC containing 0.1% SDS at 65°C. Membranes were removed from the wash solution and allowed to dry at room temperature before being wrapped in saran wrap and placed in a cassette for exposure to Biomax film (Sigma). Exposure times ranged from 6 hours to 2 weeks.

2.5 PCR-TOPO cloning

Cloning of PCR products into the vector pCR 2.1-TOPO (Invitrogen) was performed as per the manufacturer's instructions. TOPO cloning involves the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector in the absence of restriction digestion or the use of ligase. The plasmid vector is supplied linearised with single 3' thymidine (T) overhangs for TA cloning and topoisomerase I covalently bound to the vector. The principle is based on the fact that *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine to the 3' ends of the amplified products. The presence of the 3' T overhang on the vector allows ligation, driven by the topoisomerase I, to occur. The mechanism whereby topoisomerase ligates the vector to the insert is as follows: the enzyme binds to duplex DNA at specific sites, cleaving the phosphodiester bond after 5'-CCCTT in one strand. The energy from the broken phosphodiester bond is conserved by the formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr 274) of the enzyme. The phospho-tyrosyl bond between the DNA and the enzyme can be destroyed by the 5' hydroxyl of the original cleaved strand, reversing the reaction, releasing the topoisomerase and ligating the vector to the insert (Invitrogen product manual – pCR II-TOPO) (Figure 2.2).

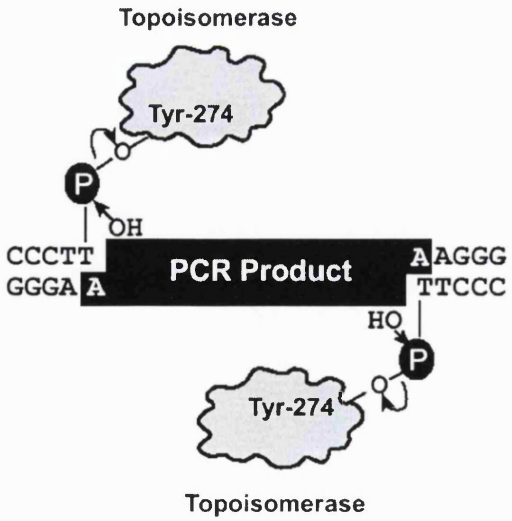
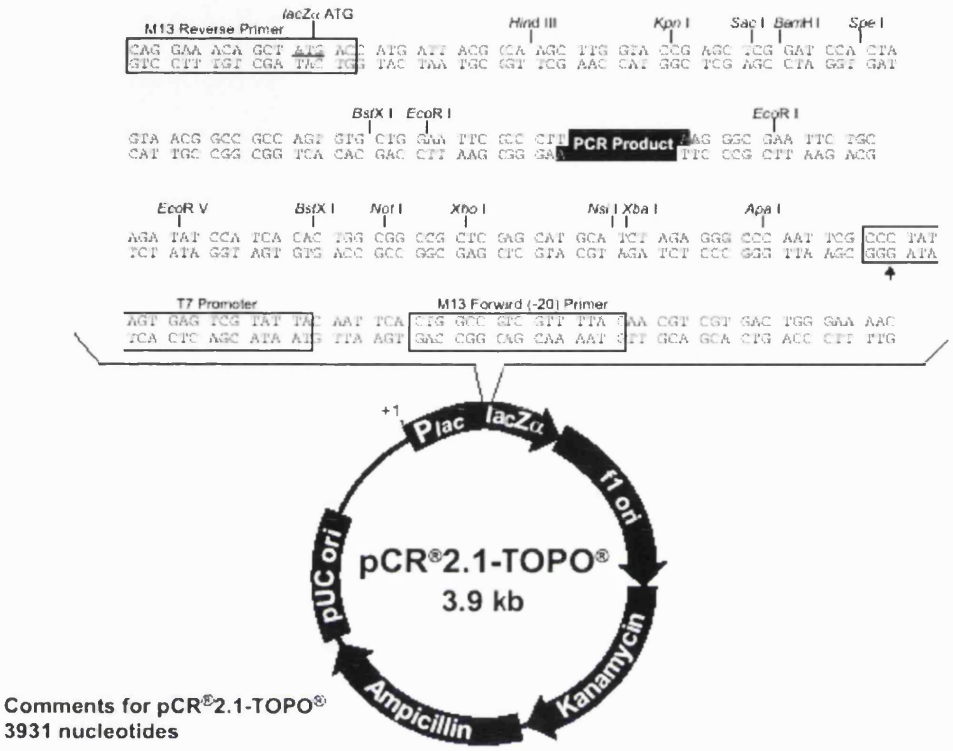


Figure 2.2 Mechanism of Topoisomerase action in TOPO TA cloning using pCR II-TOPO.



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

Figure 2.3. pCR 2.1 TOPO (Invitrogen) vector map and multiple cloning site.

The cloning reaction was prepared as follows:

Reagent	Volume
Fresh PCR product	0.5 to 4 µl
Salt solution	1 µl
Sterile water	add to total volume of 5 µl
pCR II-TOPO	1 µl
Final volume	**6 µl**

The reaction was mixed gently, incubated at room temperature for exactly 5 min, then transformed into One Shot Chemically Competent *E. coli* (as described above). Colonies were screened using restriction enzyme analysis and agarose gel electrophoresis.

2.6 Maintenance of cell cultures and transfection of mammalian cells

2.6.1 Cell culture

The Huh7 and Cos7 cell lines were maintained in Dulbecco's minimum essential medium (DMEM, Gibco BRL) supplemented with 10% serum equivalent (Hybaid) and 25 mM HEPES (Gibco BRL) (maintenance medium). Cells were passaged by trypsinisation every 3-4 days as follows: medium was discarded and monolayers were washed twice with versene (Gibco BRL). Trypsin (Sigma) was added to cells (100 µl/cm^2) and cells were incubated at 37°C for 2–3 min. Cells were dislodged from the flask by gently tapping the flask. Trypsinised cells were resuspended in maintenance medium and separated into a single cell suspension by passing the cells repeatedly through a 10 ml pipette. Cells were seeded into new flasks or plates at the desired density and incubated overnight at 37°C, 5% CO_2. Medium was replaced the following day and every two to three days as necessary.

2.6.2 Transfection

Transfection was performed using Lipofectamine 2000® (Gibco BRL) as per the manufacturer's instructions. Cells were seeded into 24 well plates or 6 well plates one day prior to transfection at an appropriate density to allow growth to 90% confluence within 24 hours (all quantities are shown below). Transfection was performed by incubating Lipofectamine 2000® with Optimem (Gibco BRL) for 5 min at room temperature. DNA was diluted in an equal volume of Optimem, combined with the Lipofectamine 2000® and incubated for 20 min at room temperature. The DNA-Lipofectamine 2000® mixture was added directly to 0.5 ml of fresh medium in each well. Cells were incubated for 24 to 48 hours to allow expression.

	24 well plate	**6 well plate**
Cos7	5 x 10^4 cells/well	2.5 x 10^5 cells/well
	50 µl optimem + 2 µl Lipofectamine 2000	250 µl optimem + 10 µl Lipofectamine 2000
	50 µl optimem + 1 µg DNA	250 µl optimem + 5 µg DNA
Huh7	2 x 10^5 cells/well	1 x 10^6 cells/well
	50 µl optimem + 2 µl Lipofectamine 2000	250 µl optimem + 10 µl Lipofectamine 2000
	50 µl optimem + 1 µg DNA	250 µl optimem + 5 µg DNA

2.7 Protein quantification

Protein quantification was performed using the Micro BCA Protein Assay Reagent Kit (Pierce). This assay is based on the use of bichinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. The purple reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}) and exhibits strong absorbance at 562nm (Smith *et al.*, 1985). The reagents provided in the kit are as follows:

Micro BCA reagent A: sodium carbonate, sodium bicarbonate and sodium tartrate (concentrations not provided by the manufacturer) in 0.2 N NaOH.
Micro BCA reagent B: Bicinchoninic acid (4%) in water
Micro BCA reagent C: Cupric sulphate pentahydrate (4%) in water

A set of diluted protein standards was prepared by dilution of the bovine serum albumin (BSA) provided in the kit (at a concentration of 2 mg/ml) to produce standards of 40, 20, 10, 5, 2.5 and 1 µg/ml. A doubling dilution series was prepared for each sample. Assays were performed in microtitre plates. A working reagent was prepared from the kit components by mixing 25 parts of Micro BCA reagent A, 24 parts of Micro BCA reagent B and 1 part of Micro BCA reagent C. A volume of 150 µl was added to 150 µl of diluted sample or standard. Plates were covered and incubated at 37°C for 2 hours, then cooled to room temperature. The absorbance was measured at 540nm. A standard curve was constructed and used to determine the protein concentration of samples.

2.8 Protein electrophoresis and detection

2.8.1 SDS polyacrylamide gel electrophoresis

Analytical electrophoresis of proteins was carried out in SDS-containing polyacrylamide gels with dimensions of 80mm by 85mm. The apparatus used was the Sigma Aldrich Mini Vertical Gel Electrophoresis Unit. Glass plates were cleaned with ethanol and assembled as per the manufacturer's instructions. Details of all solutions are provided in Appendix 1.

Resolving gel was prepared (50 ml) as follows:

Recipe for 12% resolving gel (50ml)	
Water	16.5 ml
1.5M Tris HCl pH 8.8	12.5 ml
10% (w/v) SDS	0.5 ml
Acrylamide-bisacrylamide (40%)	20 ml
10% (w/v) ammonium persulphate	500 µl
TEMED	20 µl

The resolving gel was poured between the glass plates to within 0.5 cm of the position of the combs and covered with 50 µl of water-saturated butanol. After polymerisation of the resolving gel for at least 30 min the butanol was rinsed away with distilled water. The stacking gel was prepared (10 ml) and poured onto the resolving gel.

Recipe for 5% stacking gel (10ml)	
Water	6.8 ml
1M Tris HCl pH 8.8	1.25 ml
10% (w/v) SDS	0.1 ml
Acrylamide-bisacrylamide (40%)	1.7 ml
10% (w/v) ammonium persulphate	100 µl
TEMED	10 µl

Combs were inserted gently between the glass plates and the stacking gel was allowed to polymerise for at least 30 min. Combs were removed and wells rinsed with distilled water to remove residual unpolymerised acrylamide. The plates were attached to the manifold within the tank and 500 ml SDS-PAGE running buffer (appendix 1) was added to the tank. Samples were resuspended in (or added to) 4x SDS-PAGE loading buffer (1x final concentration, see appendix 1) and heated at 100°C for 5 min, before snap cooling on ice. Samples were loaded (10 µl to 20 µl) into wells. The molecular weight marker used, of which 10 µl was loaded per well, was the Rainbow recombinant protein marker (Amersham Life Science). Gels were

run at 100 V until the dye front reached the bottom of the gel. The apparatus was disassembled and the gel used either for Coomassie Blue staining, fixation for autoradiography or for western blotting as described below.

2.8.2 Coomassie Blue staining of protein gels

Gels were stained by soaking overnight on a shaker in 0.25% Coomassie Brilliant Blue R250 (dissolved in 45% methanol, 45% distilled water, 10% glacial acetic acid). Gels were destained for several hours in 45% methanol, 45% distilled water, 10% glacial acetic acid. Stained gels were photographed using Epi Chemi II Darkroom and Labworks Image Acquisition and Analysis software (UVP).

2.8.3 Fixing SDS-PAGE gels for autoradiogaphy

Gels were fixed by soaking in 30% (v/v) methanol, 10% (v/v) acetic acid in distilled water for 30 min. Fixed gels were then placed on two dry pieces of 3MM Whatman filter paper, covered with a layer of Saran wrap and placed on the gel drier. Gels were dried at 60°C for 1 hour then placed into a cassette. Exposure of films was performed at -70°C for periods of time ranging from 12 hours to 2 weeks.

2.8.4 Western blotting

Western blotting was performed using a semi-dry blotter (Biorad). SDS-PAGE was performed as described above. The polyacrylamide gel was removed and soaked briefly in transfer buffer (39 mM glycine, 48 mM Tris HCl, 0.037% SDS and 20% methanol). The PDF membrane was cut to the same dimensions as the gel, pre-wetted in methanol and soaked in transfer buffer. Ten sheets of 3MM filter paper (also of same dimensions as gel) were also soaked in transfer buffer. The gel was placed on top of the PDF membrane and the two were inserted between a sandwich of pre-wetted (in transfer buffer) 3MM paper. The entire cassette was placed between the electrodes and blotting was performed for 1 hour at room temperature at 25 V. After protein transfer, filters were stained in 0.2% (w/v) solution of Ponceau S in order to visualise transferred proteins.

2.8.5 Antibody detection of proteins

Protein detection was performed as follows: Membranes were incubated for 2 hours in 15 ml blocking solution (10% (w/v) non-fat dried milk in PBS) to block non-specific binding of antibodies. After blocking, filters were transferred to 10 ml fresh blocking solution containing the required primary antibody at an appropriate concentration. Filters were then incubated at room temperature for 2 hours, washed three times for 10 min in Tris-saline (150mM NaCl, 50mM Tris HCl, pH 7.5) containing 0.02% Tween-20, then transferred (where necessary) to 15ml blocking solution in Tris-saline containing HRP-conjugated secondary antibody at a dilution of 1:10,000 and incubated at room temperature for a further 2 hours. Filters were then washed four times in Tris-saline. Horseradish peroxidase conjugated antibodies were detected using Amersham's Enhanced Chemiluminescence (ECL) reagents as per the manufacturer's instructions.

2.9 Sequencing

Sequencing was performed by Qiagen® Sequencing Services. DNA of high quality was obtained using the Qiagen spin miniprep kit. DNA samples (2 μg) and sequencing primers (50 pmol, at a concentration of 10 pmol/μl in distilled water) were sent to Qiagen, Germany. Results (chromatogram and sequences) were obtained by email.

2.10 Sequence analysis and primer design

All sequence analysis and primer design (for amplification and sequencing) was performed using Omiga 2.0 (Oxford Molecular) software. Sequences were inserted manually or were downloaded from Genbank.

2.11 RNA isolation and analysis

2.11.1 RNA purification

RNA was purified using the RNAgents® Total RNA Isolation System (Promega). RNase contamination was minimised by (i) treatment of all solutions, where possible, with 0.01% diethyl pyrocarbonate (DEPC) overnight at 37°C prior to autoclaving, (ii) treatment of all glassware by baking for 12 hours at 180°C and (iii) the use, wherever possible, of disposable RNase-free plasticware. Surfaces were treated with RNaseAway® (Molecular Bioproducts) before work commenced.

RNA was purified either from (i) frozen tissue, which was homogenised, using a mortar and pestle in liquid nitrogen or (ii) from cell culture monolayers, where cells were harvested by trypsinisation, snap frozen and stored at -80°C until required (no further homogenisation was performed). The procedure was as follows (volumes are given for 175 cm^2 flask of cells/ 250 mg of tissue and were adjusted for smaller or larger samples). Samples were disrupted by the addition of 3 ml chilled denaturing solution (26 mM sodium citrate, pH 4.0, 0.5% N-lauryl sarcosine, 0.125 M β-mercaptoethanol, 4 M guanidine thiocyanate). A volume of 300 µl per tube 2 M sodium acetate (pH 4) was added and the contents were mixed by inversion 3–4 times before the addition of 3 ml phenol:chloroform:isoamylalcohol per tube. Tubes were mixed gently and incubated on ice for 15 min. Samples were centrifuged at 10,000 *g* for 20 min at 4°C and the aqueous phase was removed. An equal volume of isopropanol was added to the aqueous phase and the sample was incubated at -20°C for 30 min to allow RNA precipitation. RNA was pelleted by centrifugation at 10,000 *g* for 10 min at 4°C. The RNA pellet was washed once in 2.5 ml denaturing solution, then again precipitated by the addition of isopropanol and centrifugation. The pellet was washed once with ice-cold 75% ethanol, then again pelleted at 10,000 *g* for 10 min at 4°C. Pellets were air dried for 20 min, then resuspended in 400 µl DEPC-treated water. Resuspended RNA was quantified spectrophotometrically (see below) and stored in small aliquots at -80°C.

2.11.2 RNA quantification and analysis of purity

Purified RNA (10 µl) was diluted 1/40 in distilled water and analysed spectrophotometrically at wavelengths of 230 nm, 260 nm and 280 nm.

The concentration of RNA was determined, where 1 absorbance unit (A_{260}) is equivalent to 40 µg/ml of single-stranded RNA, as follows:

RNA concentration in µg/ml = 40 x Abs_{260} x dilution factor.

Purity of RNA was assessed by obtaining the relative absorbances at 230 nm, 260 nm and 280 nm. The A_{260}/A_{280} ratio was routinely 1.7 to 1.9 indicating low levels of protein contamination. The possibility of guanidine thiocyanate contamination was discounted as the A_{260}/A_{230} ratio exceeded 1.5.

2.11.3 RNA gel electrophoresis

Analytical electrophoresis of RNA was performed in agarose gels containing formaldehyde. The agarose gel (30 ml) was prepared by dissolving 0.3 g agarose in 19 ml DEPC treated water. The agarose solution was cooled to 60°C before adding 6 ml 5x formaldehyde running buffer (0.1 M MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0) and 5.07 ml formaldehyde. The gel was cast in a fume hood to prevent exposure to formaldehyde fumes and allowed to set for 30 min at room temperature. Formaldehyde running buffer (1x) was prepared by diluting 1 part of 5x formaldehyde running buffer in 4 parts of DEPC treated water. Sample were prepared by mixing 5 µl of RNA (up to 30 µg) with 2 µl 5x formaldehyde running buffer, 3 µl formaldehyde and 10 µl formamide. Samples were heated to 60°C for 15 min, then snap cooled and briefly centrifuged. Samples were mixed with 2 µl RNA loading buffer (50% glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol FF). Prior to loading, the gel was pre-run at 75 V (5 V/cm) for 5 min. Samples were applied and electrophoresis performed at 60 V (4 V/cm) until the dye from was 1 cm from the end of the gel. Running buffer was recirculated every 30 min during electrophoresis. The gel was then either stained or used for northern blotting. Staining was performed in 0.1 M sodium acetate containing 0.5 µg/ml ethidium bromide for

45 min. Destaining was performed in 0.1 M sodium acetate overnight at room temperature. Gels were visualised and photographed using the Epi Chemi II Darkroom and Labworks Image Acquisition and Analysis software (UVP).

2.11.4 Northern blotting

RNA gels were rinsed in several changes of DEPC-treated deionised water to remove to formaldehyde. The gel was then soaked in 20x SSC for 45 min. Capillary blotting was performed in essentially the same way as Southern blotting (as described above) except that the transfer buffer used in northern blotting was 20x SSC. RNA transfer proceeded for 12-18 hours. The nitrocellulose membrane was then removed, rinsed briefly in 6x SSC, then allowed to dry at room temperature for 30 min. The dried filter was placed between two sheets of Whatman 3MM filter paper and baked at 80°C for 30 min to 2 hours in a vacuum oven. The filter was stored at room temperature until hybridisation.

2.12 Reverse transcription (using random hexamers)

Reverse transcription (RT) of RNA was primed using random hexamers. Random hexamers are short oligodeoxyribonucleotides of random sequence [$d(N)_6$] that anneal to random complementary sites on a target DNA or RNA, to serve as primers for DNA synthesis by reverse transcriptase. RT was performed in a two-step reaction, as follows:

Component	**Volume**
Random hexamers (100 ng/µl)	1 µl
10 mM dNTPs	1 µl
HepG2 RNA (1ug/µl)	5 µl
H_2O	5 µl
Total Volume	**12 µl**

Samples were incubated at 65°C for 5 min, then snap cooled on ice. Tubes were centrifuged briefly to collect samples, then the following components were added:

Component	**Volume**
5x First Strand Buffer	4 µl
0.1M DTT	2 µl
RNaseIN (ribonuclease inhibitor)	1 µl
Superscript II reverse transcriptase (Gibco BRL)	1 µl
Total Volume	**20µl**

The reaction was incubated at 42°C for 50 min, then heat inactivated at 70°C for 15 min. The addition of 1 µl RNase H (2 units), followed by incubation at 37°C for 20 min, was performed to remove RNA complementary to the cDNA. Samples were stored at -20°C until required.

CHAPTER III

CLONING OF SCCA1 AND ITS EFFECT ON BINDING OF HBV TO CELLS

3.1 INTRODUCTION

An SCCA1-like protein, HBV-BP, has been shown by De Falco *et al.* (2001b) to mediate enhanced binding of HBV to cells. These results have yet to be confirmed. Furthermore, our interest in characterising the reported effect of SCCA1 necessitated the development of the tools required for expression, purification and detection of the protein.

In order to investigate the effect of SCCA1 expression in mammalian cells on the binding of HBV to cells, a cDNA for SCCA1 was required to be cloned into mammalian expression vectors. Such vectors place a gene under the control of a heterologous promoter which is induced to express the protein upon transfection of the vector into mammalian cells. Although the expression of an unmodified protein is preferable, antibodies to SCCA1, although they exist (Cataltepe *et al.*, 2000), were not available to us. Therefore it was also necessary to clone the SCCA1 cDNA into a vector which allowed addition of a tag (the V5 polyHis tag facilitates antibody detection and purification of the protein). The tagged and untagged proteins were compared, because in some cases e.g. MAP kinase, the presence of a tag has been shown to affect protein function detrimentally (Ramage *et al.*, 2002). De Falco *et al.* (2001b) proposed that the small sequence differences of HBV-BP compared with wild type SCCA1 may be important in the effect observed on HBV-cell binding. Therefore, the sequence of the cloned cDNA was analysed and compared with both wild type SCCA1 and HBV-BP. The effect on virus-cell binding of SCCA1 expression in mammalian cell lines (whether hepatocyte-derived or not) was investigated using virus-cell binding assays.

3.2 MATERIALS AND METHODS

3.2.1 Production of SCCA1 mammalian expression constructs

3.2.1.1 Reverse transcription

Subconfluent HepG2 cells (approximately 10^7 cells) were harvested by trypsinisation, washed once in PBS and pelleted by centrifugation at 1,000 rpm for 5 min. Total RNA was isolated using RNAgents® Total RNA Isolation System (Promega), quantified spectrophotometrically and stored at –70°C. Reverse transcription of RNA was performed in a two-step reaction. The primer utilised was SCCAouter2 (5'ctg gaa gaa aaa gta cat tta tat gtg ggc 3').

Reverse transcription was performed as follows:

Component	Volume
Primer SCCAouter2 (10 pmol/µl)	1 µl
10 mM dNTPs	1 µl
HepG2 RNA (1 ug/µl)	5 µl
H_2O	5 µl
Total Volume	**12 µl**

Samples were incubated at 65°C for 5 min, then snap cooled on ice. Tubes were centrifuged briefly to collect samples, then the following components were added:

Component	Volume
5x First Strand Buffer	4 µl
0.1 M DTT	2 µl
RNaseIN (ribonuclease inhibitor)	1 µl
Superscript II reverse transcriptase	1 µl
Total Volume	**20 µl**

The reaction was incubated at 42°C for 50 min, then heat inactivated at 70°C for 15 min. The addition of 1 µl RNase H (2 units) followed by incubation at 37°C for 20 min, was performed to remove RNA complementary to the cDNA. Samples were stored at -20°C until required.

3.2.1.2 Nested PCR amplification

First round PCR was performed using primers SCCAouter1 (5' cac agg agt tcc aga tca cat cga g 3') and SCCAouter2 (5'ctg gaa gaa aaa gta cat tta tat gtg ggc 3'). Cycling was performed in the Perkin Elmer Geneamp PCR system 2400 as follows: 94°C for 1 min, 25 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min, followed by a final extension period of 72°C for 10 min. Second round PCR was performed using primers designed to introduce *XhoI* and *BstB1* restriction sites to facilitate cloning. Primers used were SCCA1-*XhoI* (5'gcg **ctc gag** ttc acc atg aat tca ctc agt gaa gcc 3', *XhoI* restriction site in bold) and SCCA1-His (5' cgc g**tt cga a**cg ggg atg aga atc tgc ca 3', *BstB1* restriction site in bold). PCR cycling was performed as above. PCR products were cloned into pCR II-TOPO (TOPO TA cloning kit, Invitrogen) to produce pCR-TOPO SCCA1. The introduction of restriction sites *XhoI* and *BstB1* was confirmed using restriction digests. During amplification, the TAG stop codon of the cDNA was not included in the amplified fragment, as stop codons within the mammalian expression constructs were used.

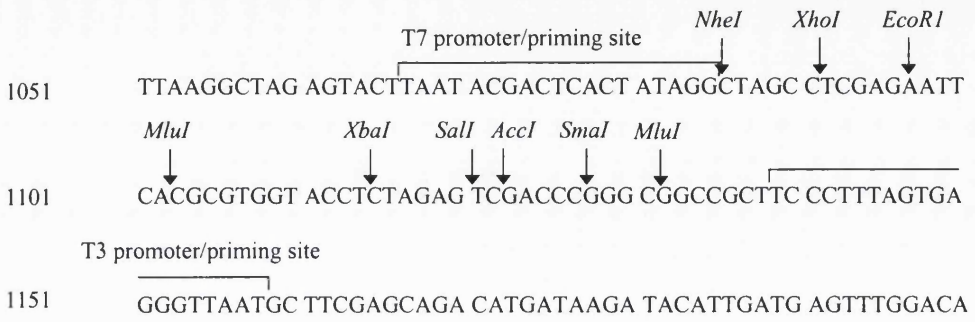
3.2.1.3 Production of pCiNeo SCCA1

SCCA1 was cloned as a *XhoI–SmaI* fragment into pCiNeo (Promega, Figure 3.1) as follows: PCR-TOPO SCCA1 was digested with *BstBI* at 65°C for 3 hours, then the DNA was purified using the Wizard® PCR preps DNA purification system (Promega). Blunt-ending was performed for 30 min at 15°C, followed by heat inactivation. DNA was then further digested with *XhoI* for 3 hours at 37°C. pCiNeo was digested with *XhoI* and *SmaI* for 3 hours and purified using the Wizard® PCR preps DNA purification system (Promega). Ligation of the digested vector and SCCA1 *XhoI*–blunt end fragment was perfomed overnight at 16°C. The ligation mixture was transformed into One-Shot® Chemically Competent *E. coli* (Invitrogen),

large-scale cultures were grown and the DNA was purified using the Endo-free Plasmid Maxi kit (Qiagen®). The original stop codon was not included, however the cloning strategy employed resulted in the use of a stop codon within the vector as shown below.

3.2.1.4 Production of pCDNA SCCA1

SCCA1 was cloned as an *XhoI–BStBI* fragment into pCDNA 3.1 V5 His C (Figure 3.2) as follows: PCR-TOPO SCCA1 was digested with *BstBI* (an isoschizomer of *SfuI)* at 65°C for 3 hours, then with *XhoI* at 37°C for a further 3 hours. The digested DNA was purified using the Wizard® PCR preps DNA purification system (Promega). The vector, pCDNA 3.1 V5 His C, was also digested with *XhoI* and *BstBI* as above. Ligation of the insert into the pCDNA vector was performed overnight at 16°C and followed by transformation into One-Shot® Chemically Competent *E. coli* (Invitrogen). DNA was purified using the Endo-free Plasmid Maxi kit (Qiagen®), quantified and stored in aliquots at –20 °C.



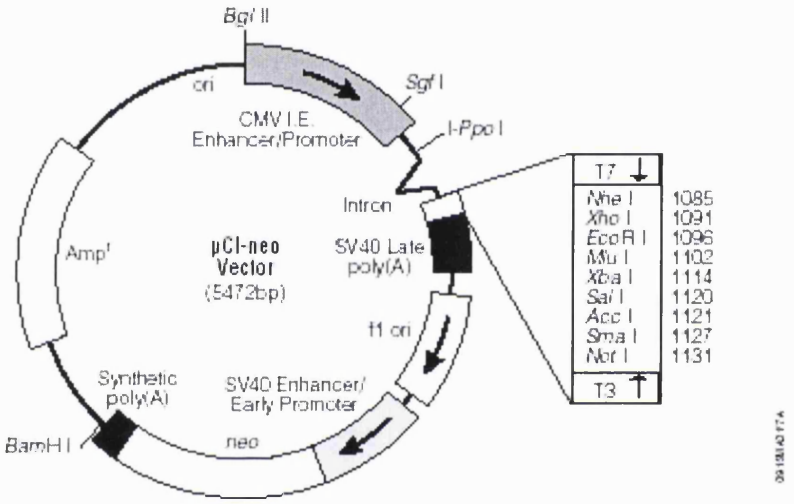


Figure 2. pCI-neo Vector circle map and sequence reference points. Neo = neomycin phosphotransferase.

pCI-neo sequence reference points.

Cytomegalovirus immediate-early enhancer	1–659
Cytomegalovirus immediate-early promoter	669–750
Chimeric intron	890–1022
T7 promoter	1067–1085
Multiple cloning region	1085–1137
T3 promoter	1140–1158
SV40 late polyadenylation signal	1167–1388
Phage f1 region	1483–1938
SV40 enhancer and early promoter	2000–2418
SV40 minimum origin of replication	2316–2381
Coding region of neomycin phosphotransferase	2463–3257
Synthetic polyadenylation signal	3321–3369
β-lactamase (Ampr) coding region	3780–4640

Figure 3.1 pCiNeo (Promega) vector circle map and multiple cloning site

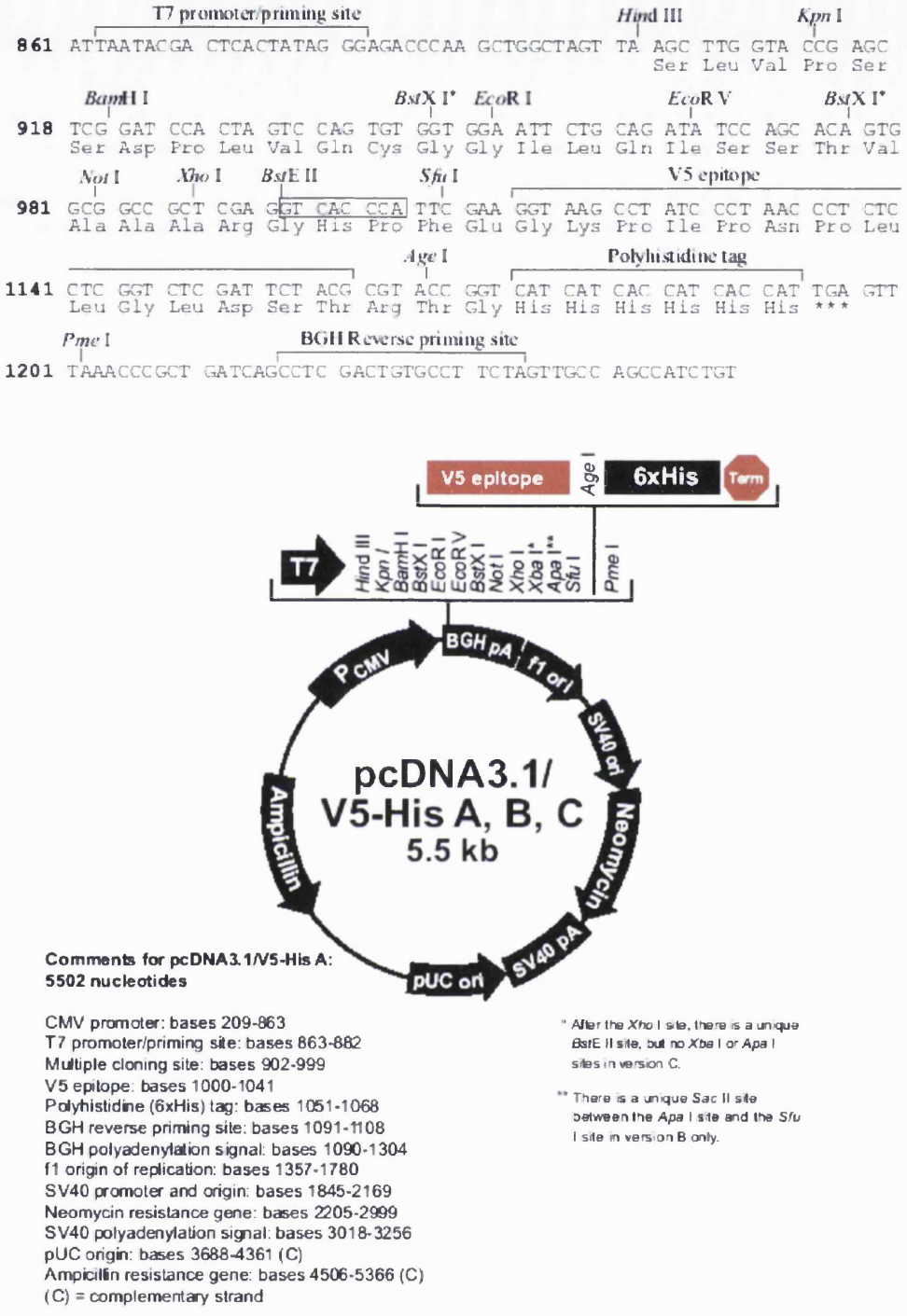


Figure 3.2 pCDNA 3.1 V5 His A, B, C (Invitrogen) plasmid circle map and multiple cloning site. *SfuI* is an isoschizomer of *BstB1*.

3.2.2 Sequencing of SCCA1

Sequencing was performed to confirm that the sequence of the SCCA1 cDNA insert in pCiNeo SCCA1 and pCDNA SCCA1 corresponded to the known wild type SCCA1 sequence. The constructs were sequenced using a series of primers designed using the published sequence of human squamous cell carcinoma antigen 1, mRNA, complete CDS (Genbank accession number U19556), as well as the standard primers T7, T3 and Bovine Growth Hormone (BGH) reverse primer (Table 3.1).

Table 3.1 Primers used for sequencing of SCCA1 constructs

Primer name	Sequence (5'–3')
SCCA210*	caa gtc aca gag aac acc aca g
SCCA598M*	aat ttc ttc tcc cac tgc cc
SCCA845M*	caa act tgt cca ttc cat c
SCCA1053M*	ctg ctc cct cct ctg taa cct c
SCCA1173M*	gga tgc tgt tgg tct tat ttt g
T7 primer	taa tac gac tca cta tag gg
T3 primer	aat taa ccc tca cta aag gg
BHG reverse primer	tag aag gca cag tcg agg

*Nomenclature of SCCA1 specific primers: The number following SCCA1 indicates the nucleotide position of the primer site on the SCCA1 cDNA, "M" indicates that the primer is in the reverse orientation

3.2.3 Cell culture and transfection

The Huh7 and Cos7 cell lines were maintained in Dulbecco's minimum essential medium (DMEM, Invitrogen) supplemented with 10% serum equivalent (Hybaid) and 25 mM HEPES (Invitrogen). Cells were passaged by trypsinisation every 3-4 days. Transient transfection of pCDNA SCCA1 and pCDNA3.1 V5 His LacZ (positive control) into Cos7 cells and Huh7 cells in 24 well plates was performed using Lipofectamine 2000 (Invitrogen). Expression of SCCA1-V5-His and Lac Z-V5

His fusion proteins was confirmed by 12% polyacrylamide gel electrophoresis followed by western blotting and detection using HRP-conjugated anti-V5 antibody (1:5000, Amersham Pharmacia) and Enhanced Chemiluminescence (Amersham Pharmacia).

3.2.4 Virus binding assays

Transfected cells were incubated for 48 hours to allow expression. Cells were washed twice with DMEM (containing no serum equivalent). Virus preparations comprising 3 ml viraemic human serum diluted in 100 ml DMEM (resulting in a final concentration of 1 pg/μl HBV DNA) were added to the cells (0.5 ml per well) and incubated overnight at 37°C. Cells were washed three times with PBS. Total DNA was harvested by the addition of 50 μl per well of PK buffer (50 mM KCl, 10 mM Tris HCl pH 8.5, 0.45% NP40) containing 0.2 mg/ml freshly-added proteinase K (Sigma). For internalisation studies, cells were harvested by trypsinisation, washed in PBS and then solubilised in PK buffer containing 0.2 mg/ml freshly-added proteinase K. Samples were incubated at 56°C for 2.5 hours, followed by inactivation of the proteinase K at 100°C for 15 min. Viral DNA was analysed by PCR using a modification of a protocol described by Kock *et al.* (1996). Briefly, PCR was performed using primers HBV1966+ (5' tgc cat ttg ttc agt ggt tcg tag ggc 3') and HBV2830- (5' ccg gca gat gag aag gca cag acg g 3'). Cycling was as follows: 94°C for 1 min, 20 cycles of 94°C for 1 min then 72°C for 3 min, followed by a final extension of 72°C for 6 min. Low cycle numbers were used to ensure amplification was limited to the exponential phase. Amplicons were separated using 1% agarose gel electrophoresis, transferred to nylon membranes by Southern blotting and detected using ^{32}P-labelled HBV genomic DNA as described in Chapter II and previously (Ling and Harrison, 1997). Comparative quantification was performed using scanning densitometry. Relative virus binding is expressed using arbitrary units, which may vary between experiments depending on background values used for normalisation. Assays were performed independently at least three times, with each experiment utilising six replicates. Results shown are in all cases derived from a single experiment which is representative of all experiments. The technique was shown to effectively discriminate between virus concentrations ranging from 1 pg/μl to 3 fg/μl.

3.3 RESULTS

3.3.1 Production of pCiNeo SCCA1 and pCDNA SCCA1

RNA purified from HepG2 cells was reverse transcribed to produce a pool of cDNA. Nested PCR using the cDNA as a template was utilised to obtain a full-length cDNA of SCCA1, to be cloned into mammalian and subsequently bacterial expression vectors. The product of the first round of the nested PCR was not detectable by agarose gel electrophoresis (AGE), but was utilised as the template for the second round PCR using primers designed to introduce restriction sites to facilitate cloning.

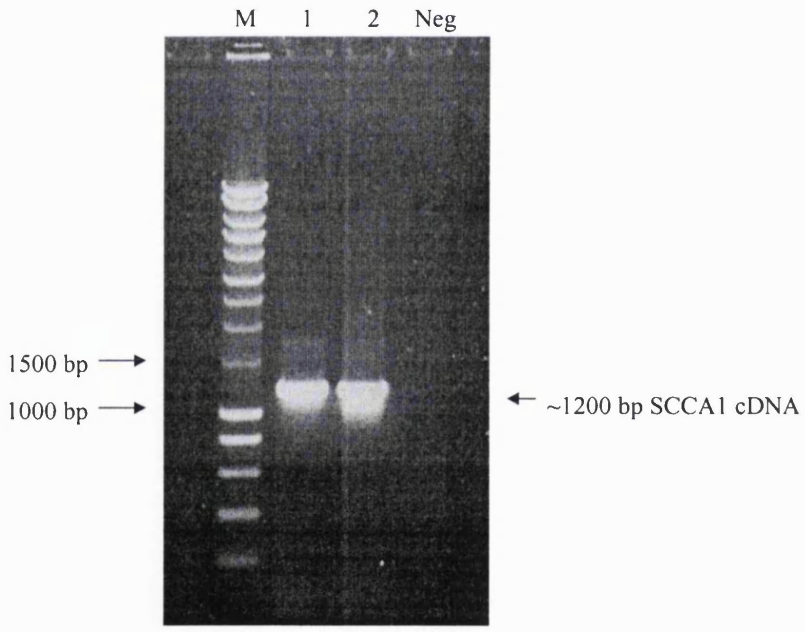
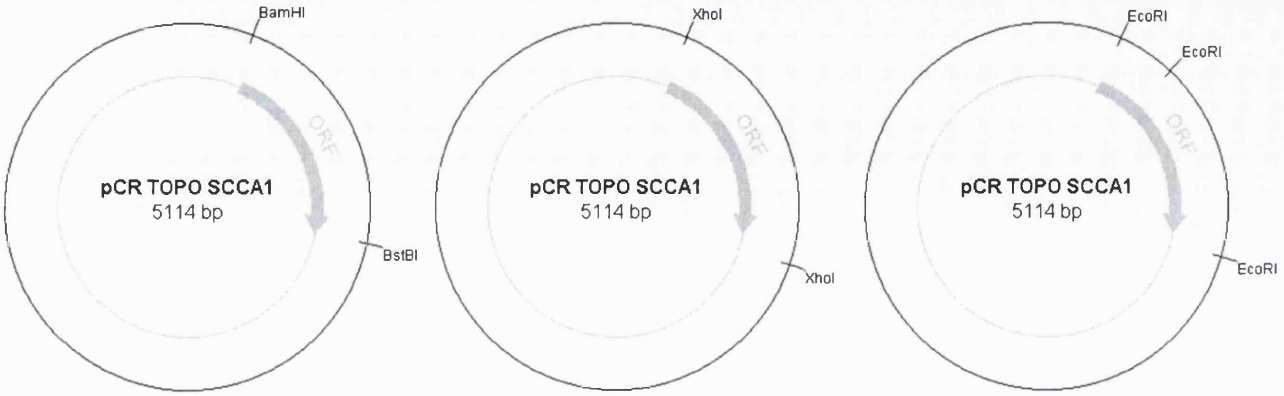


Figure 3.3 Agarose (1%) gel electrophoresis showing results of the second round PCR. M, molecular weight marker (Bioline Hyperladder I); 1 and 2, duplicate PCR reactions showing full-length SCCA1 cDNA at ~1200 bp; Neg, non-template control.

Agarose gel electrophoresis of the product of the second round PCR showed a clear product of approximately 1200 bp, the expected size for a full-length cDNA for SCCA1 (Figure 3.3). The SCCA1 cDNA was cloned into pCR II-TOPO to produce pCR-TOPO SCCA1 and restriction digest analysis was performed using two clones, clone 1 and clone 2, to confirm both the presence of the insert in pCR-TOPO and the introduction of the *Xho1* and *BstB1* sites into the termini of the cDNA.

A)



B)

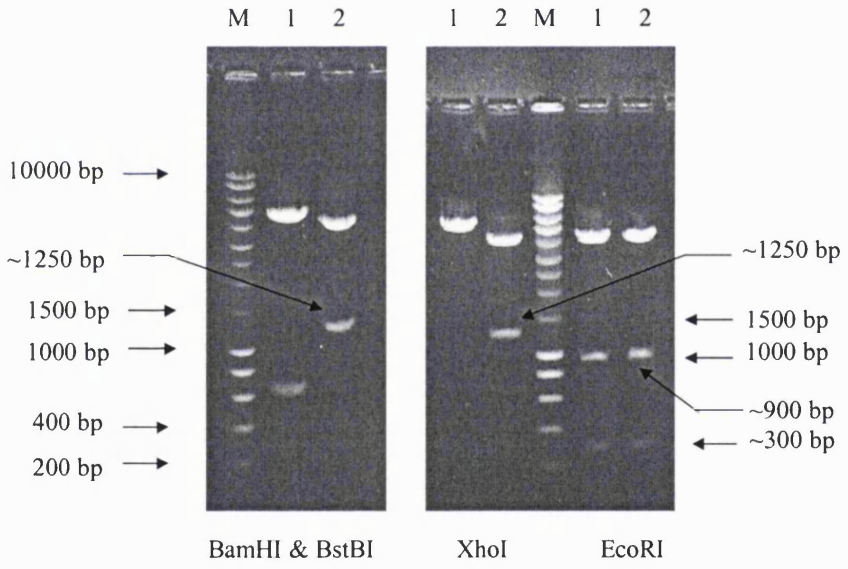


Figure 3.4 (A) Line diagrams showing predicted position of restriction sites; and (B) results of restriction digest analysis of two candidate clones of pCR-TOPO SCCA1. M, molecular weight marker (Bioline hyperladder I); 1 and 2 are individual clones of pCR-TOPO SCCA1. Digestion with *BamH1* and *BstB1* confirms introduction of a *BstBI* site has occurred in clone 2 (band at ~1250bp and vector), *XhoI* digestion confirms introduction of a *XhoI* site in clone 2 (band at ~1250 bp and vector). Digestion with *EcoRI* confirms the identity of the insert as SCCA1 (bands at ~300 bp, ~900 bp and vector).

Both clones were shown to contain an insert shown to be SCCA1 and in both clones introduction of *XhoI* and *BstBI* sites was confirmed, however, in clone 1 the insert was shown to be in the opposite orientation (Figure 3.4). Clone 2 was used for subsequent subcloning. The SCCA1 cDNA was subcloned into two mammalian expression vectors, pCiNeo and pCDNA 3.1 V5 His C (the latter allows addition of a V5 polyHis tag to the SCCA1 protein which is necessary as we did not have an antibody directed against SCCA1). SCCA1 was cloned as an *XhoI*–blunt end fragment into pCiNeo to produce pCiNeo SCCA1 and as an *XhoI–BstBI* fragment into pCDNA 3.1 V5 His C to produce pCDNA SCCA1. Transformation was perfomed and at least 6 colonies of each were inoculated into overnight cultures for restriction enzyme analysis. Colonies of pCiNeo SCCA1 were analysed for correct inserts using *BamHI* and *BglII* digestion, whereas pCDNA SCCA1 colonies were characterised using digestion with *EcoRI* and *SmaI*. Agarose gel electrophoresis of the restriction digests (Figure 3.5) showed pCDNA SCCA1 clone 9 and pCiNeo clone 1, amongst others, to be suitable candidates for further analysis.

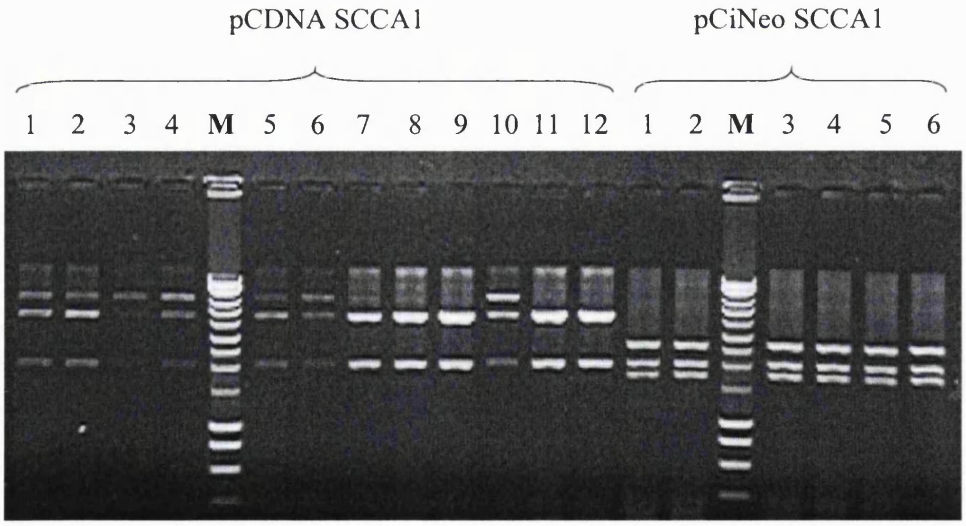


Figure 3.5 Agarose (1%) gel electrophoresis showing restriction enzyme analysis of 12 candidate clones of pCDNA SCCA1 (digested with *EcoRI* and *SmaI*) and 6 clones of pCiNeo SCCA1 (digested with *BamHI* and *BglII*). M, molecular weight marker (Bioline Hyperladder 1). Faint bands represent the products of partial DNA digestion.

3.3.2 Sequence Analysis

Sequencing was used to confirm (i) that the insert was indeed a cDNA for SCCA1 and (ii) that the junctions between the vector and the insert were as predicted (Figures 3.6 and 3.7). In both cases, use was made of stop codons within the vector. Sequence data was used to predict the amino acid sequence of the translated protein. The sequence data obtained was compared with published sequences for SCCA1 (Genbank accession number U19556) and HBV-BP (De Falco *et al.*, 2000) at both the nucleotide (Figure 3.8) and amino acid levels (Figure 3.9). Comparison of the predicted amino acid sequence of SCCA1 (complete cds, accession number U19556), HBV-BP (De Falco *et al.*, 2000b) and of the cDNA cloned into pCDNA and used in subsequent experiments showed extremely high identity (greater than 99% at the amino acid level compared with either sequence). Comparison of the nucleotide sequence of HBV-BP and pCDNA SCCA1 with the Draft Human Genome (using $Blast_p$) was performed and in both cases a unique match was found, namely SCCA1. This suggests that the SCCA1-like molecule cloned from HepG2 mRNA and utilised in the experiments below is SCCA1 rather than a variant thereof (Roijer *et al.*, 2003). The sequence of this clone has been submitted to the Genbank/EMBL database (Accession Number AJ515706). Figure 3.10 shows the predicted amino acid sequence of the cloned SCCA1 including structural motifs related to the archetypal serpin α1 proteinase inhibitor (Bartuski *et al.*, 1997) and potential N-glycosylation sites. The ov-serpins, which include SCCA1 are characterised by the absence of a signal peptide and a lack of N- and C- terminal extensions (Bartuski *et al.*, 1997).

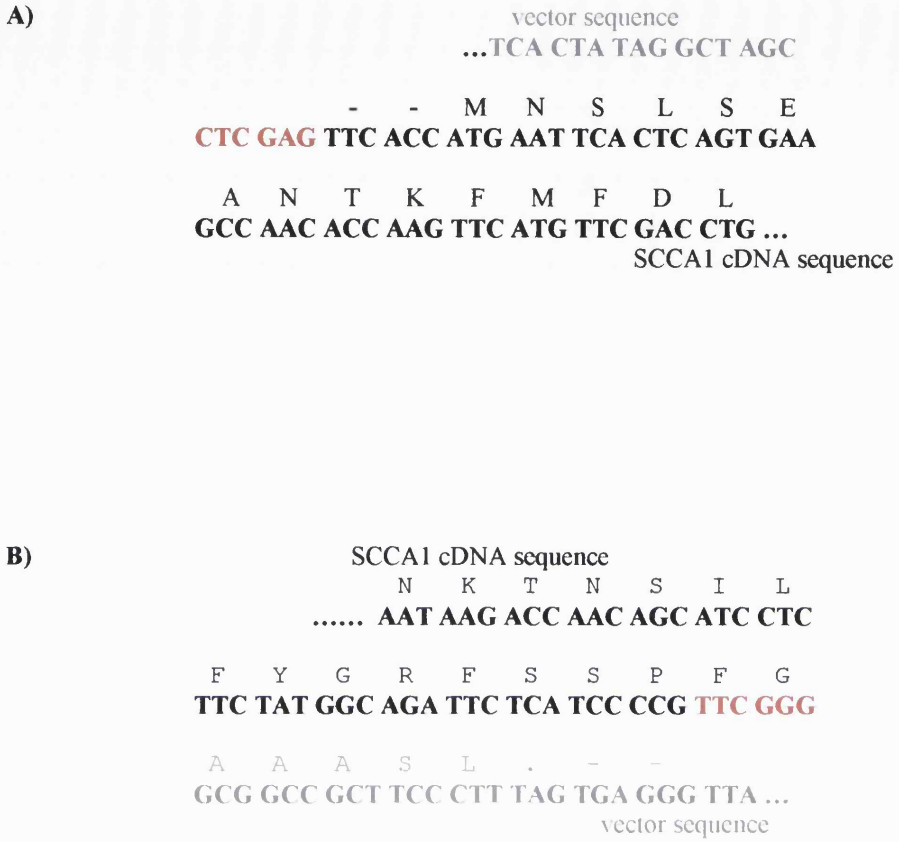


Figure 3.6 pCiNeo SCCA1 (A) The upstream junction and (B) the downstream junction between the ligated vector (pCiNeo) and the cDNA for SCCA1. Vector sequence is indicated in grey, SCCA1 sequence in black, and restriction enzyme sites (or sequence of destroyed sites) indicated in red. The stop codon for SCCA1 is indicated by a point, and is located within the vector sequence.

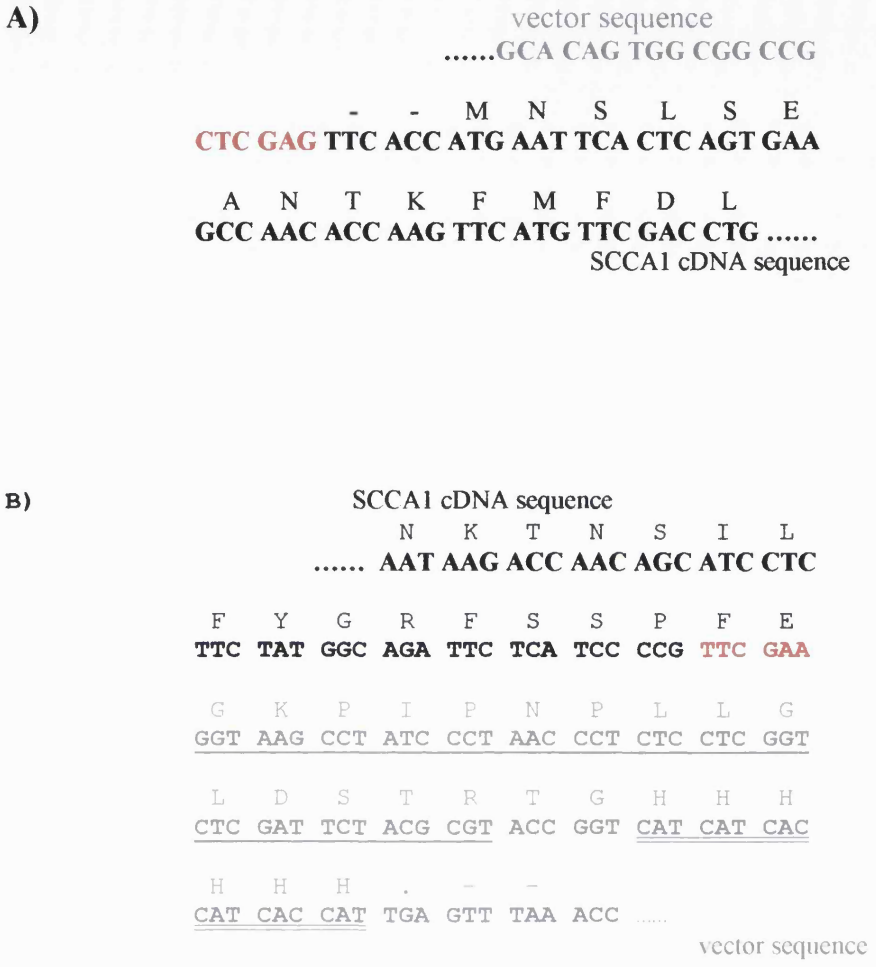


Figure 3.7 pCDNA SCCA1 (A) The upstream junction and (B) the downstream junction between the ligated vector (pCDNA 3.1 V5 His C) and the cDNA for SCCA1. Vector sequence is indicated in grey, SCCA1 sequence in black, and restriction enzyme sites (or sequence of destroyed sites) are indicated in red. The stop codon for SCCA1 is indicated by a point, and is located within the vector sequence. The V5 epitope is underlined and the polyhistidine tag is double-underlined.

```
U19556   1    CAGGAGTTCCAGATCACATCGAGTTCACCATGAATTCACTCAGTGAAGCCAACACCAAGT
AJ515706 2    ---C-CAGTGGCGG-CGC-----------------------------------------

       61     TCATGTTCGACCTGTTCCAACAGTTCAGAAAATCAAAAGAGAACAACATCTTCTATTCCC
       62     ---------------C-----------------------------------------

      121     CTATCAGCATCACATCAGCATTAGGGATGGTCCTCTTAGGAGCCAAAGACAACACTGCAC
      122     ----------------------------------------------A------------

      181     AACAGATTAAGAAGGTTCTTCACTTTGATCAAGTCACAGAGAACACCACAGGAAAAGCTG
      182     ------------------------------------------------------------

      241     CAACATATCATGTTGATAGGTCAGGAAATGTTCATCACCAGTTTCAAAAGCTTCTGACTG
      242     ------------------------------------------------------------

      301     AATTCAACAAATCCACTGATGCATATGAGCTGAAGATCGCCAACAAGCTCTTCGGAGAAA
      302     ----------------------------------------C-------------------

      361     AAACGTATCTATTTTTACAGGAATATTTAGATGCCATCAAGAAATTTTACCAGACCAGTG
      362     ------------------------------------------------------------

      421     TGGAATCTGTTGATTTTGCAAATGCTCCAGAAGAAAGTCGAAAGAAGATTAACTCCTGGG
      422     ------------------------------------------------------------

      481     TGGAAAGTCAAACGAATGAAAAAATTAAAAACCTAATTCCTGAAGGTAATATTGGCAGCA
      482     ------------------------------------------------------------

      541     ATACCACATTGGTTCTTGTGAACGCAATCTATTTCAAAGGGCAGTGGGAGAAGAAATTTA
      542     ------------------------------------------------------------

      601     ATAAAGAAGATACTAAAGAGGAAAAATTTTGGCCAAACAAGAATACATACAAGTCCATAC
      602     ------------------------------------------------------------

      661     AGATGATGAGGCAATACACATCTTTTCATTTTGCCTCGCTGGAGGATGTACAGGCCAAGG
      662     ------------------------------------------------------------

      721     TCCTGGAAATACCATACAAAGGCAAAGATCTAAGCATGATTGTGTTGCTGCCAAATGAAA
      722     ------------------------------------------------------------

      781     TCGATGGTCTCCAGAAGCTTGAAGAGAAACTCACTGCTGAGAAATTGATGGAATGGACAA
      782     ------------------------------------------------------------

      841     GTTTGCAGAATATGAGAGAGACACGTGTCGATTTACACTTACCTCGGTTCAAAGTGGAAG
      842     ------------------------------------------------------------

      901     AGAGCTATGACCTCAAGGACACGTTGAGAACCATGGGAATGGTGGATATCTTCAATGGGG
      902     ------------------------------------------------------------

      961     ATGCAGACCTCTCAGGCATGACCGGGAGCCGCGGTCTCGTGCTATCTGGAGTCCTACACA
      962     ------------------------------------------------------------

     1021     AGGCCTTTGTGGAGGTTACAGAGGAGGGAGCAGAAGCTGCAGCTGCCACCGCTGTAGTAG
     1022     ------------------------------------------------------------

     1081     GATTCGGATCATCACCTACTTCAACTAATGAAGAGTTCCATTGTAATCACCCTTTCCTAT
     1082     A-----------------------------------------------------------

     1141     TCTTCATAAGGCAAAATAAGACCAACAGCATCCTCTTCTATGGCAGATTCTCATCCCCGT
     1142     ------------------------------------------------------------

     1201     AG
     1202     --
```

Figure 3.8 Comparison of the nucleotide sequence for wild type SCCA1 (Accession number U19556) with the cloned cDNA for SCCA1 (Accession number AJ515706).

```
U19556   1    MNSLSEANTKFMFDLFQQFRKSKENNIFYSPISITSALGMVLLGAKDNTAQQIKKVLHFD
HBV-BP   1    ------------------------------------------------------------
AJ515706 1    --------------S-----------------------------N-------------

       61     QVTENTTGKAATYHVDRSGNVHHQFQKLLTEFNKSTDAYELKIANKLFGEKTYLFLQEYL
       61     ------------------------------------------------------------
       61     ---------------------------------------------T--------------

      121     DAIKKFYQTSVESVDFANAPEESRKKINSWVESQTNEKIKNLIPEGNIGSNTTLVLVNAI
      121     ------------------------------------------------------------
      121     ------------------------------------------------------------

      181     YFKGQWEKKFNKEDTKEEKFWPNKNTYKSIQMMRQYTSFHFASLEDVQAKVLEIPYKGKD
      181     ------------------------------------------------------------
      181     ------------------------------------------------------------

      241     LSMIVLLPNEIDGLQKLEEKLTAEKLMEWTSLQNMRETRVDLHLPRFKVEESYDLKDTLR
      241     ------------------------------------------------------------
      241     ------------------------------------------------------------

      301     TMGMVDIFNGDADLSGMTGSRGLVLSGVLHKAFVEVTEEGAEAAAATAVVGFGSSPTSTN
      301     ------------------------------------------------A---------
      301     ------------------------------------------------------------

      361     EEFHCNHPFLFFIRQNKTNSILFYGRFSSP
      361     ------------------------------P-
      361     --------------------------------
```

Figure 3.9 Comparison of the predicted protein sequence for the prototype SCCA1 (Accession number U19556) with HBV-BP (De Falco *et al.*, 2001b) and the cloned cDNA for SCCA1 (Accession number AJ515706). The reactive site loop is underlined, with the putative cleavage site at P1 P1' double underlined.

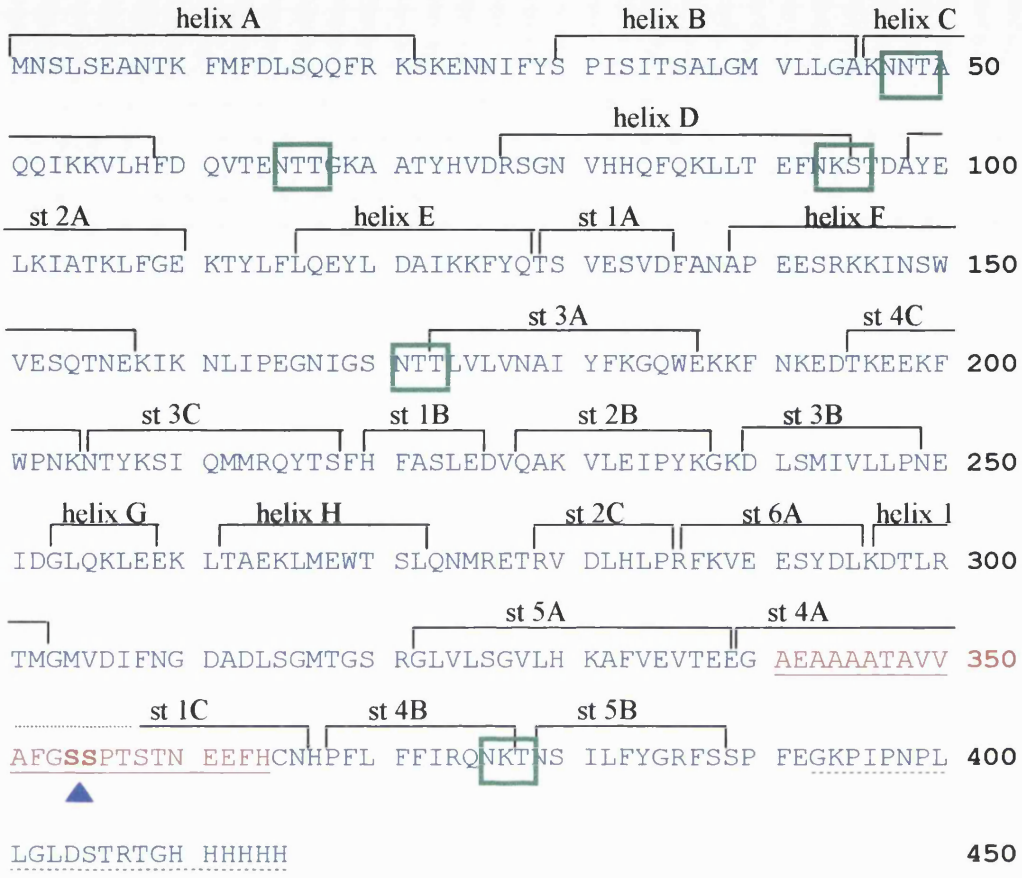


Figure 3.10 The predicted amino acid sequence for the cloned cDNA for SCCA1 (Accession number AJ515706) showing (superimposed above the sequence) structural motifs related to the archetypal serpin α1 proteinase inhibitor (Bartuski *et al.*, 1997). The reactive site loop is underlined and in red, with the putative scissile bond at P1 P1' marked by a solid triangle. Locations of potential N-glycosylation sites are shown by green boxes. The dotted underlining indicates the position if the V5 polyhistidine tag.

3.3.3 Confirmation of expression of SCCA1 in transfected cells

In order to confirm expression of SCCA1 in cell culture, highly purified endotoxin-free pCDNA SCCA1 was transiently transfected into Cos7 cells and compared with untransfected Cos7 cells and cells transfected with pCDNA 3.1 V5 LacZ (a control plasmid supplied by Invitrogen). Cells were harvested 48 hours post-transfection and subjected to SDS-PAGE and western blotting with an anti-V5 monoclonal antibody and a secondary HRP-conjugated anti-mouse antibody which was detected using ECL. pCDNA SCCA1 and pCDNA LacZ were expressed at high levels in Cos7 cells (Figure 3.11). Similar experiments were performed in HepG2 cells and Huh7 cells (both hepatocyte-derived cell lines). In both cases expression was approximately 100-fold less than Cos7 cells and only barely detectable using western blotting techniques.

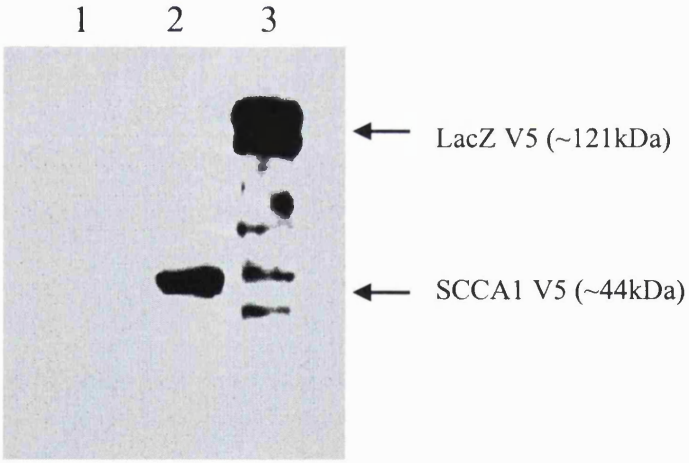


Figure 3.11 Western blot confirming expression of pCDNA SCCA1 and pCDNA 3.1 V5 LacZ in Cos7 cells. Lane 1: mock transfected Cos7 cells; lane 2: pCDNA SCCA1 transfected Cos7 cells; Lane 3: pCDNA 3.1 V5 LacZ transfected Cos7 cells. Protein sizes were determined by comparison with a pre-stained molecular weight marker (Amersham).

3.3.4 Effect of SCCA1 transfection on the binding of HBV to host cells

A previous report by de Falco *et al.* (2001b), has shown that transfection of a protein described as a homologue of SCCA1 (and referred to as HBV-BP by the authors) resulted in enhanced virus-cell binding. This report has thus far not been confirmed by other groups. Similar experiments were performed in order to confirm these observations.

3.3.4.1 Validation of PCR-based assay of HBV DNA levels

Preliminary experiments were performed in order to confirm the efficacy of the virus-cell binding assay used below to determine relative levels of cell-associated virus DNA. Template HBV DNA ranging from 1 pg/µl to 1 fg/µl was subjected to PCR amplification, Southern blotting and densitometry as described above. The results, shown in Figure 3.12 below, confirm the semi-quantitative nature of the virus-cell binding assay.

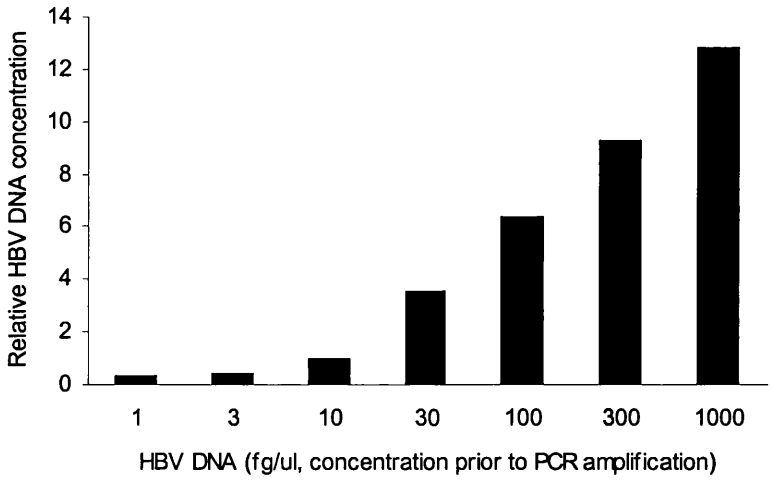


Figure 3.12 Validation of the semi-quantitative nature of the virus-cell binding assay. Increasing amounts of template DNA ranging from 1fg/µl to 1 pg/µl resulted in increased densitometry values.

3.3.4.2 Comparison of the effect of pCiNeo SCCA1 and pCDNA SCCA1 on virus-cell binding

A hepatocyte-derived cell line, Huh7, was transfected with both pCiNeo SCCA1 and pCDNA SCCA1 using lipofectamine 2000. Expression of SCCA1 continued for 48 hours and was followed by cell-virus binding assays. The amount of virus bound to cells was quantified relative to controls using PCR analysis followed by Southern blotting and densitometry.

SCCA1 transfection resulted in enhanced binding of HBV to cells compared with mock transfected cells (Figure 3.13). This enhanced virus-cell binding was observed for both pCiNeo SCCA1 and pCDNA SCCA1, indicating that the addition of a V5 and polyhistine tag to SCCA1 did not compromise this effect (although enhanced cell virus binding was slightly reduced when transfection was performed using tagged SCCA1). Having confirmed that the addition of a V5 polyhistidine tag to SCCA1 did not affect the results significantly, the pCDNA SCCA1 construct was used for subsequent studies as the addition of the tag allowed closer investigation of the transfected protein and its role in enhanced virus-cell binding.

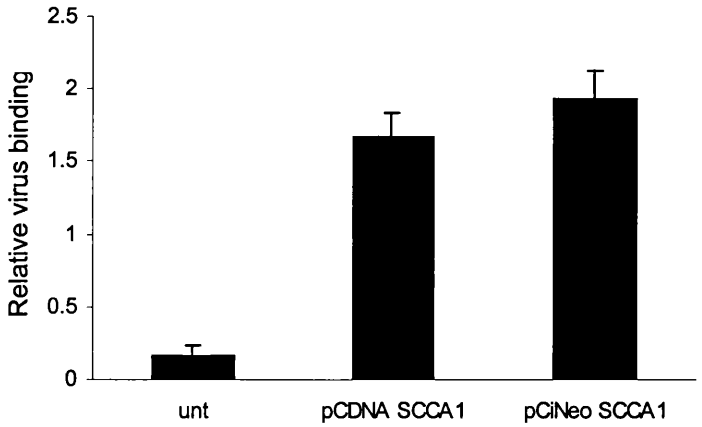


Figure 3.13 Effect of transfection of pCiNeo SCCA1 and pCDNA SCCA1 on virus-cell binding using Huh7 cells. Unt, untransfected control. Relative virus binding is assessed by scanning densitometry (n=6, mean ± standard deviation).

3.3.4.3 SCCA1 transfection in hepatocyte-derived cells compared with non-hepatocyte cells

In order to investigate whether the enhanced cell-virus binding observed in SCCA1 transfected cells was specific to hepatocyte-derived cells, comparisons were performed between transfected Huh7 cells (hepatocyte-derived) and Cos7 cells (African Green Monkey kidney). Transient transfection of pCDNA SCCA1 into these cell lines was followed by cell-virus binding assays. Expression of SCCA1 in Cos7 cells resulted in a small increase in cell-virus binding (approximately a two-fold increase) (Figure 3.14). However the enhancement of virus binding was significantly lower in Cos7 cells compared with Huh7 cells where approximately a five-fold increase in bound particles was observed compared with untransfected cells. This observation is not due to transfection or expression efficiency – indeed, SCCA1 expression (monitored using western blotting) was considerably higher in Cos7 cells, where it had less effect on virus binding, than in Huh7 cells.

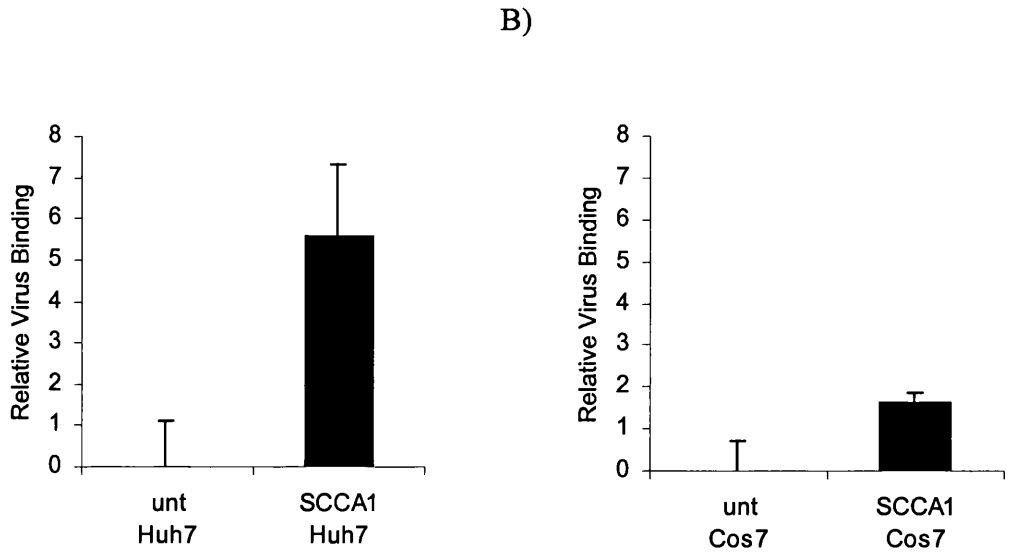


Figure 3.14 Effect of SCCA1 transfection on virus-cell binding in (A) Huh7 cells and (B) Cos7 cells (n=6, mean ± standard deviation). Values are normalised to background virus-cell binding separately for untransfected Cos7 cells and Huh7 cells.

3.3.5 Effect of cell culture conditions on SCCA1-mediated binding

Therefore, transient SCCA1 expression results in enhanced binding of HBV to the surface of transfected cells. In order to assess the significance of these results, the effect of SCCA1 transfection on virus-cell binding was compared with that of various cell culture conditions reported to provide the optimal conditions for binding of HBV to hepatocyte-derived cells. These conditions include the addition to cell culture medium of dimethyl sulfoxide (DMSO) and the effect of serum starvation on cells (Glebe *et al.,* 2001). Both SCCA1 transfected and mock transfected SCCA1 cells were exposed to the reported optimal cell culture conditions, after which virus-cell binding assays were performed. The results show that SCCA1 expression in Huh7 cells results in markedly higher virus binding than is observed either in DMSO treated cells or serum-starved cells, or a combination of the two.

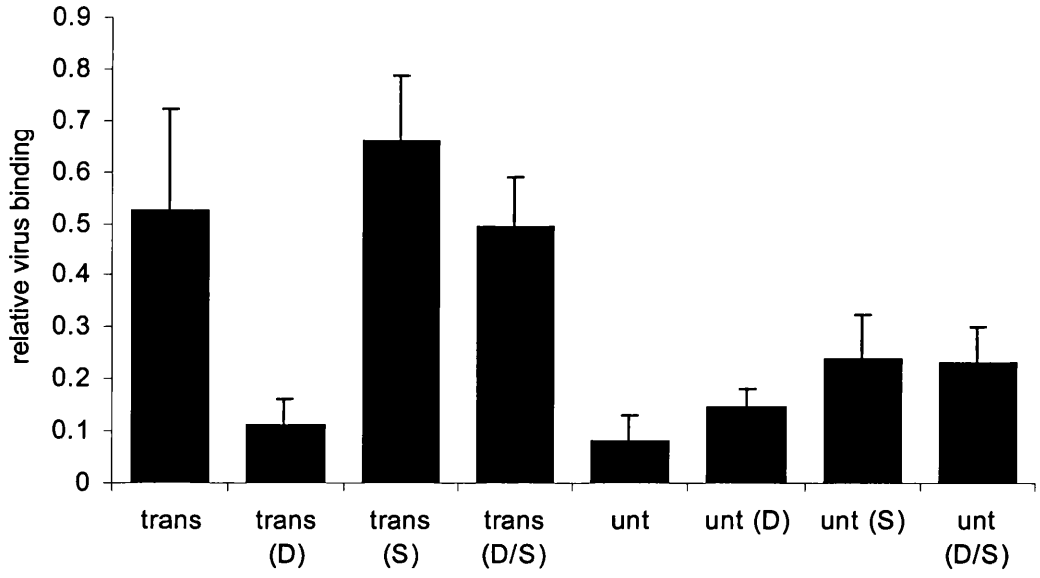


Figure 3.15 Comparison of DMSO and/or serum starvation on the relative levels of virus bound to transfected (trans) or untransfected (unt) cells. D, DMSO treatment, S, serum starvation, D/S, combination of DMSO treatment and serum starvation (n=6, mean ± standard deviation).

Furthermore, in transfected cells, serum starvation resulted in a small further increase in virus binding. Interestingly, DMSO treatment of SCCA1 transfected cells almost completely abolished the effect of SCCA1 transfection on virus-cell binding (Figure 3.15). The effect of SCCA1 on virus binding was therefore found to be greater, in our hands, than that of previously reported cell culture conditions.

3.3.6 SCCA1-mediated virus internalisation and replication

The cell-virus binding assays utilised in this study merely detect total viral DNA associated with cells and do not provide information regarding the possible internalisation of HBV particles or subsequent replicative steps. In order to assess viral internalisation, use was made of the observation by Qiao *et al.* (1994), that HBV which is bound to the cell surface, but not internalised by the host cell (e.g. in adsorption experiments performed at 4°C to prevent possible internalisation), can be removed from the host cell by treatment with trypsin, was utilised. Transient transfection was performed therefore, expression allowed to proceed for 48 hours, followed by virus-binding overnight at 37°C. Comparisons were made between cells harvested by trypsinisation (where cell-associated virus which has not been internalised would be removed) compared with those harvested by scraping the cells and bound virus (whether internalised or not) into a lysis buffer. This comparison was performed for both SCCA1 transfected and mock-transfected cells. Virus particles bound to SCCA1 transfected cells was found to be significantly protected from trypsin compared with virus particles associated with untransfected Huh7 cells (Figure 3.16).

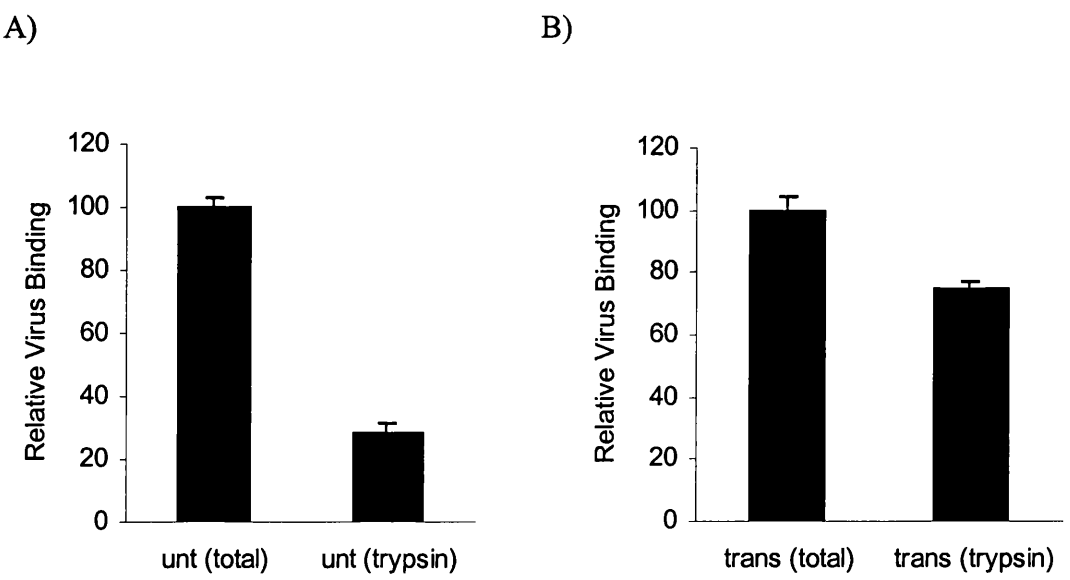


Figure 3.16 Analysis of the effects of trypsinisation on cell-associated virus DNA in (A) SCCA1 untransfected Huh7 cells as compared to (B) SCCA1 transfected Huh7 cells (n=6, mean ± standard deviation).

Internalisation of the virus raises the question whether increased virus binding and internalisation is associated with replication of viral DNA. Attempts were made to purify replicative intermediates directly from transfected cells exposed to HBV. Despite incubation for long periods of time, up to three weeks, to allow for the accumulation of replicative intermediates (Tuttleman *et al.,* 1986), it has not been possible to demonstrate convincingly the presence of replicative intermediates in SCCA1 transfected cells by purification of replicative intermediates. Furthermore, attempts were made to use a PCR-based approach (Kock et al., 1996) reported to differentiate between rcDNA and the cccDNA intermediates (this methods relies on the use of two sets of primers, the first set amplifying rcDNA and cccDNA equally efficiently and the second set which amplifies that region of the cccDNA which is unique to the replicative intermediate, having been formed by repair of the rcDNA

molecule). Although preliminary results showed a PCR product indicative of cccDNA, subsequent experiments suggested that high levels of rcDNA resulted in atefactual PCR amplification "across the gap" of the rcDNA. Therefore the PCR-based results suggesting replication of HBV in SCCA1-transfected cells was not felt to be reliable in this case.

3.4 DISCUSSION

A cDNA encoding SCCA1 was amplified by nested reverse transcription PCR and cloned into two mammalian expression vectors to produce pCiNeo SCCA1 and pCDNA SCCA1. Sequencing of the cDNA was performed and comparisons were made between this cDNA, the prototype SCCA1 and HBV-BP (De Falco *et al.*, 2001b) at both the nucleotide and amino acid level. At the nucleotide level only 4 bases differed from the prototype (Accession Number U19556), three of which resulted in coding changes. Comparison of all three amino acid sequences also revealed an extremely high degree of identity. Interestingly, in this predicted protein sequence residue Gly351, which forms part of the catalytic reactive site loop, is conserved with the prototype, whereas in HBP-BP there is a Gly351Ala substitution. De Falco *et al.* (2001b) proposed the possibility that the Gly351Ala mutation may be significant in the effect of SCCA1 on virus-cell binding. The results presented here show that this is not the case.

These results confirm that transfection of SCCA1 into cell lines, whether hepatocyte-derived (Huh7) or non-hepatocyte derived (Cos7), results in increased binding of HBV particles to cells compared with mock transfected controls. The addition of a V5 polyhistidine tag had no significant effect on virus binding. More intriguingly, the enhanced virus-cell binding is considerably more marked in cells of hepatocyte origin (Huh7, more than 5 fold increase) than Cos7 cells (2 fold increase). These results suggest that SCCA1-mediated virus binding is specific to hepatocyte-derived cells and support the hypothesis that a dual receptor system is required for virus binding, the (as yet unknown) second component being expressed endogenously in hepatocyte-derived cells. If this is the case, i.e. SCCA1 acts as a co-receptor, then non-hepatocyte derived cells expressing SCCA1 may be a valuable tool for identifying the second (unknown) component of a putative receptor complex.

SCCA1 transfection not only enhanced virus-cell binding but also protected bound virus from trypsin treatment, suggesting the possibility that there is virus

internalisation. Numerous attempts were made to detect possible replicative intermediates. A selective PCR technique, which is reported to detect cccDNA specifically, a replicative intermediate, and not rcDNA (Kock *et al.*, 1996), was used to analyse cell-associated viral DNA. Although initial results suggested the presence of cccDNA in SCCA1 transfected cells but not in mock-transfected cells, the technique was found to be unreliable. The presence of increased levels of rcDNA was sufficient to produce false positive cccDNA bands. Direct purification of replicative intermediates (using non-PCR based methods) was unsuccessful. Therefore, these data suggest that SCCA1 transfection results in enhanced cell-virus binding and internalisation, but the virus progresses no further along the infection pathway. This may be due to a further block downstream of internalisation, either in the transport of viral DNA to the nucleus, or entry of the DNA through the nuclear membrane.

These results confirm the possibility that SCCA1 functions as the cellular receptor for HBV, a hypothesis which will be further investigated in the next chapter.

CHAPTER IV

STUDIES USING RECOMBINANT SCCA1

4.1 INTRODUCTION

The results described in the previous chapter show that transfection of a cDNA for SCCA1 into mammalian cells results in enhanced binding of HBV to cells. Furthermore, cell-associated virus DNA is significantly protected from trypsin, indicating that there is virus internalisation. These results raise the obvious possibility that SCCA1 functions as the cellular receptor for HBV.

A cellular receptor for a virus can be expected to interact directly with a viral ligand. SCCA1, as a cellular receptor, fulfils this requirement as the protein has been reported to interact directly with preS1, the virus protein which is thought to interact with the cellular receptor (Neurath *et al.*, 1986; Pontisso *et al.*, 1989). Indeed SCCA1 was originally identified using a tetramer of preS1 peptides in an affinity chromotography system (De Falco *et al.*, 2001b) relying on a tight association between SCCA1 and the preS1 domain.

There remain at least three further requirements to demonstrate convincingly a role for SCCA1 as the cellular receptor for HBV:

1. A receptor would normally be expressed on the surface of the cell in order to facilitate the virus-cell interaction.

2. It has been proposed that the extreme hepatotropism exhibited by HBV may be the result of liver-enhanced or liver-specific expression of the cellular receptor. Although this is not necessarily the case, as liver-specific cellular transcription factors have also been implicated in the viral hepatotropism, conventionally the receptor would be required to be expressed in the liver.

3. Soluble forms of the candidate receptor have been shown in many viruses to compete for the binding of viral particles to cells e.g. Human Immunodeficiency Virus (Dimitrov, 1997).

These three questions remain unanswered regarding SCCA1-mediated binding of HBV to cells. Therefore, cell surface biotinylation studies were performed to address the question of cell surface expression of SCCA1 expressed from transiently transfected pCDNA SCCA1. Secondly, soluble recombinant SCCA1 was expressed in a bacterial system and used in competitive binding assays. Lastly, reverse-transcription PCR used to assess the expression of SCCA1 in liver tissue and hepatocyte-derived cells lines.

4.2 MATERIALS AND METHODS

4.2.1 Localisation of SCCA1 in transfected cells

4.2.1.1 Cell surface biotinylation

Cells were washed twice with ice cold PBS. Cell surface biotinylation was performed by adding 2 ml PBS containing 67 µl of a stock solution of 30 mg/ml biotin N-hydroxysuccinimide (Sigma Aldrich) in DMSO (final concentration 2 mg/ml) to each well (the negative control was PBS containing DMSO alone). Plates were incubated at 4°C for 1 hour with gentle rocking. Labelling was terminated by washing cells twice with 0.1 M glycine in PBS and twice with PBS alone.

4.2.1.2 Analysis of biotinylated SCCA1

Cos7 cells were trypsinised and seeded into a 6 well plate at a concentration of 2.5 x 10^5 cells/well in 2 ml DMEM10. Cells were incubated overnight at 37°C in 5% CO_2. Cells in two wells were transfected with pCDNA SCCA1 as described previously. Two wells were utilised as untransfected controls. Cells were incubated for 48 hours to allow expression of SCCA1. Cell surface biotinylation of one transfected and one untransfected well was performed as described above (the remaining two wells were mock biotinylated). After washing, cells were solubilised in 200µl RIPA buffer (50 mM Tris HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA). Twenty microlitres was set aside for analysis of total lysate by western blotting. The remaining 180 µl was diluted with a further 820 µl RIPA buffer and 50 µl streptavidin-agarose (Sigma). Samples were incubated on a rotator at 25°C for 30 min to allow biotinylated molecules to bind to the streptavidin moiety. The streptavidin-agarose beads were washed 5 times with RIPA buffer, then resuspended in 50 µl 1x SDS-PAGE loading buffer (appendix 1) and heated to 100°C for 15 min. Protein analysis was performed using SDS-PAGE and western blotting using an anti-V5 antibody as described above.

4.2.2 Bacterial expression of recombinant SCCA1

4.2.2.1 Construction of pGEX 4T 3 SCCA1 polyHis

The SCCA1 gene was subcloned from pCDNA SCCA1 into pGEX 4T 3 (Amersham Pharmacia) as follows: the SCCA1 (V5 polyHis) cDNA was excised from pCDNA SCCA1 by *XhoI* and *PmeI* digestion followed by purification from a low melting point agarose gel. The vector was digested with *NotI*, blunt-ended and then further digested with *SalI* (to utilise the compatible cohesive ends resulting from *SalI* and *XhoI* digestions). The *XhoI–PmeI* SCCA1 insert was ligated in frame into the vector to produce a gene encoding a GST-SCCA1 V5 polyHis fusion construct. Restriction enzyme digestion analysis and DNA sequencing confirmed that insertion had been successful.

4.2.2.2 Preparation of competent *E. coli* cells and transformation with pGEX 4T 3 SCCA1 polyHis

A single colony of *E. coli* BL21 cells was inoculated into LB and incubated overnight at 37°C, 225 rpm. This culture (500 µl) was used to inoculate 100 ml LB and incubated at 37°C, 225 rpm until the culture reached an OD_{600} of 0.45 (ensuring the culture remained in the exponential phase). Cells were pelleted for 5 min at 3,000 rpm and resuspended in 25 ml ice-cold 0.1 M $MgCl_2$. Cells were pelleted for a further 5 min at 3,000 rpm, then resuspended in 2 ml ice cold 0.1 M $CaCl_2$. The cells were pelleted again and resuspended in 2 ml ice cold 0.1 M $CaCl_2$. The cells were incubated on ice for 1 hour, then 200 µl per transformation was aliquoted into 15 ml Falcon tubes. pGEX 4T 3 SCCA1 (10 ng) was added to the cells (no DNA was added to negative control) and gently mixed. Tubes were stored on ice for 30 min, heat shocked at 42°C for exactly 30 sec and further incubated on ice for 2 min. LB (800 µl per tube) was added to the cells, which were then incubated at 37°C, 225 rpm for 45 min, before 100 µl was plated onto LB plates containing ampicillin. Plates were incubated overnight at 37°C.

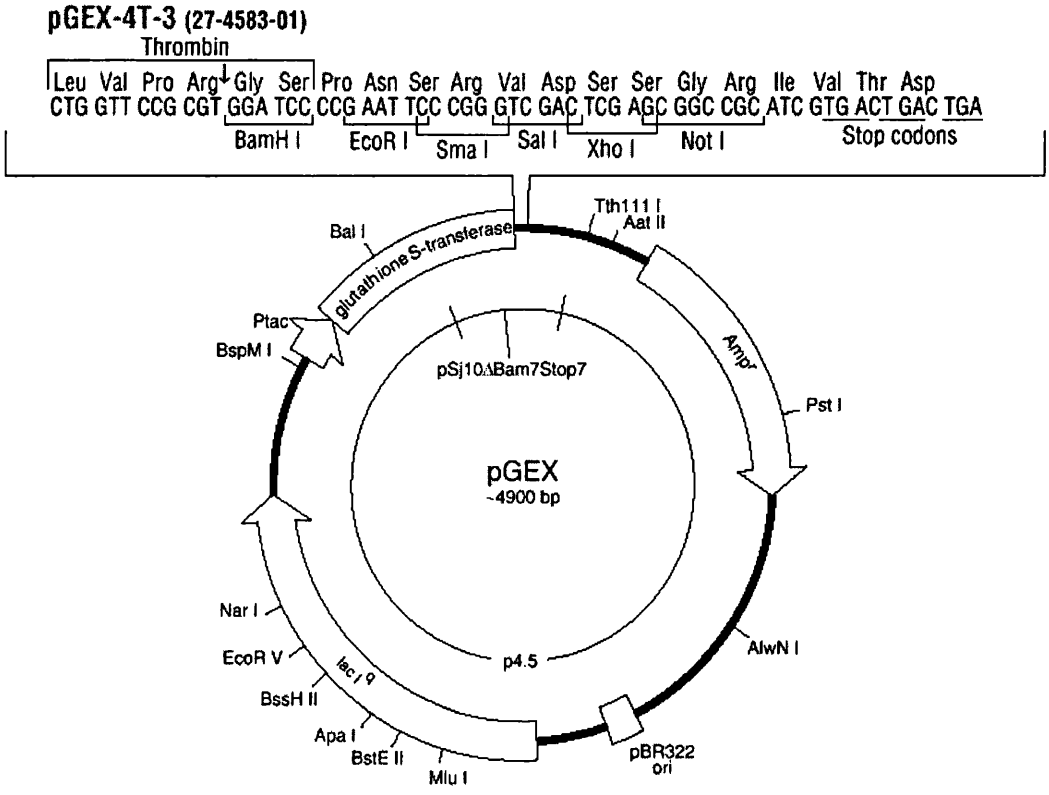


Figure 4.1 pGEX 4T 3 (Amersham Pharmacia) plasmid circle map and multiple cloning site.

4.2.2.3 Expression of a GST SCCA1 polyHis fusion protein in *E. coli*

Pilot studies were performed to confirm bacterial expression of the GST SCCA1 polyHis fusion protein in *E. coli* BL21 (this strain is protease deficient and is therefore useful for high levels of expression of recombinant proteins). *E. coli* colonies transformed previously with pGEX 4T 3 SCCA1 were used to inoculate 5 ml cultures of LB containing ampicillin (the controls comprised untransformed *E. coli* BL21 grown in antibiotic deficient L-broth and uninduced *E. coli* BL21 transformed with pGEX 4T 3 SCCA1). Cultures were incubated at 37°C, 225 rpm for four hours (to ensure that cells were in the exponential phase of growth). Recombinant protein expression was induced by the addition of 100 mM IPTG to a final concentration of 100 nM and induction continued at 30°C, 225 rpm for 90 min. Analysis was performed using pelleted cells (1.5 ml) resuspended in 200 µl SDS-PAGE loading buffer and resolved on a 12% polyacrylamide gel. Coomassie Blue staining and western blotting (using an anti-V5 antibody) was used to confirm recombinant protein expression.

4.2.2.4 Batch expression of GST SCCA1 polyHis

A single colony of *E. coli* BL21 cells was inoculated into LB containing ampicillin and incubated overnight at 37°C, 225 rpm. This culture (500 µl) was used to inoculate 100 ml LB which was incubated at 37°C, 225 rpm until the culture reached an OD_{600} of 0.6. Cells were induced to express the fusion protein by the addition of 100 µl of 100 mM IPTG (to a final concentration of 100 nM), followed by incubation at 30°C for 3 hours. Bacteria were pelleted at 3,000 rpm for 10 min and the pellet stored overnight at -20°C.

4.2.2.5 Purification of GST SCCA1 polyHis

Cells were resuspended in 10 ml binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and lysed using sonication with a Soniprep 150 (MSE) (10 cycles of increasing amplitude ranging from 5 to 10 microns). The crude bacterial extract was centrifuged to pellet insoluble debris (3,000 rpm, 15 min). Purification was performed using Probond Resin (Invitrogen) equilibrated in binding buffer and packed into 1 ml

spin columns. The clarified bacterial lysate was applied to the column and incubated in the presence of protease inhibitors (1 mM PMSF) for 30 min at 4°C. Columns were washed with washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) until the OD_{280} was less than 0.06, then proteins were eluted step-wise using 50 mM, 200 mM, 350 mM and 500 mM imidazole (Sigma) in washing buffer. Fractions were followed spectrophotometrically (OD_{280}) and screened by SDS-PAGE and western blotting. GST SCCA1 polyHis was found to have been eluted by 200 and 350 mM imidazole. The purified fusion protein was concentrated to 1 mg/ml using Vivaspin 20ml centrifugal concentrators (Vivascience).

4.2.2.6 Proteolysis assays

The proteolysis assay utilised is based on a shift in fluorescence of 7-amino-4-methoxy coumarin (AMC), which upon cleavage from the substrate undergoes a shift in fluorescence detectable using a fluorescent plate reader. Z-Phe-Arg-AMC.HCl, a fluorogenic substrate for papain, was obtained from Bachem. Papain was obtained from Sigma. The reaction buffer used for all assays was 0.1 M phosphate buffer, pH 6.8, containing 1 mM EDTA and 4 mM dithiothreitol (DTT). Recombinant SCCA1 (wild type or mutants in later work, 20 μg) was incubated with papain (20 nM) in the reaction buffer for 30 min at 25°C to allow formation of a serpin enzyme complex. Inhibition of papain was measured by the addition of the fluorogenic substrate (10 μM) to the reaction. The amount of AMC liberated from the substrate was determined fluorometrically, using a Cytofluor 4000 multi-well plate reader (Perseptive Biosystems) with excitation and emission wavelengths of 360 nm and 460 nm respectively.

For kinetics studies, the above experiment was performed using a range (from 2.5 μM to 10 μM) of substrate concentrations, [S]. These data were used to obtain values for V_o (the slope of the linear portion of the curve). A graph was plotted of $1/V_o$ against 1/[S] to determine the kinetics of inhibition.

4.2.3 Endogenous expression of SCCA1 in liver and hepatocyte-derived cells

Total RNA was isolated from HepG2 and Huh7 cells (approximately 10^7 cells each), pCDNA SCCA1 transfected Huh7 cells (48 hours post-transfection, approximately 10^6 cells), and total resected liver (approximately 250 mg, obtained with informed consent and kindly provided by Dr Clare Selden). Reverse transcription was performed priming with random hexamers (as described in Chapter II). PCR amplification of SCCA1 cDNA was performed using primers 210 (5' caa gtc aca gag aac acc aca g 3') and 598M (5' aat ttc ttc tcc cac tgc cc 3'). The house-keeping gene control was GAPDH, amplified using primers GAPDHfor (5' tga tga cat caa gaa ggt ggt gaa g 3') and GAPDHrev (5' tcc ttg gag gcc atg tgg gcc at 3'). For confirmation of the identity of SCCA1 PCR products, Southern blotting was performed, using a ^{32}P dCTP random labelled SCCA1 fragment which had been excised from pCDNA SCCA1 using *XhoI* and *BstBI* restriction enzyme digestion, gel purified and radiolabelled as described previously.

4.3 RESULTS

4.3.1 Localisation of SCCA1 in transfected cells

The possible role of SCCA1 in virus-cell binding suggests a requirement for transiently expressed SCCA1 to be localised on the surface of transfected cells. There is a great of deal of controversy as to whether SCCA1 (*in vivo*) is retained within the cell, or whether it is secreted into plasma (Cataltepe *et al.*, 2000a; Uemura *et al.*, 2000). The approach used here was an attempt to take a "snap-shot" of the proteins expressed on the cell surface at a single time point.

Transient transfection studies were performed, followed by cell surface biotinylation. The presence of biotinylated SCCA1 (with appropriate and rigorous controls) would then indicate that SCCA1 had been available for biotinylation and was therefore located at the cell surface. Total biotinylated protein was subsequently purified from the total cellular protein using streptavidin-agarose, resolved using SDS-PAGE and analysed using western blotting (with an anti-V5 antibody) and ECL (Figure 4.2). The results show that a proportion of SCCA1 resulting from transient expression of pCDNA SCCA1 is localised to the cell surface where it could be available to interact with virus particles. An alternative interpretation of these results is that SCCA1 is tightly associated with a surface protein and is removed by streptavidin as part of a protein complex.

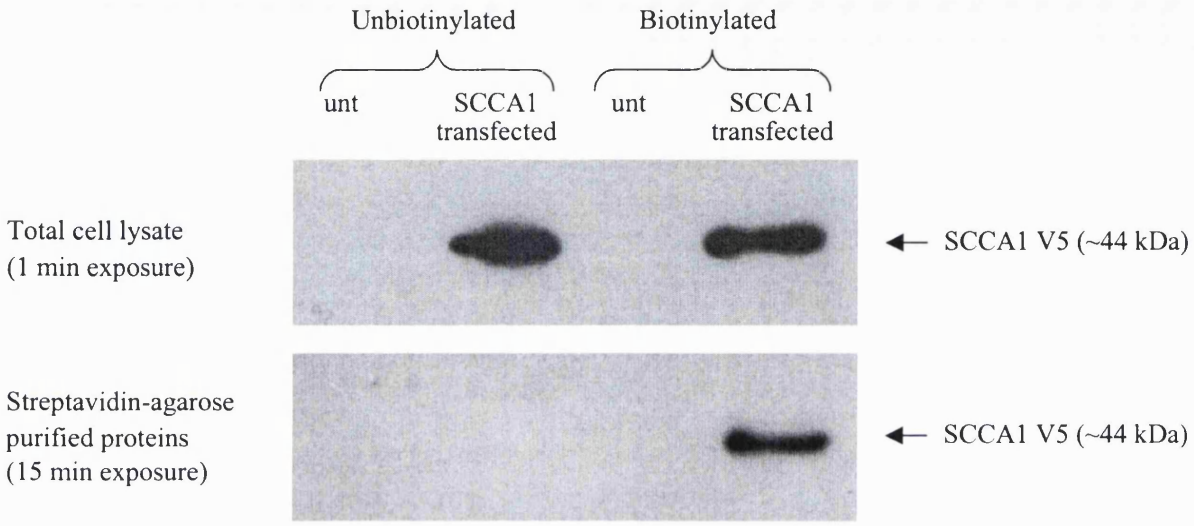


Figure 4.2 Western blot showing the presence of SCCA1 in biotinylation studies. The top panel shows confirmation that SCCA1 was equally well transfected and expressed in both the biotinylated and unbiotinylated wells. The bottom panel shows that a proportion of the SCCA1 in transfected and biotinylated cells is purified using streptavidin-agarose, indicating that SCCA1 was biotinylated (the negative control for unbiotinylated transfected cells shows no purification of SCCA1 using streptavidin-agarose).

4.3.2 Bacterial expression of recombinant SCCA1

4.3.2.1 Production of pGEX 4T 3 SCCA1

The complete cDNA for SCCA1 was inserted into a bacterial expression vector, pGEX 4T 3, using standard cloning techniques. Essentially, the cDNA for SCCA1 (with the additional poyhistidine/V5 tag) was excised from pCDNA SCCA1 by restriction enzyme digestion using *XhoI* and *PmeI*. The excised cDNA was inserted in frame with the GST into the multiple cloning site of pGEX 4T 3 which had been digested with *NotI*, then blunt-ended and further digested with *SalI*. The resulting construct, pGEX SCCA1 therefore encoded a GST fusion protein, GST SCCA1 V5 polyHis (Figure 4.3).

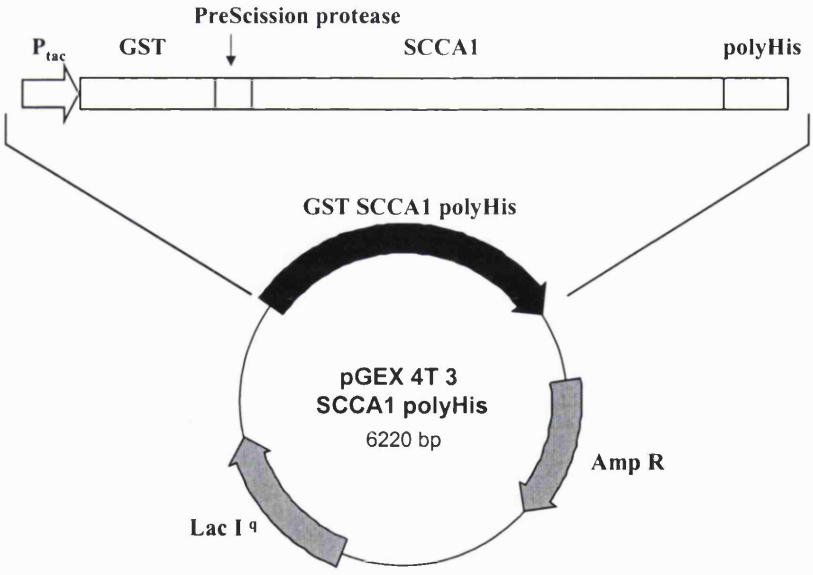


Figure 4.3 Structure of pGEX 4T 3 SCCA1 V5 polyHis showing detail of the recombinant fusion protein. The V5 tag is located adjacent to the polyHis region.

The successful insertion of the SCCA1 insert was confirmed using restriction enzyme digestion with *EcoRI* and *PstI* which was predicted to result in fragments of the following sizes: 268 bp, 761 bp, 1182 bp and 3985 bp. (Figure 4.4). DNA sequencing was performed to ensure that insertion in frame had been successful.

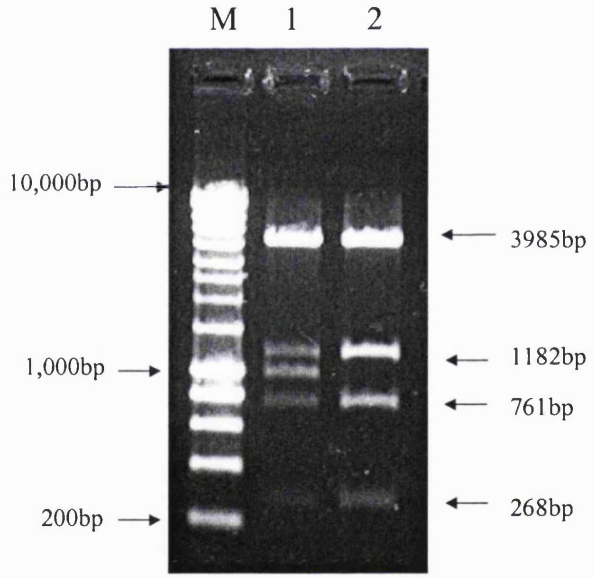


Figure 4.4 Agarose gel electrophoresis showing results of digestion of two clones of pGEX SCCA1 with *EcoRI* and *PstI*. M, molecular weight marker (Bioline Hyperladder I); 1 partial digestion of clone 1 of pGEX SCCA1; 2, digestion of clone 2 of pGEX SCCA1. The unlabelled band in lane 1 is partial digestion product (1029 bp) resulting from combination of the 761 bp fragment and the 268 bp fragment.

4.3.2.2 Expression and purification of recombinant SCCA1

The bacterial expression construct was then transformed into *E. coli* strain BL21. A number of transformed clones were examined to determine expression levels of recombinant SCCA1 (rSCCA1). These pilot studies were performed using small volume cultures inoculated with single bacterial colonies, induced to express recombinant protein by the addition of IPTG and analysed by SDS-PAGE and Coomassie Blue staining. The single clone which produced the highest levels of rSCCA1 as determined by protein staining was selected for batch production of larger amounts of rSCCA1.

Large volume culture, fusion protein expression and purification of rSCCA1 by imidazole elution from ProBond resin was performed. Recombinant protein (purified) was analysed using SDS-PAGE, Coomassie Blue staining and western blotting utilising an antibody directed at the carboxyterminal V5 tag. The results showed high levels of rSCCA1 expressed and purified to near homogeneity (Figure 4.5).

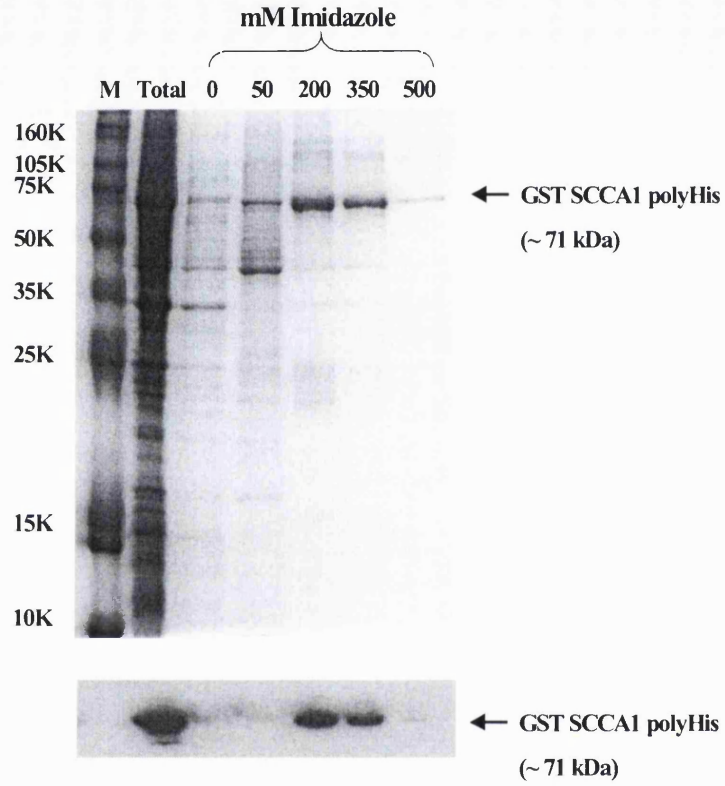


Figure 4.5 SDS-PAGE was followed by Coomassie Blue staining (upper panel) and western blot analysis (lower panel) of expressed and purified GST SCCA1 V5 polyHis from crude bacterial extract by imidazole elution from ProBond resin. Western blotting was performed using an anti-V5 antibody. M, molecular weight marker (Rainbow recombinant protein marker, Amersham). Total, crude bacterial lysate prior to purification procedure.

4.3.2.3 Functional studies of recombinant SCCA1

The addition of terminal tags to proteins often can have detrimental effects on their ability to maintain biological function (Ramage *et al.*, 2002). It was necessary therefore to ensure that the rSCCA1 produced in this system maintained the ability to function as a protease inhibitor. We tested this ability using a fluorescent proteolysis assay which measures the rate of proteolysis of a fluorescent substrate by a cysteine protease, in this case papain, in the presence and absence of the inhibitor. Papain is a known target protease for SCCA1 *in vitro*, although much less potently inhibited than other proteases such as cathepsins S, K and L (not available in this study) (Luke *et al.*, 2000). Recombinant SCCA1 was therefore incubated with papain to allow the formation of serpin-enzyme complexes. The uncomplexed free papain was then measured using the proteolysis assay (Figure 4.6). In order to confirm that the kinetics of the reaction were those of a competitive inhibitory reaction the proteolysis assay was performed over a range of substrate concentrations (Figure 4.7). The results were used to determine the rate of reaction for each concentration and subsequently to derive the kinetics of the reaction by plotting $1/V_0$ versus 1/[S] (Figure 4.8).

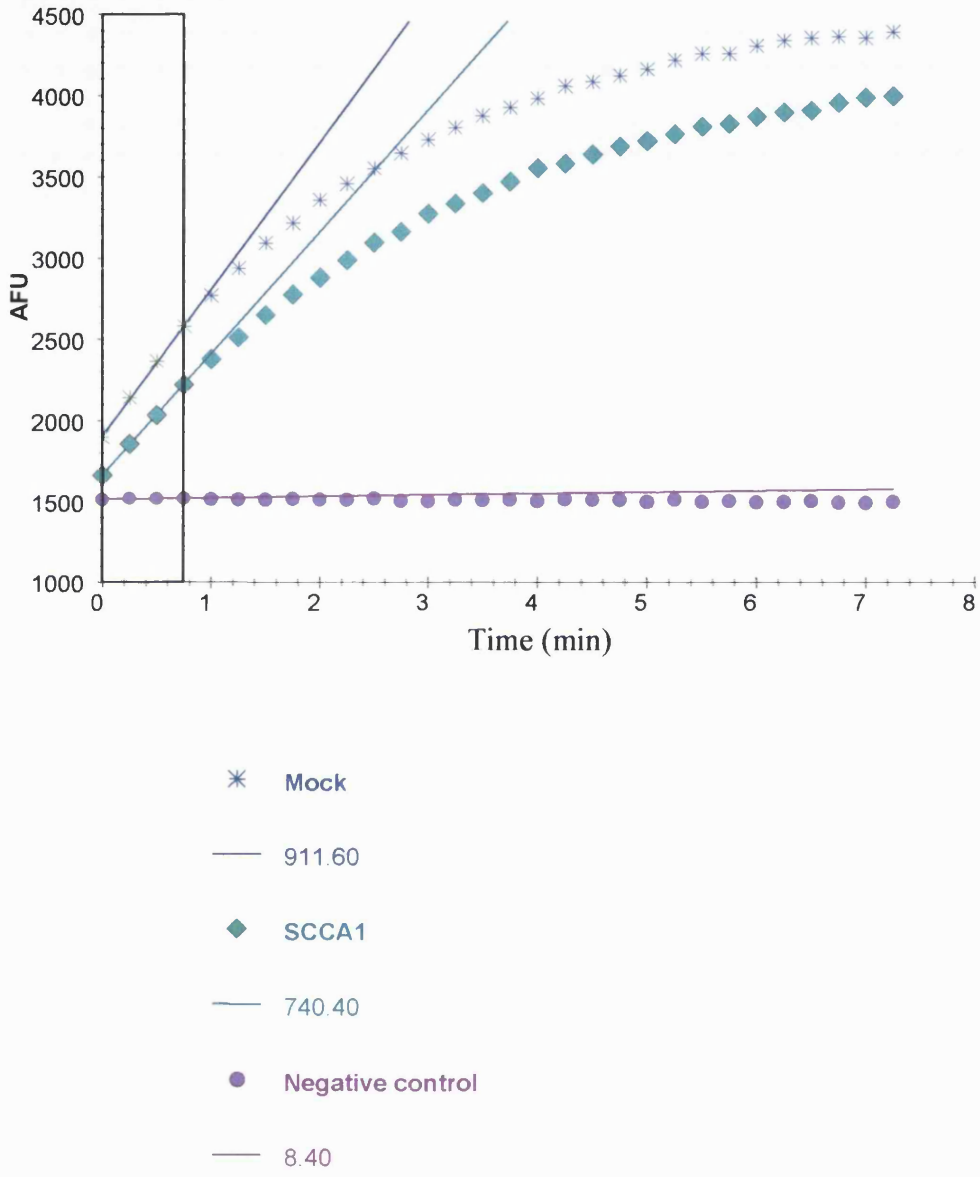


Figure 4.6 Proteolysis assay showing rSCCA1 (◇) inhibition of papain (20 mM) proteoysis of a fluorescent substrate (10 nM) (compared with the negative control (o), containing no papain, and the mock preparation (*) purified from untransformed *E. coli* BL21). AFU is a measure of fluorescence. Trendlines are a measure of initial velocity of the reaction, with values provided in the legend above.

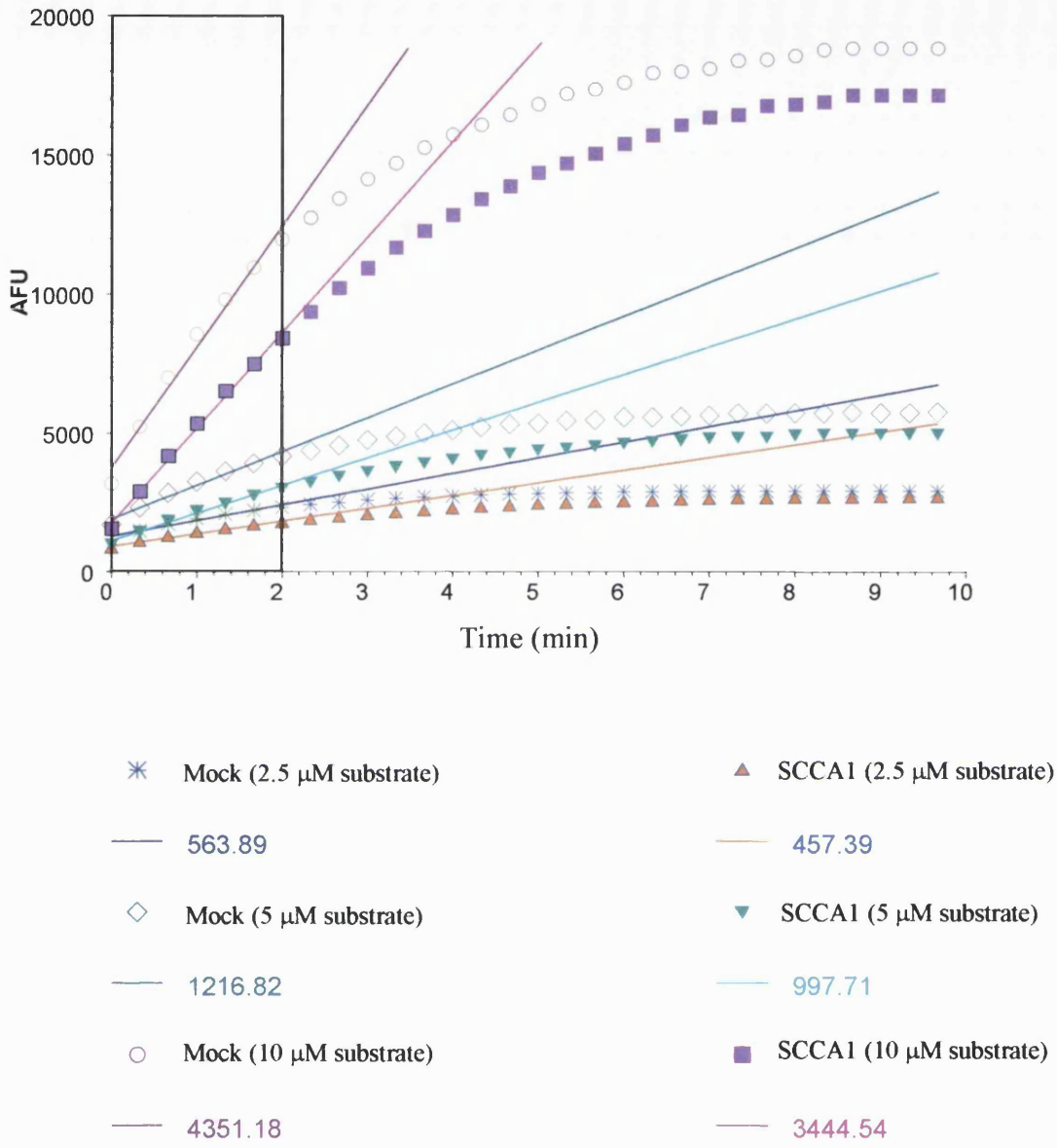


Figure 4.7 Proteolysis assay to determine the kinetics of rSCCA1 inhibition of papain. Recombinant SCCA1 (or mock preparation purified from untransformed *E. coli* BL21) was incubated with 20 nM papain, 20 μg rSCCA1 and with increasing amounts of fluorescent substrate ranging from 2.5 μM to 10 μM. AFU is a measure of fluorescence. Trendlines are a measure of initial velocity of the reaction, with values provided in the legend above.

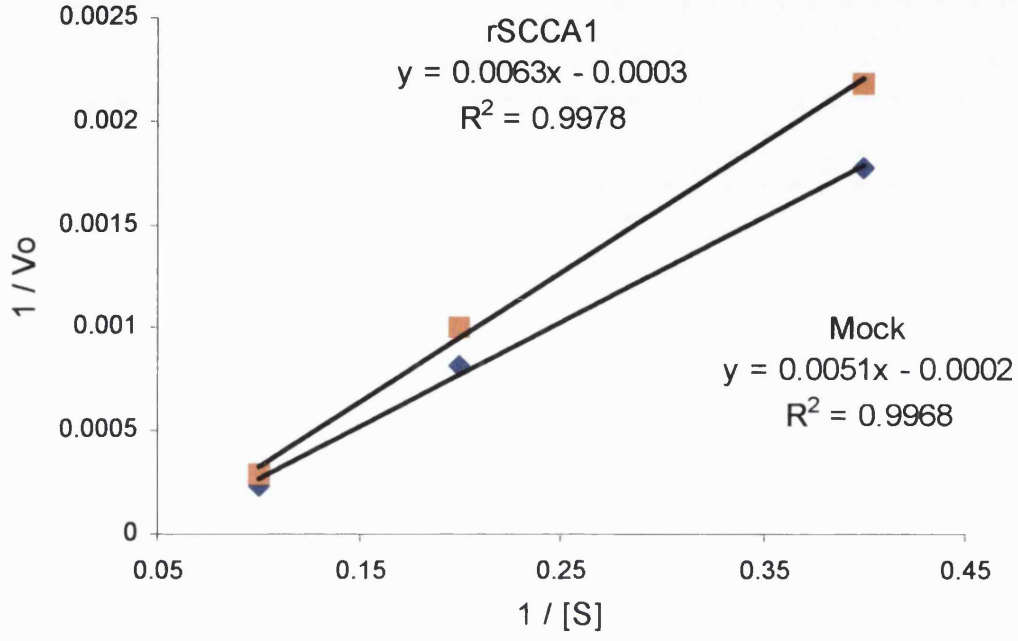


Figure 4.8 Kinetics of SCCA1 (o) inhibition of papain (compared with mock-purified rSCCA1 (◇) from untransformed cells). The graph shows that SCCA1 functions as an inhibitor of papain.

4.3.2.4 Effect of recombinant SCCA1 on virus-cell binding in pCDNA SCCA1 transfected cells

Soluble rSCCA1 was utilised to perform competitive binding assays in which increasing amounts of rSCCA1 were used to attempt to reduce virus-cell binding. Cells were transiently transfected with pCDNA SCCA1 and expression permitted to continue for 48 hours. After that period, virus infectivity studies were performed as previously, except that amounts of rSCCA1 ranging from 0.2 μg to 10 μg per well were added 10 min prior to the addition of the virus. Virus-cell binding was analysed using PCR, Southern blotting and scanning densitometry, as described above.

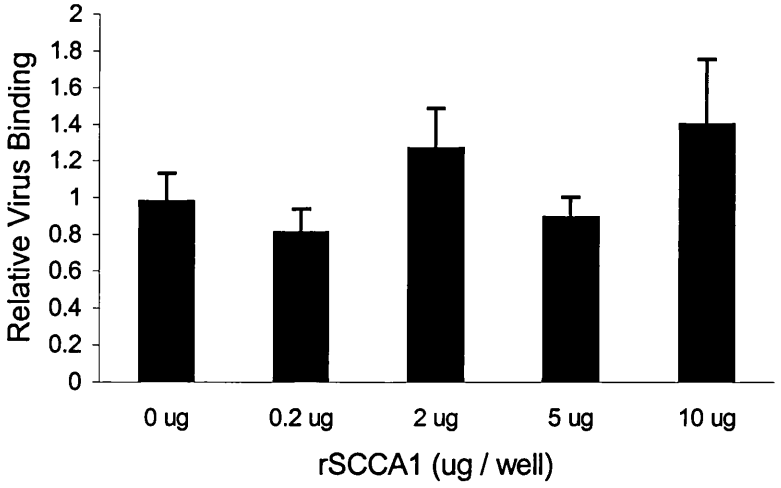


Figure 4.9 Results of competitive binding assays in which increasing amounts of rSCCA1 (0.2 μg to 10 μg) were added to wells containing pCDNA SCCA1 transfected Huh7 cells prior to the addition of virus preparations. No significant differences were observed compared with the transfected cell controls to which rSCCA1 was not added (n=6, mean ± standard deviation).

The results showed no significant difference between control transfected cells (which had not been exposed to rSCCA1) and those which had been exposed to increasing amounts of rSCCA1. Therefore, there appears to be no competition between cellular SCCA1 (expressed from the transfected construct) and rSCCA1 in the binding of virus particles. Interesting results were obtained in experiments in which the virus particles were pre-incubated with rSCCA1 (using increasing amounts ranging from 0 μg to 10 μg rSCCA1) (Figure 4.10). After pre-incubation of virus with rSCCA1 cell-virus binding assays were performed as described above using transiently transfected Huh7 cells. The results showed enhanced binding of HBV to transfected cells after pre-incubation with rSCCA1 (Figure 4.10). These results suggest the possibility that SCCA1 functions as a "bridge" between the virus and the cellular receptor.

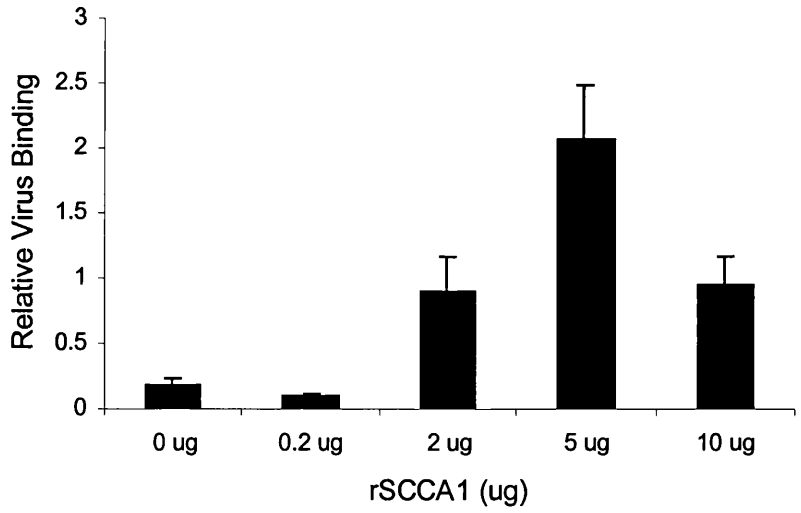


Figure 4.10 Results of competitive binding assays in which increasing amounts of rSCCA1 (0.2 μg to 10 μg) were pre-incubated with virus preparations prior to the addition of virus to pCDNA SCCA1 transfected Huh7 cells (n=6, mean ± standard deviation).

4.3.3 Endogenous expression of SCCA1 in liver and hepatocyte-derived cells

The endogenous expression of SCCA1 in liver and in two hepatocyte-derived cell lines, Huh7 and HepG2 cells, as well as SCCA1 transfected Huh7 cells, was investigated using RT-PCR performed on total RNA. In samples containing equivalent amounts of GAPDH mRNA, SCCA1 mRNA was not detected either in total liver or in hepatocyte-derived cell lines (although a PCR product was obtained from liver mRNA, Southern blotting confirmed that this was not SCCA1). As expected, there were high levels of SCCA1 mRNA in Huh7 cells transfected with pCDNA SCCA1 (Figure 4.11).

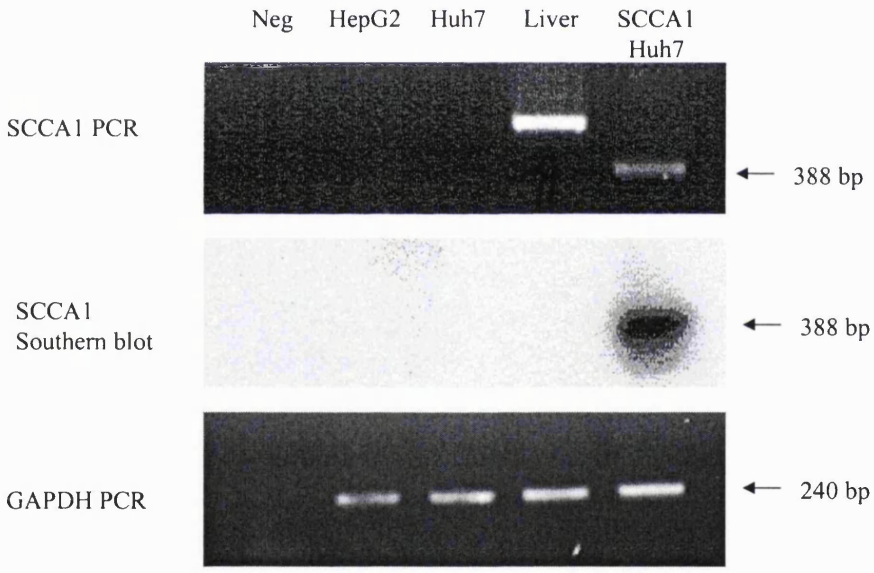


Figure 4.11 Expression of SCCA1 as determined by RT-PCR using 30 cycles of amplification. Top panel: PCR for SCCA1 (expected PCR product size is 388 bp); Middle panel: Southern blot of SCCA1 PCR products (diluted 1/100) using ^{32}P labelled DNA probe for SCCA1; Lower panel: PCR for GAPDH (house-keeping gene control) showing that equivalent amounts of RNA were utilised.

4.4 DISCUSSION

The potential role of SCCA1 as a cellular receptor for HBV was investigated further with reference to the questions posed in the introduction to this chapter. Cell surface biotinylation studies showed clearly that SCCA1, transfected into mammalian cells, was purified using strepatavidin and was therefore available for cell surface biotinylation. This would indicate that SCCA1 transfected into cells is expressed on the cell surface and would therefore be easily available to interact with the viral preS1 ligand. However, an alternative explanation, which these results cannot discount, is that expressed SCCA1 becomes tightly associated with a surface protein.

Competition assays in which recombinant soluble SCCA1 was used to block the binding of HBV to cells showed no significant difference in virus binding in the absence or presence of varying amounts of rSCCA1. This result was surprising and seems to be inconsistent with the idea of SCCA1 as the cellular receptor for HBV. These results also contradict those obtained previously (De Falco *et al.*, 2001b). The possibility that these results were due to the absence of post-translation modifications, which are required for SCCA1 function and which occur in mammalian expression systems, but not in bacterial expression systems, was eliminated by functional studies of rSCCA1. Recombinant SCCA1 was shown to inhibit proteolysis of a labelled peptide by a cysteine protease, papain. Although papain is inhibited by SCCA1 only weakly, to approximately 80% activity as compared with cathepsin S, which is inhibited far more dramatically (Luke *et al.*, 2000), inhibition was obvious and consistent. This discounts the possibility that the lack of effect of rSCCA1 on virus binding is due to loss of function in bacterially-expressed protein.

A further inconsistency with the proposed role of SCCA1 as a virus receptor was the confirmation that SCCA1 is not expressed at high levels either in liver tissue or in hepatocyte-derived cells. This is in agreement with the reported distribution of SCCA1, as detected by RT-PCR and immunohistochemistry, in a wide range of tissues, but not in the liver (Cataltepe *et al.*, 2000b). However, although SCCA1 is

evidently not expressed in hepatocyte-derived cells at levels detectable by single-round PCR, the SCCA1 clone utilised in these studies was derived from HepG2 cells after nested PCR (with the number of cycles exceeding 60 in total). Therefore, it is possible that SCCA1 is expressed in such tissues at minute levels. Certainly SCCA1 levels in Huh7 cells transfected with pCDNA SCCA1 are considerably higher than physiological levels.

Taken together, the results in this and the previous chapter confirm that SCCA1 over-expression in mammalian cells affects the binding of HBV to cells. However, the lack of competition by soluble SCCA1 and the negligible expression of SCCA1 in the target cells of the virus raises difficulties in the understanding of the mechanism whereby SCCA1 enhances virus-cell binding. A possible explanation for both these observations, that SCCA1 is not expressed in the liver and that rSCCA1 does not block virus-cell binding is suggested by the mechanism of action of serine protease inhibitors in their inhibition of proteases. As discussed in Chapter I, the mechanism whereby serpins irreversibly inhibit proteases is by the formation of a serpin-enzyme complex (SEC). This complex is removed from circulation rapidly via unknown receptors which are almost exclusively expressed by hepatocytes. Although uncleaved serpins are removed from circulation at low level, serpin removal is enhanced considerably by the cleavage of the RSL that occurs during the formation of a serpin enzyme complex. Therefore it is possible that the function of SCCA1 as a protease inhibitor is critical in enhanced virus-cell binding, and that the virus subverts the hepatic clearance of SECs in order to access hepatocytes. In this scenario, the rSCCA1 which has an uncleaved RSL, and therefore does not bind to the hepatic receptors responsible for SEC removal, would not affect enhanced virus-cell binding. This hypothesis is supported by the observation that virus pre-incubated with rSCCA1 shows enhanced binding to transfected cells, suggesting the possibility that rSCCA1 "bridges" the interaction of HBV with the cell. This would possibly require a conformational change in the rSCCA1 after interaction with the virus, and focussed attention on the reactive site loop of the protein. These hypotheses will be further explored in the next chapter.

CHAPTER V

THE EFFECT OF MUTATIONS WITHIN THE REACTIVE SITE LOOP OF SCCA1 ON VIRUS-CELL BINDING

5.1 INTRODUCTION

The understanding of the biological role of SCCA1 *in vivo*, and the mechanism whereby the molecule functions, is very limited and is largely based on studies involving related serpins. However, the unusual cross-class activity of SCCA1 (which inhibits cysteine proteases despite being termed a serpin) makes extrapolation from other members of the serpin family difficult. Despite this limitation, the reactive site loop of SCCA1 has been characterised thoroughly and has been shown to be essential for the inhibition of target cysteine proteases (Schick et al., 1998). As described in detail in Chapter I, it has been demonstrated for SCCA1 that the formation of a serpin-enzyme complex (SEC) and protease inhibition rely on RSL cleavage and insertion into the body of the molecule. Therefore, the SEC formed by SCCA1 is thought to be analogous to those observed in the inhibition of serine proteases by serpins (Irving et al., 2002). Based on these observations, and considering the hypotheses presented in the previous chapter, the focus of research shifted to the RSL of SCCA1 and its importance in enhanced binding of HBV to cells.

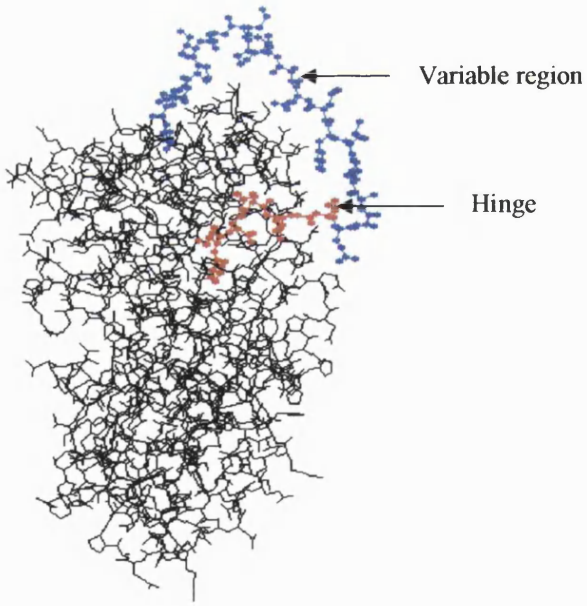


Figure 5.1 Schematic showing the RSL projecting from the body of the serpin molecule. The hinge region is indicated in red and variable region in blue. Schematic produced using Genbank Accession Numbers 1HP7 (alpha 1 antitrypsin, uncleaved).

The RSL consists of a loop projecting from the body of the protein, comprising a hinge region and a variable reactive centre loop (Figure 5.1). There have been several studies utilising site-directed mutagenesis to examine the role of individual residues within the RSL (Luke et al., 2000; Perani et al., 2000; Schick et al., 1998). Mutations within the hinge region have been shown to affect RSL mobility and the rate at which the RSL inserts into the body of the serpin. Mutation of the P14 residue from alanine to arginine has been shown to block RSL insertion and abrogate inhibitory activity and, although the protein still functions as a substrate i.e. the enzyme is capable of binding to the serpin, cleavage of the RSL does not result in a SEC (Schick et al., 1998). Mutation of the variable region has shown that the P3 residue is critical in the interaction between cathepsin S and SCCA1. Mutation of the P3 phenylalanine to alanine results in a loss of cathepsin S inhibition (Schick et al., 1998).

The cleavage site within the RSL is serine/serine (P1/P1') and, for the formation of an SEC, cleavage is required absolutely. Conversion of the serine residues to proline residues results in abrogation of the inhibitory effect of serpins (Prof. David Lomas, CIMR, personal communication). Site-directed mutagenesis offers a powerful tool to analyse the importance of the RSL in SCCA1-mediated virus-cell binding. In this chapter, a variety of functional and deletion mutations will be introduced, using site-directed mutagenesis, into the RSL of the cloned SCCA1 constructs. The effect of transfection of RSL mutants on virus-cell binding will be assessed for comparison with wild type SCCA1.

5.2 MATERIALS AND METHODS

5.2.1 Construction of SCCA1 reactive site loop mutants

A series of different site-directed mutatgenesis techniques were utilised to produce seven RSL mutants from the wild type pCDNA SCCA1 construct for comparison with the wild type protein.

5.2.1.1 Production of SCCA1 PheP3Ala and AlaP14Arg RSL mutants

Site-directed mutagenesis was performed using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) (Figure 5.2) as per the manufacturer's instructions. The template DNA (pCDNA SCCA1) was denatured and the single-stranded molecule annealed to two oligonucleotides:

(i) a mutagenic oligonucleotide which encodes the desired changes in the RSL as well as the destruction of a *PstI* site (which does not affect the amino acid sequence) to facilitate selection of mutants (Table 5.1) and

(ii) a selection oligonucleotide which encodes mutations that alter the ampicillin resistance gene, creating a new additional resistance to a antibiotic selection mixture provided by the manufacturer.

Subsequent DNA synthesis and ligation using T4 DNA polymerase and T4 ligase links the two oligonucleotides and the DNA is transformed into the repair minus *E. coli* strain BMH 71-18 *mutS*, where selection is facilitated by the altered antibiotic resistance.

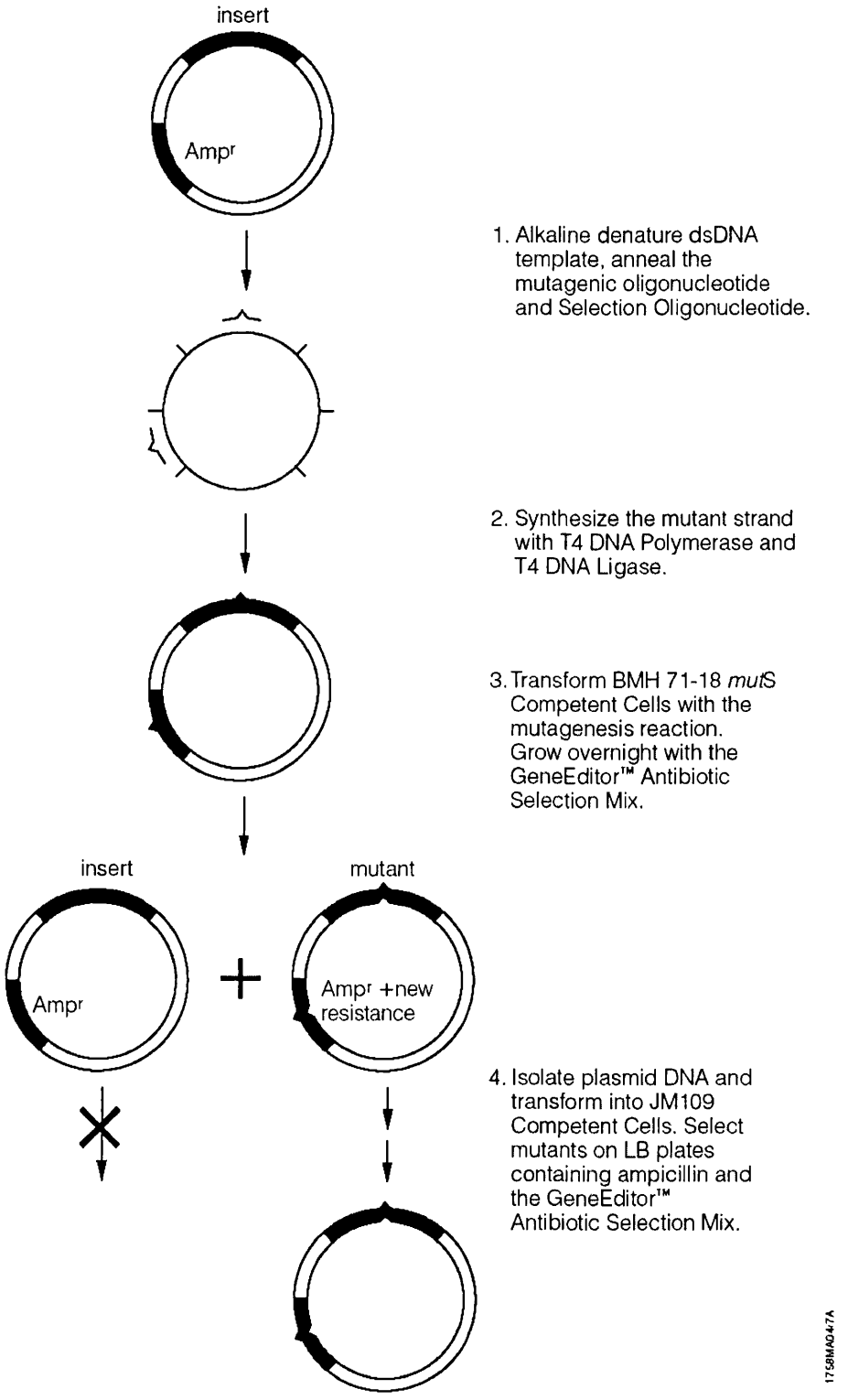


Figure 5.2 Schematic diagram of the GeneEditor *in vitro* mutagenesis procedure (Promega).

Table 5.1 Mutagenic Oligonucleotides used for the generation of SCCA1 P3 and P14 RSL mutations

Oligonucleotide name	Sequence
AlaP14Arg	5' gag gag gga **cg**a gaa gct gc**t** gct gcc acc gc 3'
PheP3Ala	5' gaa gct gc**t** gct gcc acc gct gta gta gca **gc**c gga tca tca cc 3'

Bold: Mutation in *PstI* site to facilitate identification of mutants (no change in amino acid sequence). **Bold and Underlined**: Introduction of coding changes.

Mutants were transformed subsequently into the final host strain, *E. coli* JM109 (which is not repair negative). DNA was extracted from minipreps derived from single colonies. Selection of clones with desired mutations coded by the mutagenic oligonucleotides was carried out by restriction digestion using *PstI* (successful mutagenesis having destroyed a *PstI* site present in pCDNA SCCA1). DNA sequencing was performed to verify the presence of the desired mutations.

5.2.1.2 Production of the SCCA1 SerP1Pro, SerP1'Pro RSL mutant (Pro-Pro)

Mutation of the putative cleavage site from P1 serine P1' serine to P1 proline P1' proline was performed, as shown in Figure 5.3, using the following mutagenic oligonucleotide: ProPro 5' gta gta ***gca ttc*** gga **cca cca** cct act tc 3'. This oligonucleotide contains the modified putative cleavage site P1 P1' (in bold) and also encompasses a *BsmI* restriction site (in bold italics) to facilitate replacement of a portion of the wild type RSL with the Pro-Pro mutant.

The wild type SCCA1 construct, pCDNA SCCA1, was used as the template for mutagenic PCR. The PCR reaction mixture was prepared as follows:

Component	**Volume**
Water	33 µl
dNTPs (10 mM)	5 µl
10x *Pfu Turbo* PCR buffer	5 µl
Primer 1 RSL-rev (10 pmol/µl)	2.5 µl
Primer 2 ProPro (10 pmol/µl)	2.5 µl
DNA template 3 ng/µl (pCDNA SCCA1)	1 µl
Pfu turbo DNA polymerase	1 µl
Total Volume	**50 µl**

PCR was performed in the Perkin Elmer 2400 (Applied Biosystems) with cycling parameters as follows:

Step 1: 94°C for 5 min

Step 2: 94°C for 30 secs
55°C for 30 secs
72°C for 30 secs
} 30 cycles

Step 3: 72°C for 5 min

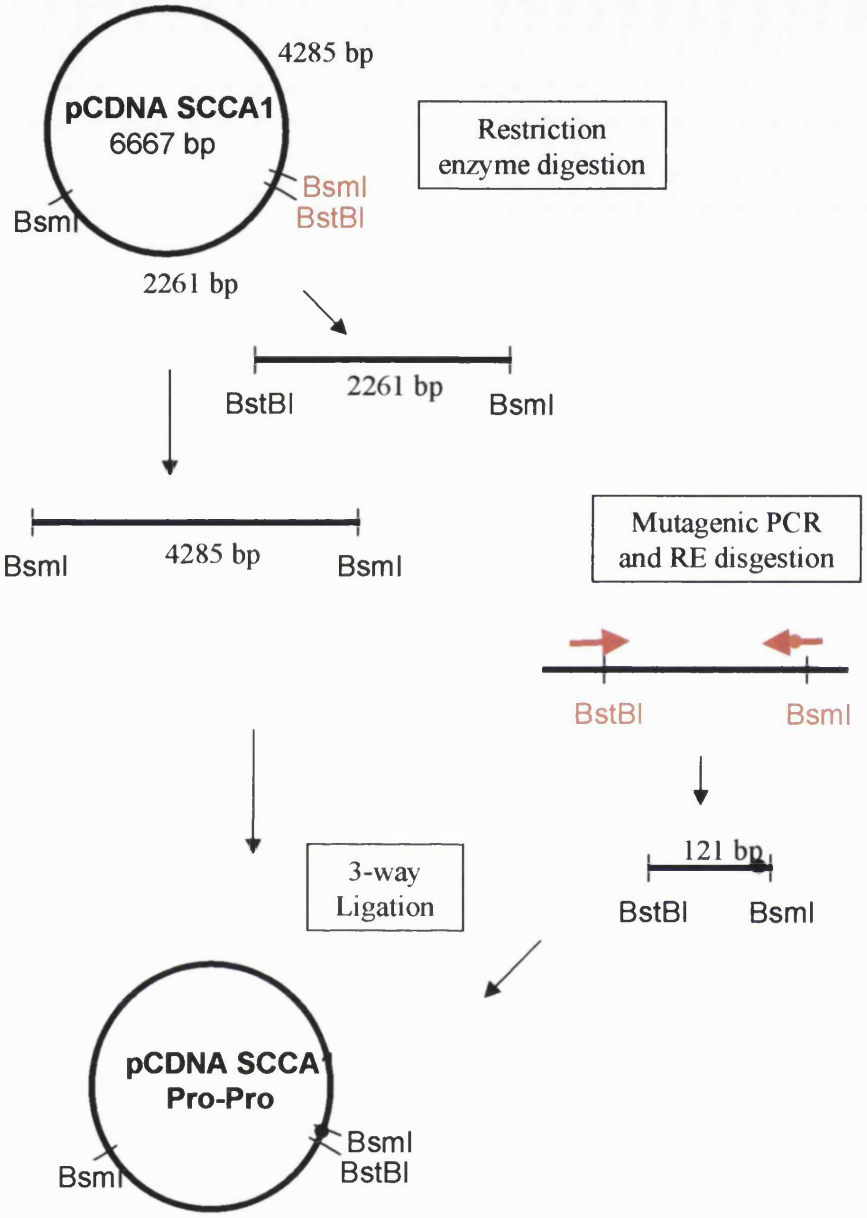


Figure 5.3 Mutagenesis strategy for the production of the Pro-Pro RSL mutant from wild type pCDNA SCCA1. The mutation is indicated, in the mutagenic oligonucleotide and the expression construct, by a dot.

Agarose gel electrophoresis was performed to confirm that amplification had been successful and then restriction digestion of the amplicon and of the parental plasmid was performed as follows:

Component	**pCDNA SCCA1**	**ProPro PCR product**
pCDNA SCCA1 (3 μg/μl)	5 μl	-
ProPro PCR product	-	20 μl
BSA (1 mg/ml)	3 μl	3 μl
Buffer NE4 (New England Biolabs)	3 μl	3 μl
BsmI	1.5 μl	1.5 μl
BstB1	1.5 μl	1.5 μl
H_2O	16 μl	1 μl
Total volume	**30 μl**	**30 μl**

Restriction enzyme digestion was performed at 65°C for 4 hours (optimal temperature for *BsmI* and *BstBI*) then low melting point agarose gel electrophoresis was used to purify the two fragments from the parental pCDNA vector (2261 bp and 4285 bp fragments) and the 121 bp digested Pro-Pro PCR product. Ligation of the three purified fragments was performed as follows:

Reaction component	**Volume**
pCDNA SCCA1 2261 bp fragment (*BsmI* to *BstBI*)	1 μl
pCDNA SCCA1 4285 bp fragment (*BsmI* to *BstBI*)	1 μl
ProPro PCR product (*BsmI* to *BstBI*)	6 μl
10x ligase buffer	1 μl
T4 DNA Ligase (New England Biolabs)	1 μl
Total volume	**10 μl**

Ligation was performed overnight at 16°C and ligated DNA was transformed into chemically competent InvαF' *E. coli* cells as described previously. Successful ligation and mutagenesis was confirmed by restriction digestion and by sequencing.

5.2.1.3 Production of SCCA1 RSL deletion mutants (work performed by Sarah Ong under my supervision)

Deletion mutants were constructed using a PCR based technique utilising pairs of reverse complementary primers (Figure 5.4). The wild type construct pCDNA SCCA1 was digested with *KspI* and *BstB1* as follows:

Component	Volume
pCDNA SCCA1 (3 μg/ul)	2 μl
KspI	2.5 μl
BstB1	2.5 μl
BSA (1 mg/ml)	5 μl
10x buffer L (Roche)	5 μl
H_2O	33 μl
Total Volume	**50 μl**

DNA was digested at 37°C for 90 min (optimal temperature for *KspI*), followed by 90 min at 65°C (optimal temperature for *BstBI*). The resulting 220 bp fragment was purified using low melting point agarose gel electrophoresis, excision of the template DNA and recovery of the DNA from the agarose using the Wizard DNA Purification system (Promega). The *KspI–BstBI* fragment of pCDNA SCCA1 was used as template for PCR (the RSL-for primer spans the *KspI* site and RSL-rev spans the BstBI site to facilitate re-insertion of the modified RSL mutants into the vector backbone). PCR was performed to generate fragments on either side of the deletions using the sets of reverse complementary primers together with either RSL-for and RSL-rev.

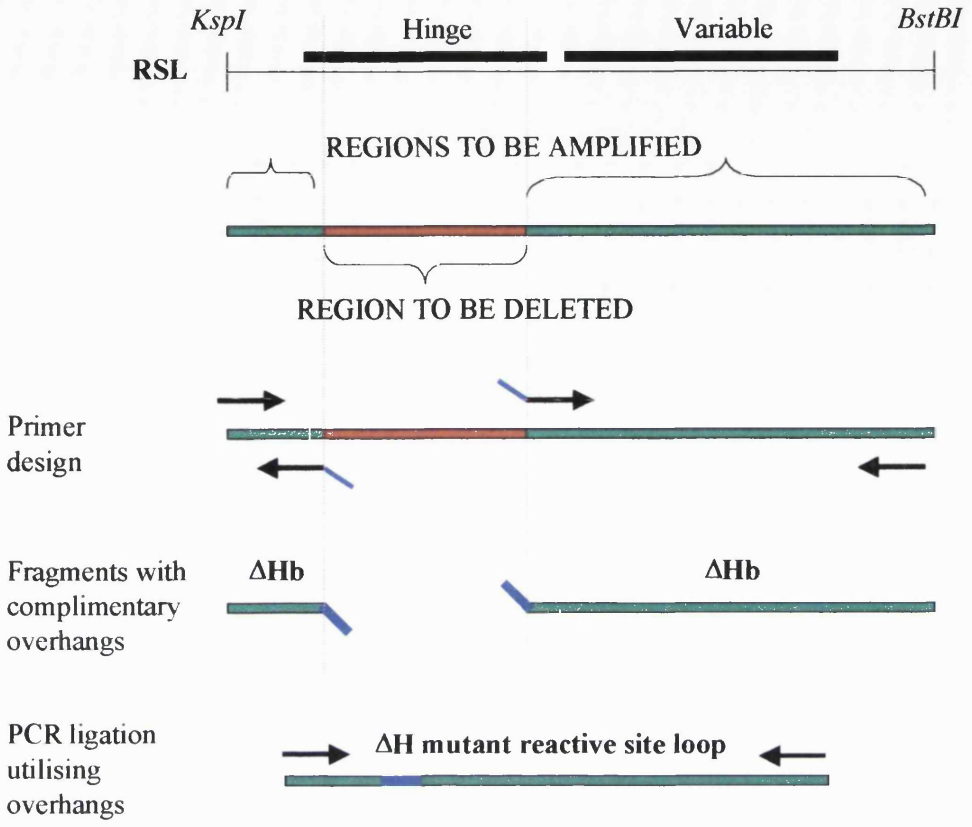


Figure 5.4 Schematic showing the approach used in the construction of RSL deletion mutants from the parental pCDNA SCCA1 vector. The construction of ΔH is shown as an example.

PCR was performed using the primers shown in Table 5.2. In the first round PCR eight reactions were prepared as shown in Figure 5.5. The reaction mixtures were prepared as follows:

Component	Volume
Water	33 µl
dNTPs (10 mM)	5 µl
10x PCR buffer	5 µl
Primer 1 [RSL-for/RSL-rev] (10 pmol/µl)	2.5 µl
Primer 2 (10 pmol/µl)	2.5 µl
DNA template (pCDNA SCCA1 *KspI* to *BstB1)*	1 µl
Pfu turbo DNA polymerase	1 µl
Total Volume	**50 µl**

Cycling conditions for the first round PCR were as follows: 94°C for 5 min, followed by thirty cycles of 94°C for 30 secs, 55°C for 30 secs and 72°C for 30 secs. There was a final extension at 72°C for 5 min.

Table 5.2 Primers utilised for the production of RSL deletion mutants.

Primer	Sequence (5' – 3')
RSL-for	GAC CGG GAG CCG CGG TCT CG
RSL-rev	AGG CTT ACC TTC GAA CGG G
ΔV-for	GAA GCT GCA GCT GCC AAT GAA GAG TTC C
ΔV-rev	GGA ACT CTT CAT TGG CAG CTG CAG CTT C
ΔH-for	GAG GTT ACA GAG GAG ACC GCT GTA GTA GC
ΔH-rev	GCT ACT ACA GCG GTC TCC TCT GTA ACC TC
Δ10-for	GGA GGG AGC AGA AGC ATT CGG ATC ATC
Δ10-rev	GAT GAT CCG AAT GCT TCT GCT CCC TCC
Δ20-for	GAG GTT ACA GAG GAG AAT GAA GAG TTC C
Δ20-rev	GGA ACT CTT CAT TCT CCT CTG TAA CCT C

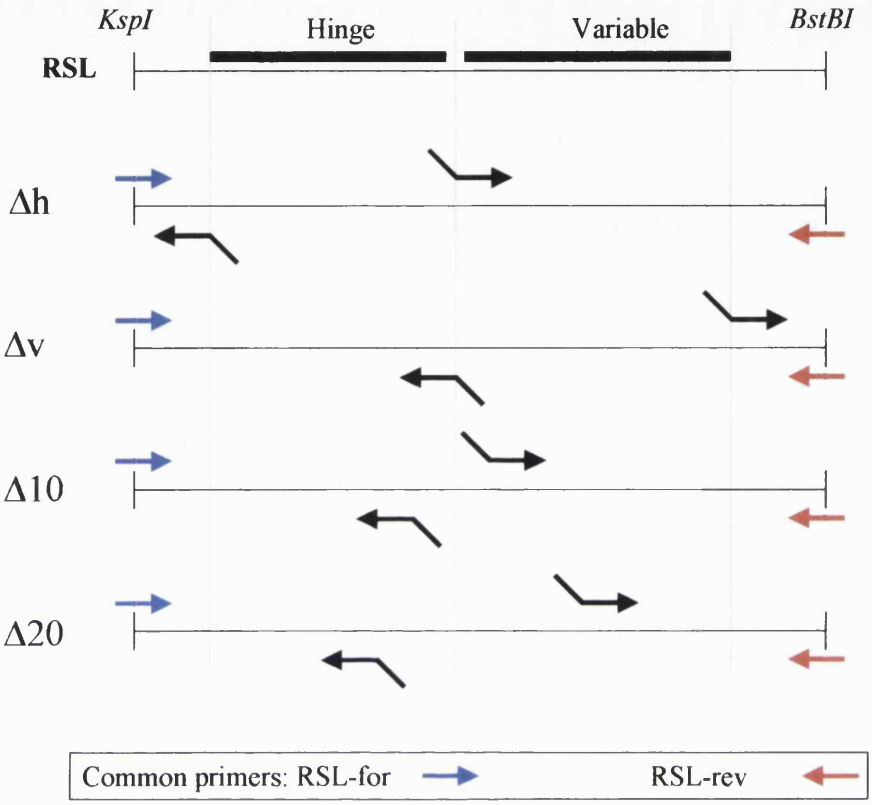


Figure 5.5 Schematic showing the arrangement of primers used in the construction of RSL deletion mutants from the parental pCDNA SCCA1 vector.

The PCR products were electrophoresed in a 2% agarose gel for 1 hour at 100V to confirm that PCR had been successful. Amplicons were purified using the Wizard® PCR preps DNA purification system (Promega) to remove primers and fragments A and B were diluted 1/50 and combined as template for a second PCR reaction which was primed by RSL-for and RSL-rev. PCR conditions were identical to those used in the first round PCR. Fragments of appropriate lengths were digested with *KspI* and *BstB1* and re-ligated into the parental pCDNA SCCA1 vector (vector fragment digested with *KspI* and *BstB1*). DNA sequencing was performed to verify the presence of desired mutations.

5.2.2 Purification of rSCCA1 produced in Cos7 cells

Cos7 cells were seeded into 12.5 cm^2 flasks (1.2 x 10^6 cells/flask) and incubated overnight at 37°C, 5% CO_2. Cells were transfected as described previously and incubated for 48 hours to allow expression of SCCA1. Cells were washed three times with PBS and harvested using versene (no trypsin). Cell pellets were stored at -20°C until rSCCA1 purification. Cells were resuspended in 2 ml binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) and lysed using sonication. The crude cell extract was centrifuged to pellet insoluble debris (3,000 rpm, 15 min). Batch purification was performed using Probond Resin (Invitrogen) equilibrated in binding buffer and incubated with the cell lysate in the presence of protease inhibitors (1 mM PMSF) for 30 min at 4°C. The resin was washed five times with washing buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) then proteins were batch eluted using 500 mM imidazole in washing buffer. Successful elution was confirmed using SDS-PAGE and western blotting using an anti-V5 antibody.

5.3 RESULTS

5.3.1 Construction of SCCA1 RSL mutants

The template used for the construction of the RSL mutants was pCDNA SCCA1, which encodes the wild type protein. The amino acid sequence of the SCCA1 encoded by this construct is shown below (Figure 5.6) as is a detailed schematic of the RSL which forms the focus of this chapter.

A)

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MNSLSEANTK FMFDLSQQFR KSKENNIFYS PISITSALGM VLLGAKNNTA 50
QQIKKVLHFD QVTENTTGKA ATYHVDRSGN VHHQFQKLLT EFNKSTDAYE 100
LKIATKLFGE KTYLFLQEYL DAIKKFYQTS VESVDFANAP EESRKKINSW 150
VESQTNEKIK NLIPEGNIGS NTTLVLVNAI YFKGQWEKKF NKEDTKEEKF 200
WPNKNTYKSI QMMRQYTSFH FASLEDVQAK VLEIPYKGKD LSMIVLLPNE 250
IDGLQKLEEK LTAEKLMEWT SLQNMRETRV DLHLPRFKVE ESYDLKDTLR 300
TMGMVDIFNG DADLSGMTGS RGLVLSGVLH KAFVEVTEEG AEAAAATAVV 350
AFGSSPTSTN EEFHCNHPFL FFIRQNKTNS ILFYGRFSSP FEGKPIPNPL 400
LGLDSTRTGH HHHHH                                      450
```

B)

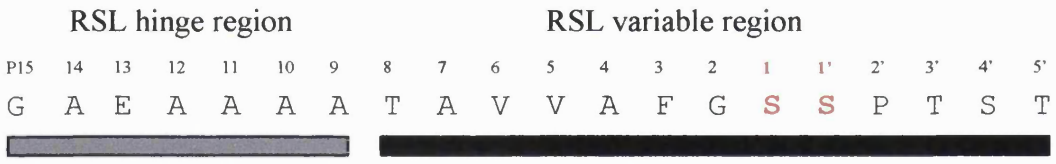


Figure 5.6 (A) Amino acid sequence of the SCCA1 cDNA in pCDNA SCCA1. The RSL (reactive site loop) is underlined, with the putative reactive site P1 P1' indicated in red. The dotted line indicates the position if the V5 polyhistidine tag. (B) Detail showing the RSL hinge and variable region and the putative cleavage site (serine serine). (Schechter and Berger Numbering System (Schechter and Berger, 1967), but, for comparative purposes, numbering relative to conserved P15Gly).

5.3.1.1 Construction of the SCCA1 PheP3Ala and AlaP14Arg RSL mutants

Site-directed mutagenesis was performed using the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega) as per the manufacturer's instructions. The template used for mutagenesis, pCDNA SCCA1, was subjected to alkaline denaturation using sodium hydroxide. Agarose gel electrophoresis was performed to confirm that denaturation had occurred (Figure 5.7). Following denaturation, the mutagenic oligonucleotides were annealed to the single-stranded template and mutant strand synthesis and ligation was performed. The double-stranded molecule was then transformed into *E. coli* BMH 71-18 *mutS* cells and grown overnight in the presence of selective antibiotics. DNA purified from these cultures was then retransformed into a stable host (*E. coli* JM109) for long-term maintenance of the plasmid.

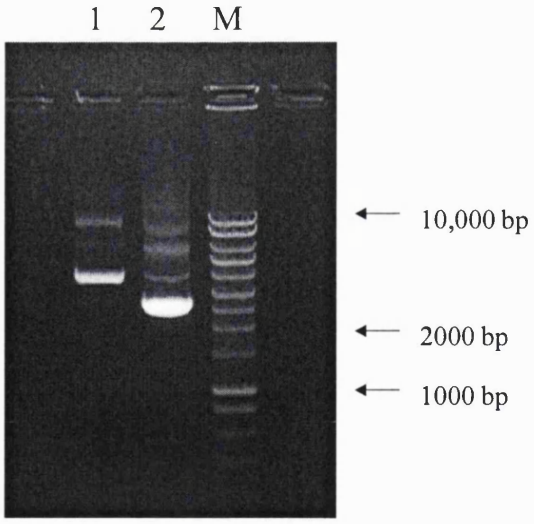


Figure 5.7 Agarose gel electrophoresis showing alkaline denaturation of pCDNA SCCA1. Lane 1: Untreated covalently closed pCDNA SCCA1; Lane 2: Alkaline denatured pCDNA SCCA1; Lane 3: Molecular weight marker (5µl Bioline Hyperladder 1).

Transformants were analysed by growing overnight mini-cultures, extracting plasmid DNA and subjecting the DNA to restriction enzyme analysis using *PstI* (Figure 5.8). Successful mutagenesis would result in the loss of a *PstI* site, allowing selection of transformants to be analysed further by sequencing. P14 (9) and P3 (7) were selected for sequencing analysis. Sequencing was performed using the 1173M primer and sequence data was compared with the wild type SCCA1 to confirm that the desired changes had been made successfully (Figure 5.9).

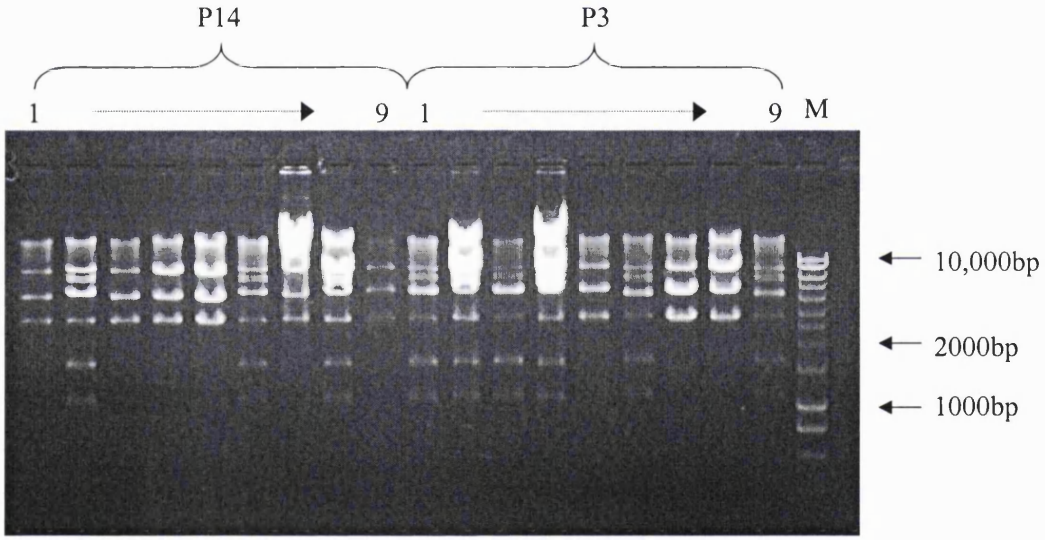


Figure 5.8 Agarose gel electrophoresis showing restriction enzyme analysis of P14 clones 1-9 and P3 clones 1-9. The destruction of a *PstI* site results in the loss of bands at approximately 1,200 bp and 1,700 bp. Clones P14 1, 3, 4, 5, 7 and 9 show loss of the *PstI* site, as do clones P3 5, 7 and 8.

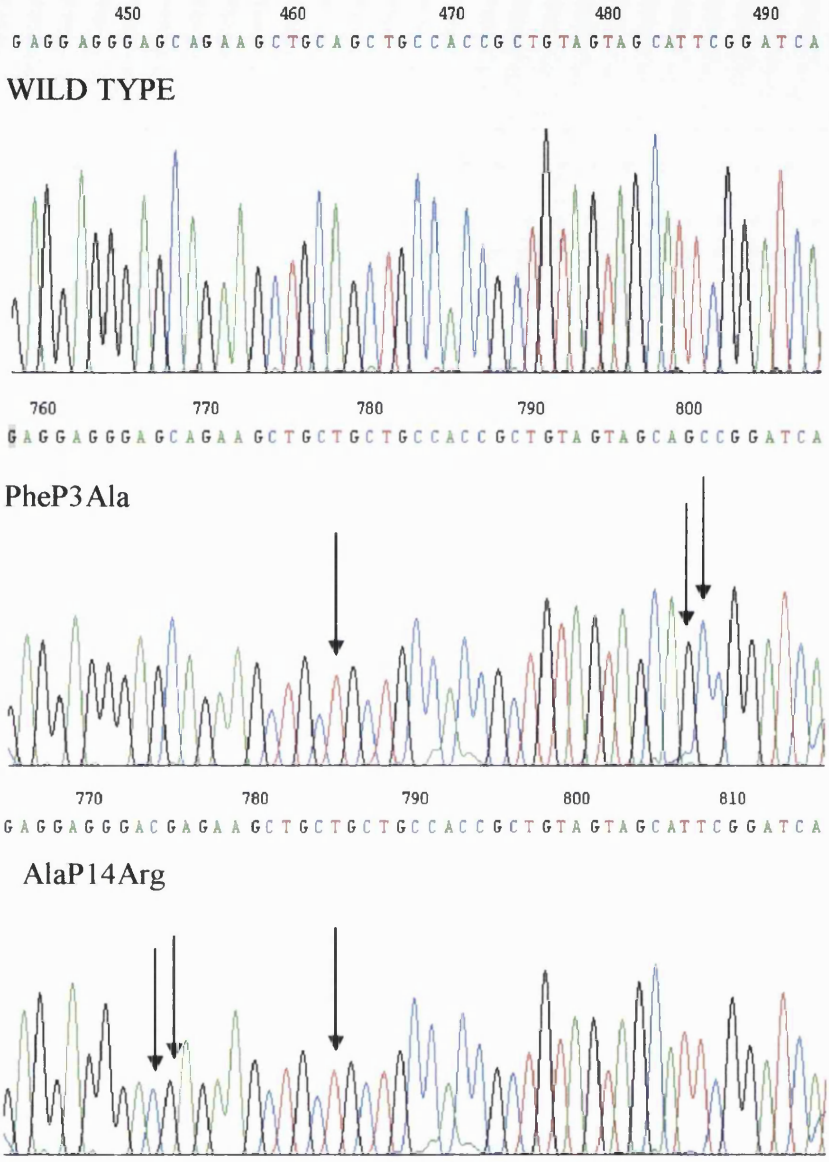


Figure 5.9 Sequencing data (chromatogram) confirming that mutagenesis was successful in the P3 and P14 RSL mutants by comparison with the wild type SCCA1 construct (arrows indicate changes).

5.3.1.2 Functional assays of inhibitory activity of SCCA1 PheP3Ala and AlaP14Arg

Both the PheP3Ala and AlaP14Arg constructs were transfected into Cos7 cells which over-express proteins. Expression of both proteins of the correct sizes was confirmed by SDS-PAGE and western blotting, using an anti-V5 antibody, as described in Chapter III.

In order to circumvent the necessity for subcloning the mutants into a bacterial expression system, expressed protein was purified from transfected Cos7 cells, making use of the polyHistidine tag at the carboxytermini of proteins expressed by pCDNA 3.1 V5 His. Purification was performed using imidazole elution from ProBond resin, in a manner similar to that used for purifiation of bacterially-expressed proteins. Function of the RSL mutants and wild type SCCA1, expressed in Cos7 cells, was assessed using proteolysis assays to measure the inhibition of papain proteolysis of a fluorescent peptide substrate. The results confirmed that while Cos7 expressed wild type SCCA1 showed inhibition of proteolysis by papain, neither the PheP3Ala mutant not the AlaP14Arg mutant SCCA1 proteins showed any detectable inhibition of proteolysis by papain (Figure 5.10).

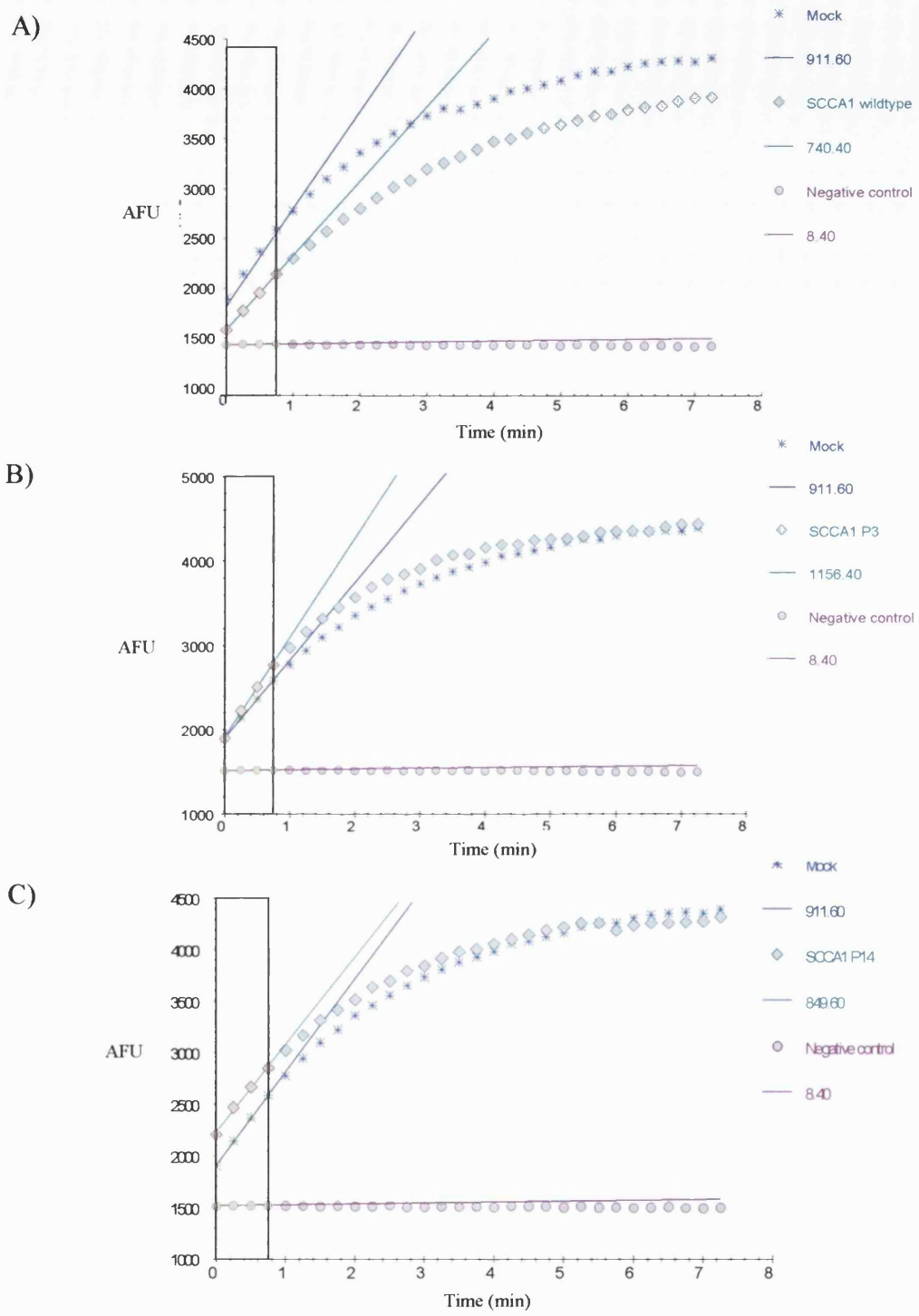


Figure 5.10 Comparison of the protease inhibitory activity of wild type SCCA1 (A) with that of PheP3Ala SCCA1 (B) and AlaP14Arg SCCA1 (C). In each graph negative control contains no papain, mock means mock purified SCCA1 from untransfected Cos7 cells). Trendlines are a measure of the initial velocity.

5.3.1.3 Virus-cell binding assays using RSL functional mutants

The construction of functional mutants, focussing exclusively on the reactive site loop of SCCA1, provided a tool for assessing the role, if any, of the RSL in the enhanced binding of HBV to cells reported in previous chapters. Virus-cell binding assays were therefore utilised to compare the effect of transient transfection of the PheP3Ala SCCA1 and the AlaP14Arg SCCA1 constructs on the level of virus-cell binding. Transient transfection of equal amounts of each construct into Huh7 cells was performed and expression was allowed to proceed for 48 hours. Virus-cell binding assays were performed as described previously. The data showed that neither the P3 nor the P14 RSL mutant constructs resulted in abrogated enhanced virus-cell binding (Figure 5.11). Although both RSL mutants exhibited slightly greater enhancement of virus binding, this increase was not significantly different from that observed for the wild type SCCA1 transfected cells. These results suggested that the function of SCCA1 as a specific inhibitor of certain known proteases, including papain and cathepsins S, K and L (all of which have been reported previously to be affected by alterations to the P3 and P14 residues), is not required for enhanced virus-cell binding.

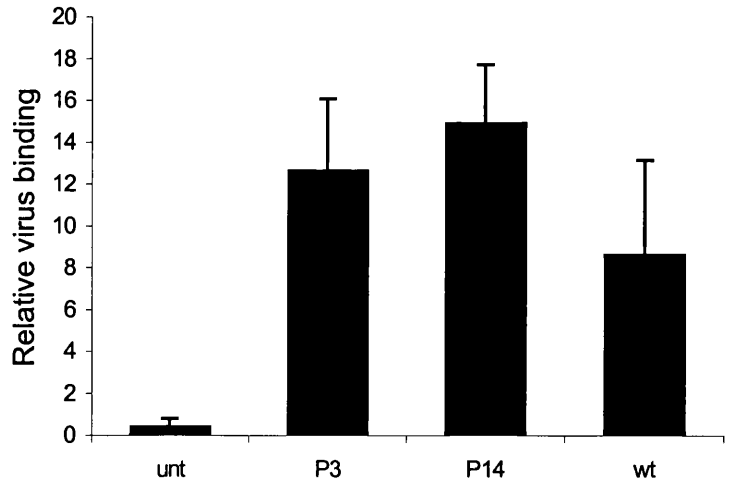


Figure 5.11 Comparison of the effect of transient transfection of wild type SCCA1 with PheP3Ala and AlaP14Arg constructs on virus-cell binding (n=6, mean ± standard deviation).

5.3.1.4 Construction and effect on virus-cell binding of the SCCA1 SerP1Pro SerP1'Pro RSL double mutant

Very little is known about the biological role of SCCA1 *in vivo*. Those proteases known to be inhibited *in vitro* by SCCA1 may not necessarily be the "real" biological target of the serpin *in vivo*. For this reason, the putative cleavage site, P1 P1', which is postulated to be cleaved proteolytically during the binding of target proteases to the RSL, was investigated. Mutagenesis was performed to change the P1 and P1' residues from serine to proline. PCR mutagenesis was performed using the wild type pCDNA SCCA1 as a template. The resulting PCR product of 121 bp and referred to as the Pro-Pro cassette (Figure 5.12) was digested with *BsmI* and *BstB1*, as was the parental vector pCDNA SCCA1 (resulting in 2 vector fragments of 2261 bp and 4285 bp and the excised RSL cassette) . The two large vector fragments were ligated to the Pro-Pro cassette, to form a Pro-Pro mutant of pCDNA SCCA1 and transformed into *E. coli* InvαF'. Transformants were screened by sequencing to obtain a clone in which the putative cleavage site was modified from P1 serine P1' serine to P1 proline P1' proline (Figure 5.13).

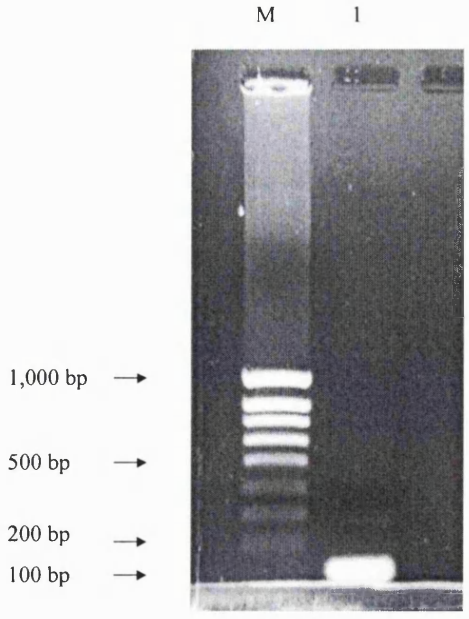


Figure 5.12 Mutagenic PCR to produce the SerP1Pro, SerP1'Pro RSL (Pro-Pro) cassette of 121 bp. M (lane 1), molecular weight marker (Bioline Hyperladder I).

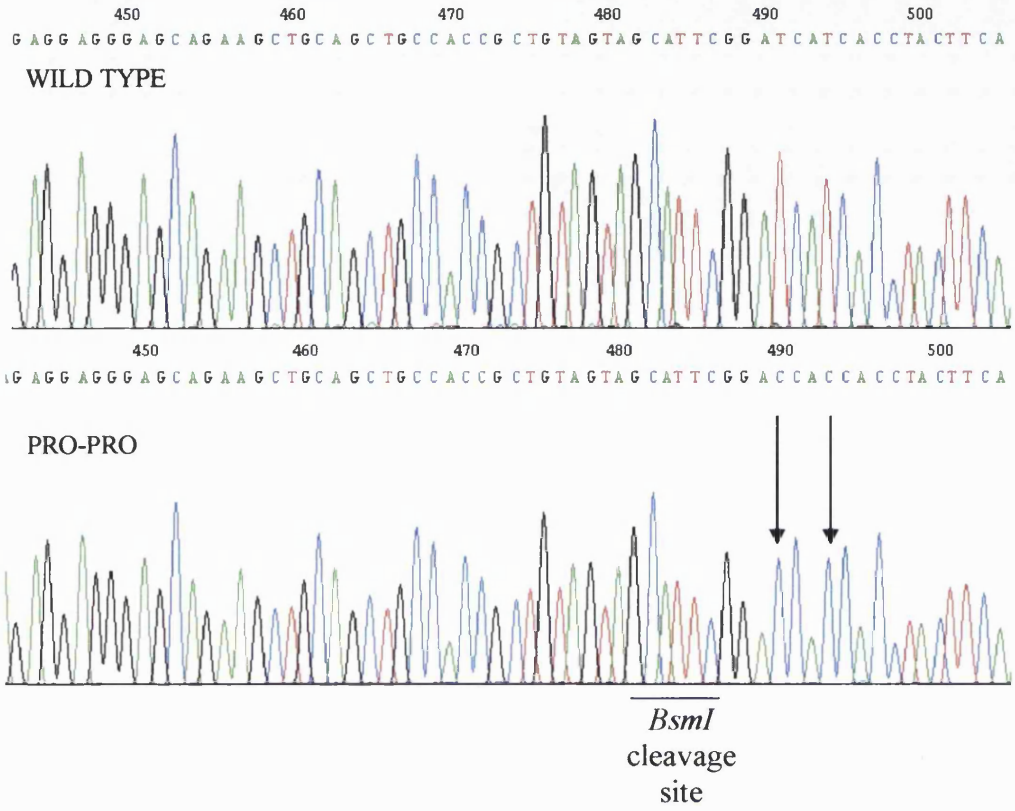


Figure 5.13 Sequencing data confirming that mutagenesis successfully resulted in the production of the Pro-Pro RSL mutant by comparison with the wild type SCCA1 construct (arrows indicate changes, the *Bsm*I restriction enzyme recognition site is underlined).

As with the P3 and P14 mutants, expression of constructs in Cos7 cells was used to confirm by western blotting that expression was consistent with the expected size of the modified protein (data not shown). Cos7 expressed protein also was used in proteolysis assays to confirm that the modified protein was no longer inhibitory for papain (data not shown). Virus-binding assays were performed to assess the effect of the mutant protein compared with wild type protein on virus-cell binding. The results showed no significant difference in virus-cell binding comparing cells expressing wild type SCCA1 and those expressing the Pro-Pro mutant SCCA1 (Figure 5.14).

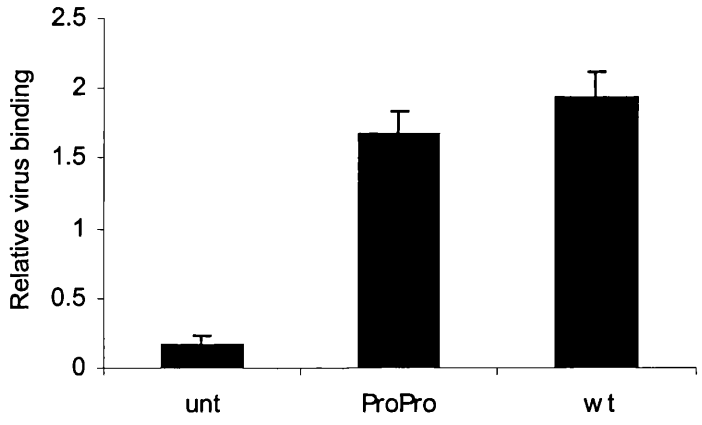


Figure 5.14 Comparison of the effect of transfection of wild type SCCA1 with Pro-Pro mutant SCCA1 constructs on virus-cell binding (n=6, mean ± standard deviation).

5.3.1.5 Construction and effect on virus-cell binding of the RSL deletion mutants

A more dramatic approach was applied to investigating the possible role of the reactive site loop of SCCA1. A series of four RSL deletion mutants was constructed in order to investigate further the role, if any, of the reactive site loop in enhanced binding of HBV to cells. RSL deletion mutants were constructed using sequential PCR reactions and pairs of primers incorporating complementary overhangs. The template for all PCR reactions was the *KspI–BstB1* fragment of pCDNA SCCA1. The template was obtained by digestion of the parental vector (Figure 5.15) and low melting point agarose gel electrophoresis.

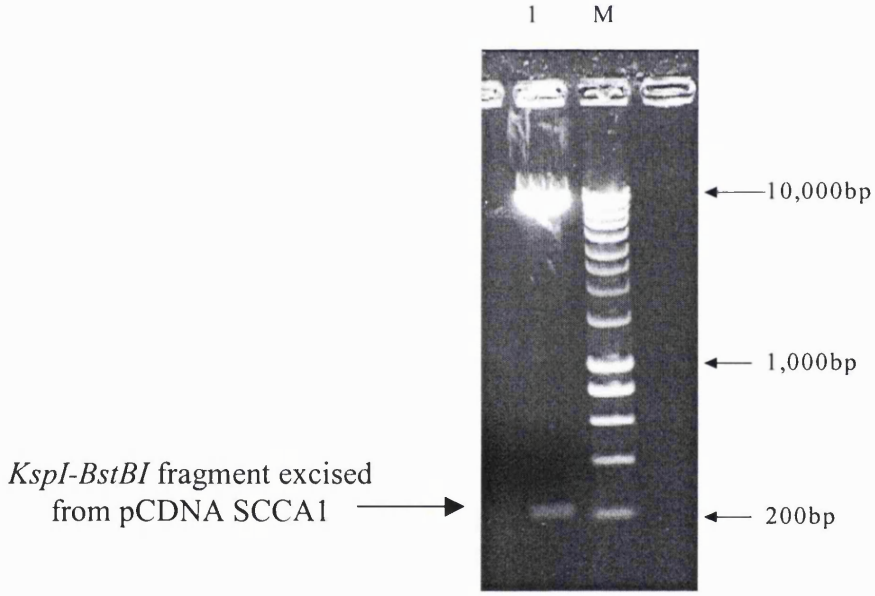


Figure 5.15 Agarose gel electrophoresis showing excision of the *KspI – BstB1* fragment of pCDNA SCCA1 (lane 1). M, molecular weight marker (Bioline Hyperladder 1).

PCR was performed to generate the fragments (A and B, defined below in Table 5.3) on either side of the deletions (fragments A and B also contain reverse complementary overhangs). Fragments were constructed with primers as shown in Table 5.3 below. PCR products from all reactions were subjected to agarose gel electrophoresis and the product sizes compared with those expected (Figure 5.16). In all cases, PCR products of the expected size were obtained.

PCR products from the above reactions were purified using low melting point agarose gel electrophoresis and diluted 1/50 for use as templates in the subsequent PCR ligation step. Fragments A and B flanking the deletions were ligated together using PCR with an equimolar mixture of the fragments. Products of the PCR ligation were subjected to agarose gel electrophoresis (Figure 5.17) and product sizes again compared with the sizes predicted for ligated products (Figure 5.16 C).

Table 5.3 Primers used in the amplification of each sub-fragment

Fragments A	**Forward primer**	**Reverse primer**	**Fragments B**	**Forward primer**	**Reverse primer**
Δh A	RSL-for	Δh-rev	Δh B	Δh-for	RSL-rev
Δv A	RSL-for	Δv-rev	Δv B	Δv-for	RSL-rev
Δ10 A	RSL-for	Δ10-rev	Δ10 B	Δ10-for	RSL-rev
Δ20 A	RSL-for	Δ20-rev	Δ20 B	Δ20-for	RSL-rev

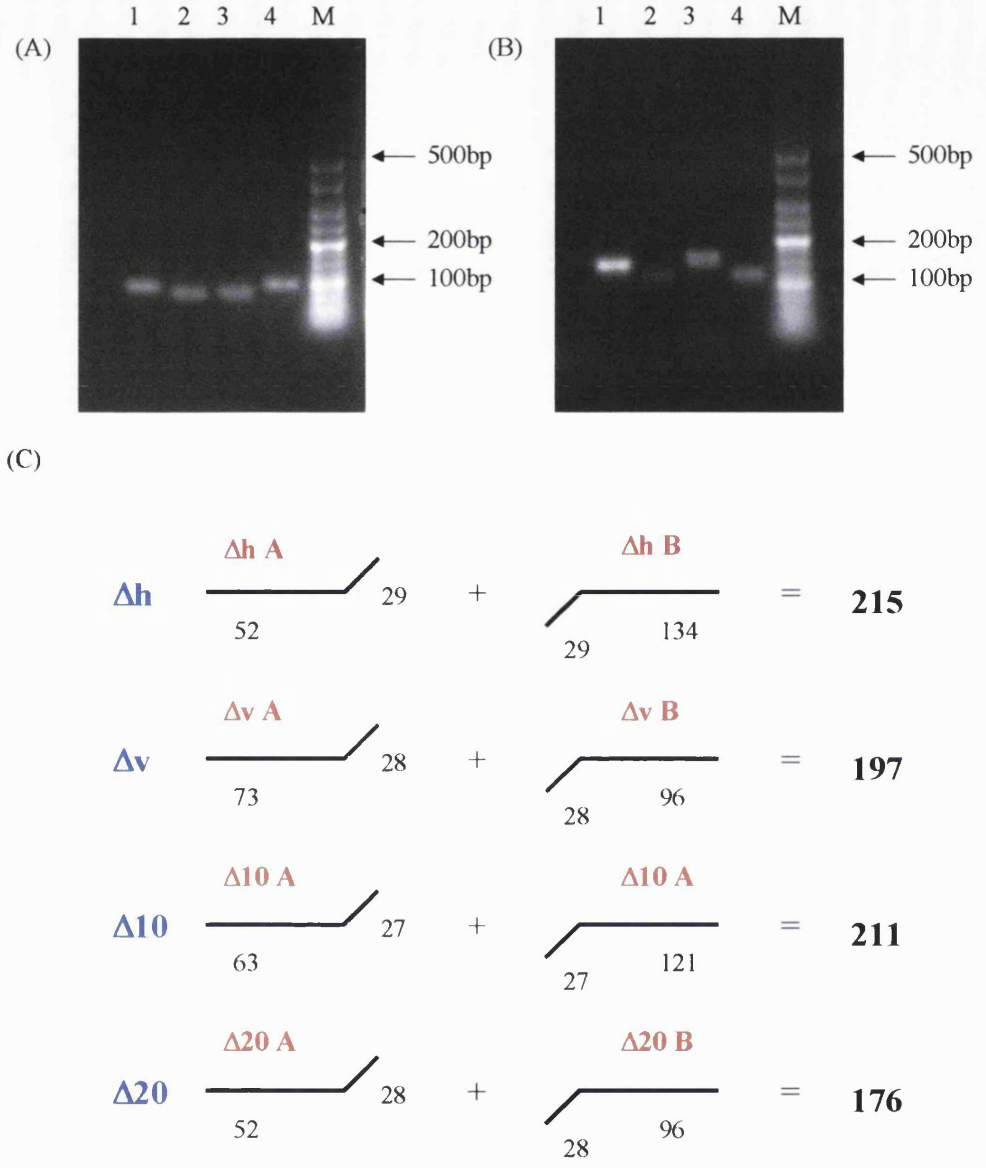


Figure 5.16 Agarose gel electrophoresis (2%) of PCR products (10μl per well). (A) Products of PCR using RSL-for and corresponding reverse primers. Lane 1, Δh A; Lane 2, Δv A; Lane 3, Δ10 A; Lane 4, Δ20 A; M, Bioline hyperladder V. (B) Products of PCR using RSL-rev and corresponding forward primers. Lane 1, Δh B; Lane 2, Δv B; Lane 3, Δ10 B; Lane 4, Δ20 B; M, Bioline hyperladder V. (C) Predicted sizes of PCR products.

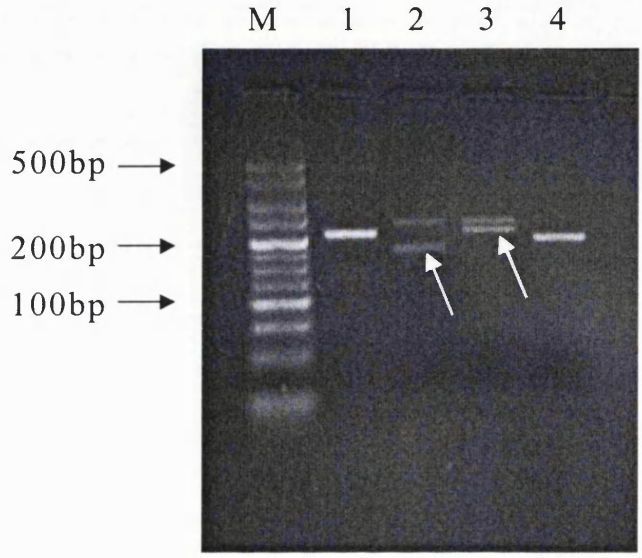


Figure 5.17 Agarose gel electrophoresis (4%) of the products of PCR ligation (10µl per well). M, molecular weight marker (Bioline Hyperladder V), Lane 1, Δ10; Lane 2, Δ20; Lane 3, Δh; Lane 4, Δv. Arrows indicate the position of products of the expected size.

In two reactions, Δv and Δ10, single products of the predicted size were obtained. However, for Δh and Δ 20, doublets resulting from non-specific amplification were observed. In each case the lower band (indicated by arrows in Figure 5.17) corresponded to the predicted size of the PCR ligation product. These lower bands and the single bands for Δv and Δ10 were gel purified using low melting point agarose gel electrophoresis. Purified PCR products and the parental vector (pCDNA SCCA1) were subjected to restriction enzyme digestion using *BstBI* and *KspI* and then the four shortened RSL cassettes were re-ligated into the digested vector. Transformation was performed for all four deletion mutants and minipreps screened for successful ligation by restriction enzyme digestion using *BstBI* and *SacII* (an isoschizomer of *KspI*).

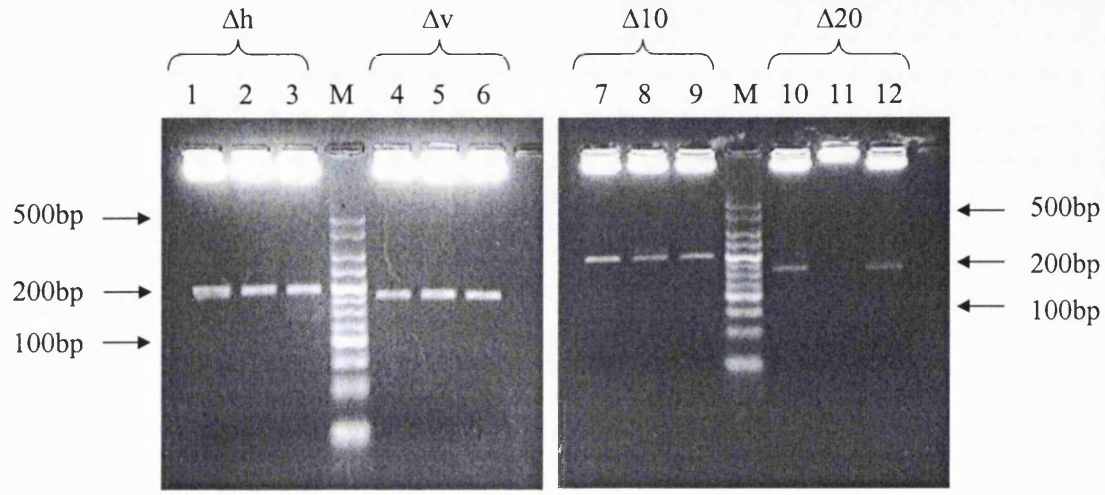


Figure 5.18 Screening of deletion mutant minipreps, using restriction enzyme digestion with *BstBI* and *SacII*, to identify transformants for each of the four deletion mutants.

One transformant for each deletion mutant was identified and maxipreps were performed on large-scale cultures. For each deletion mutant, restriction enzyme analysis was again performed to confirm that appropriate clones had been expanded (Figure 5.19). Each of the RSL deletion mutants contained shortened RSL cassettes of predicted length and were subsequently sequenced and compared to the wild type sequence to confirm the specificity of the mutagenesis (Figure 5.20).

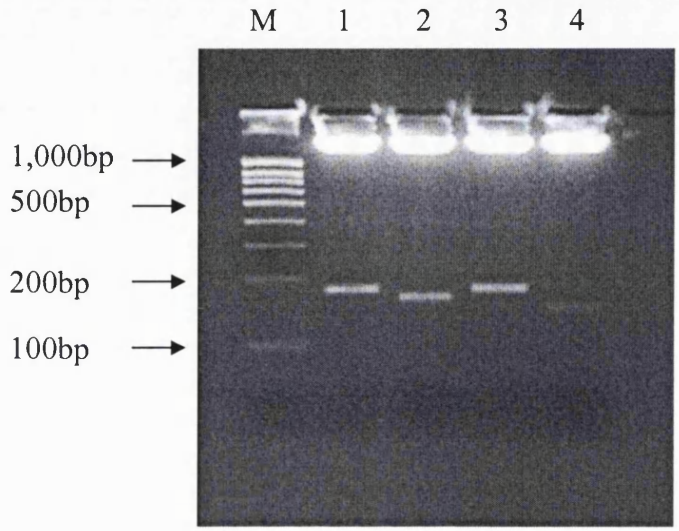


Figure 5.19 Restriction enzyme analysis using *BstBI* and *SacII* of DNA purified from large-scale overnight cultures. M: molecular weight marker (Bioline Hyperladder IV, 5µl), Lane 1: Δh, Lane 2: Δv, Lane 3: Δ10, Lane 4: Δ20.

Table 5.4 Amino acid sequences (wt residues 329 – 375) of the RSL deletion mutants created from pCDNA SCCA1.

wild type	**LH KAFVEVTEEG AEAAAATAVV AFGSSPTSTN EEFHCNHPFL FFIRQ**
Δ hinge	LH KAFVEVTEE- ------TAVV AFGSSPTSTN EEFHCNHPFL FFIRQ
Δ variable	LH KAFVEVTEEG AEAAAA---- ---------N EEFHCNHPFL FFIRQ
Δ 10	LH KAFVEVTEEG AE-------- AFGSSPTSTN EEFHCNHPFL FFIRQ
Δ 20	LH KAFVEVTEE- ---------- ---------N EEFHCNHPFL FFIRQ

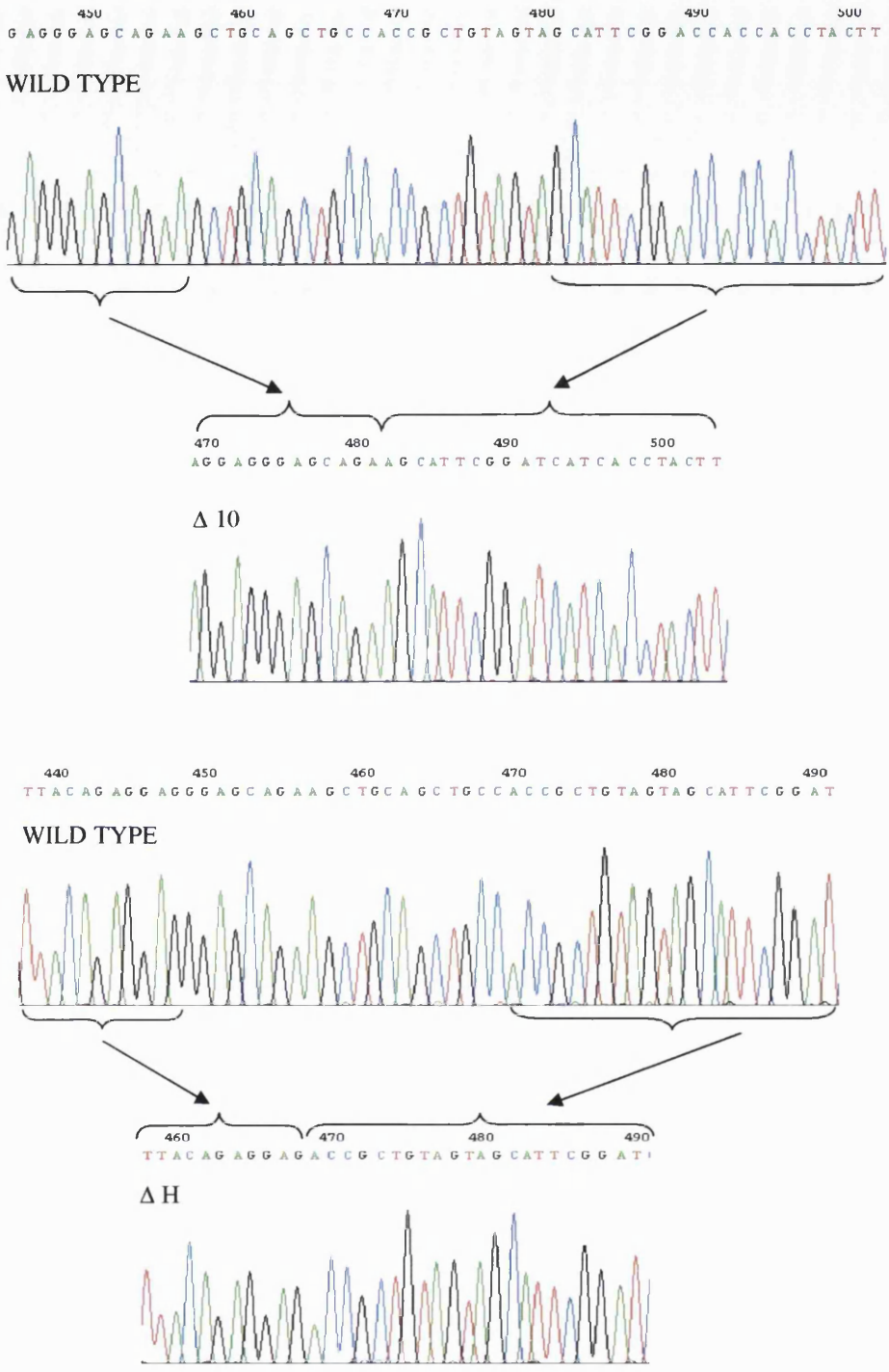
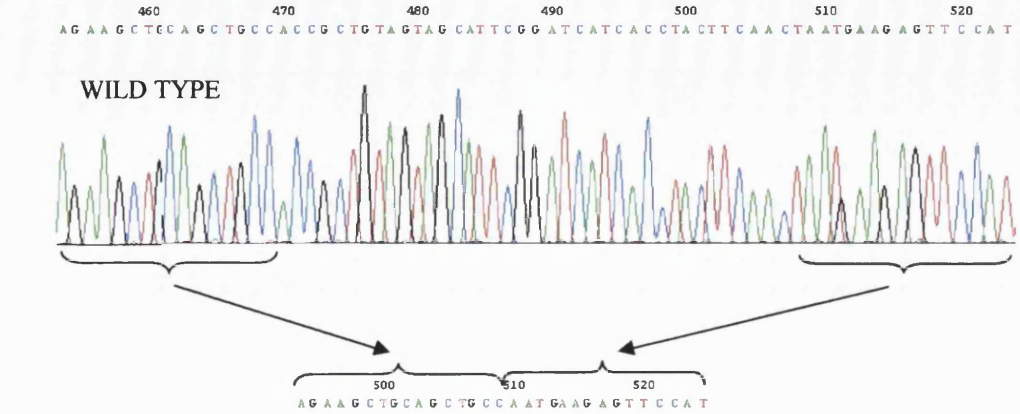


Figure 5.20 Sequencing data (chromatograms) confirming deletions were introduced into the RSL of wild type SCCA1 (continued on next page) producing four constructs - ΔH, ΔV, Δ10 and Δ 20.



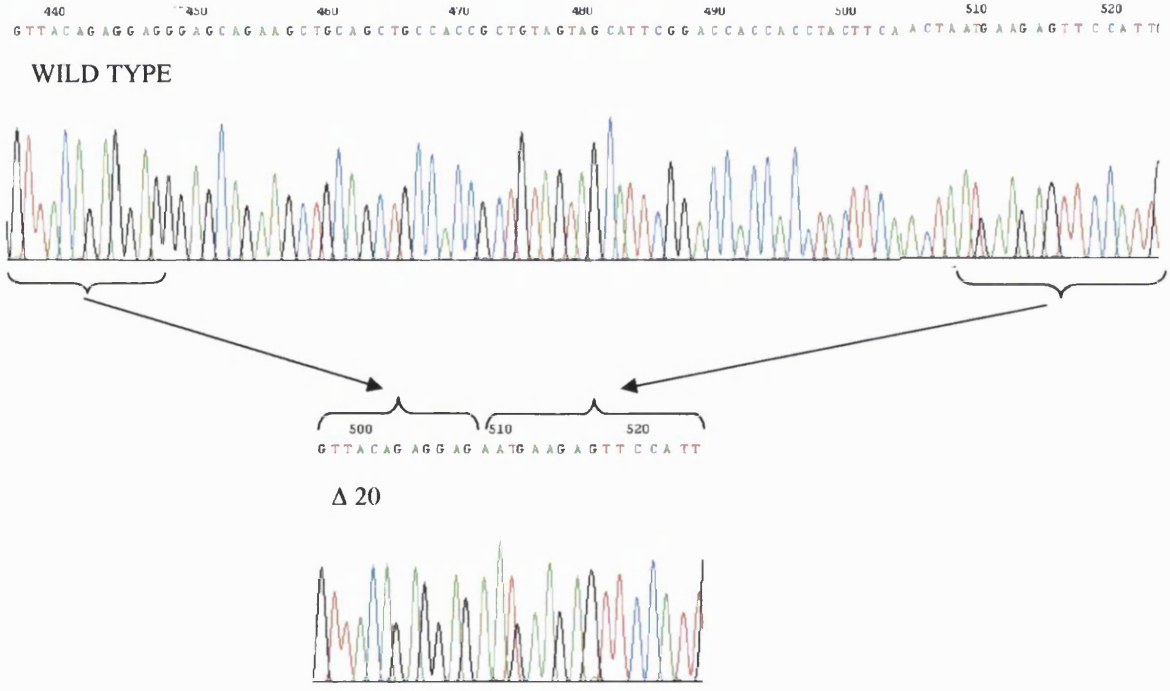


Figure 5. 20 contd.

As described above, transfection of constructs into Cos7 cells was used to confirm by western blotting that protein expression was consistent with the expected size of the modified protein (data not shown). Cos7 expressed protein also was used in proteolysis assays to confirm that the modified protein was no longer inhibitory for papain (data not shown). As with the RSL functional mutants, virus-binding assays were performed comparing wild type SCCA1 with the RSL deletion mutants. Transient transfection of equivalent amounts of each construct into Huh7 cells was performed and expression allowed to proceed for 48 hours before virus-binding assays were performed. In no case did deletion of any region within the RSL result in abolition of the enhanced virus-cell binding observed for wild type SCCA1 transfected cells (Figure 5.21). More surprisingly, in the cases of the Δ10 and Δ20 constructs, there was further enhancement of viral binding which was statistically significantly different from the wild type SCCA1 transfected cells.

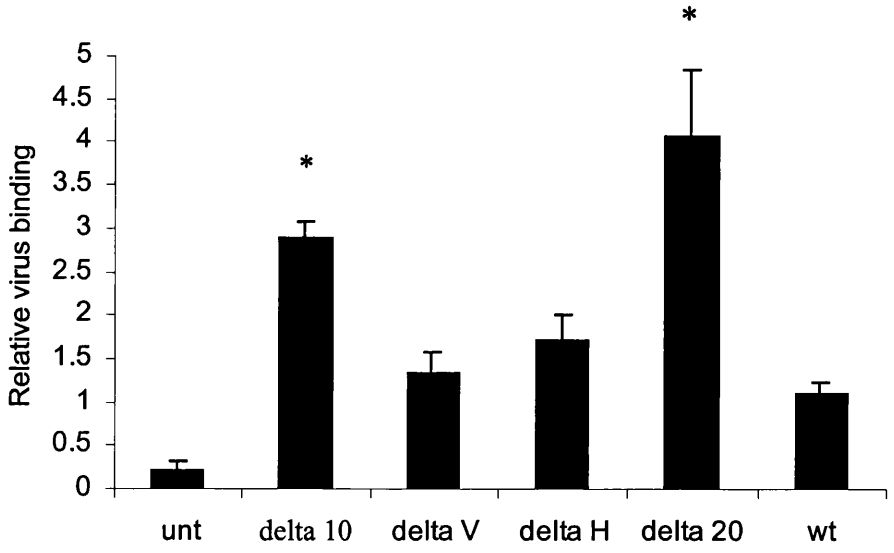


Figure 5.21 Comparison of the effect of transfection of wild type SCCA1 with the RSL deletion constructs on virus-cell binding (n=6, mean ± standard deviation). * indicates statistically significant increase compared with wild type SCCA1 as determined using students t-test ($p<0.05$).

5.4 DISCUSSION

The role of the RSL of SCCA1 in enhanced virus-cell binding was investigated by the introduction of various mutations into the RSL of the wild type SCCA1 molecule used in previous experiments. Substitutional mutagenesis was used to create three mutants, PheP3Ala, AlaP14Arg and SerP1Pro, SerP1'Pro. These mutants were tested for their ability to mediate enhanced binding of HBV to cells compared with untransfected cells and pCDNA SCCA1 (wild type) transfected cells. Although the mutations introduced were shown to abrogate inhibition of proteases by the recombinant protein, no reduction was observed in virus-cell binding compared with the wild type. These results show that the known activity of SCCA1 as an inhibitor of proteases is not required for the enhanced virus-cell binding observed post-transfection.

In order to confirm that the RSL of SCCA1 is not required for enhanced virus-cell binding, a more dramatic approach to mutagenesis was employed. A PCR-based deletional mutagenesis procedure was developed to construct four mutants in which regions of the RSL were removed. Comparison of the RSL mutants with wild type SCCA1 confirmed that the RSL does not play a role in the enhancement of virus-cell binding. Interestingly, in two cases, the Δ10 and Δ20 constructs, even further enhancement of virus-cell binding was observed compared with the wild type. The reasons for the further enhancement are not yet known, but may be due to the altered conformation of the SCCA1 molecule resulting from the large deletions that have been introduced, and may suggest that another region of the SCCA1 protein interacts with HBV.

The complex between serpins and proteases is one of many interactions in which serpins may be involved (Janciauskiene, 2001). The hypothesis proposed in the previous chapter favoured a critical role for the RSL, which is not the case. However there remain a variety of alternative possible explanations for SCCA1-mediated virus-cell binding. These include the possibility, discussed in the next chapter, that

the effect of SCCA1 on the binding of HBV to cells is an indirect effect i.e. transfection of SCCA1 into hepatocyte derived cells results in alteration of the expression of a second protein which mediates enhanced virus-cell binding.

CHAPTER VI

THE ROLE OF THE LDL RECEPTOR RELATED PROTEIN (LRP) IN SCCA1-MEDIATED VIRUS CELL BINDING

6.1 INTRODUCTION

The data presented in the previous chapters is based on the assumption that the enhanced binding of HBV to cells expressing SCCA1 (whether hepatocyte-derived or not) is a result of a direct interaction between SCCA1 and HBV. This was assumed to be the case largely because SCCA1 was identified initially using an affinity chromatography approach which relied on the direct binding of SCCA1 to tetramers of the HBV preS1 domain (De Falco et al., 2001b). Although the consistent ability of functionally deficient variants of SCCA1 to enhance virus-cell binding is not incompatible with that hypothesis (particularly if a domain other than the RSL mediates virus-cell binding), another possibility is that the enhanced binding of HBV to cells transfected with SCCA1 constructs is an indirect effect of SCCA1 expression. An alternative hypothesis was therefore that transfection of SCCA1 constructs into cells resulted in the expression of a second protein, which could either mediate enhanced virus-cell binding alone or in concert with SCCA1 (which may itself either be cell-associated or secreted into medium to facilitate the formation of "bridging" complexes). In the absence of gene array data indicating what the secondary molecule(s) might be, a hypothesis-driven approach was adopted.

Basu et al. (2001) observed that a squamous cell carcinoma cell line, UV6139, showed enhanced binding of alpha 2 macroglobulin (A2M) compared with a variety of other cell lines and primary tissue. The A2M receptor (Ashcom et al., 1990) is a cell surface glycoprotein now recognised as being the same protein as the low-density lipoprotein (LDL) receptor related protein (LRP) (Strickland et al., 1990; Kristensen et al., 1990). This protein is an attractive candidate for the putative unknown secondary protein responsible for mediation of enhanced virus-cell binding for a variety of reasons which will be discussed below. LRP is a member of the low density lipoprotein receptor gene family (LRF) (Figure 6.1). These proteins are type I transmembrane proteins with common structural features such as epidermal growth factor (EGF)-like repeats, cysteine-rich complement-like repeats, YWTD-containing repeats which are arranged in propeller-like structures (Springer, 1998), a

transmembrane domain and a cytoplasmic domain (Hussain et al., 1999). They are an evolutionarily conserved group of cell-surface receptors which are expressed in both vertebrates and invertebrates (Nykjaer and Willnow, 2002). These receptors bind several unrelated ligands, a function that is probably determined largely by electrostatic potential rather than primary sequence. Ligands are delivered via clathrin-coated pits to endosomes for degradation through receptor-mediated endocytosis (RME) (Hussain et al., 1999). After internalisation and degradation of ligands, the LRP recycles to the plasma membrane (Herz and Strickland, 2001). The functional significance of the structural domains with regard to RME has been elucidated. The complement-like repeats have been shown to be the site of ligand binding, while the cytoplasmic tails encode recognition sites for cytosolic adaptor proteins (Trommsdorff et al., 1998; Gotthardt et al., 2000; Goretzki and Mueller, 1998; Stockinger et al., 2000; Barnes et al., 2001), including motifs that regulate internalisation via clathrin-coated pits (Owen and Evans, 1998; Li et al., 2000). The EGF-like repeats appear to be essential for the pH-dependent release of bound ligands in the endosomes (Howell and Herz, 2001; Davis et al., 1987).

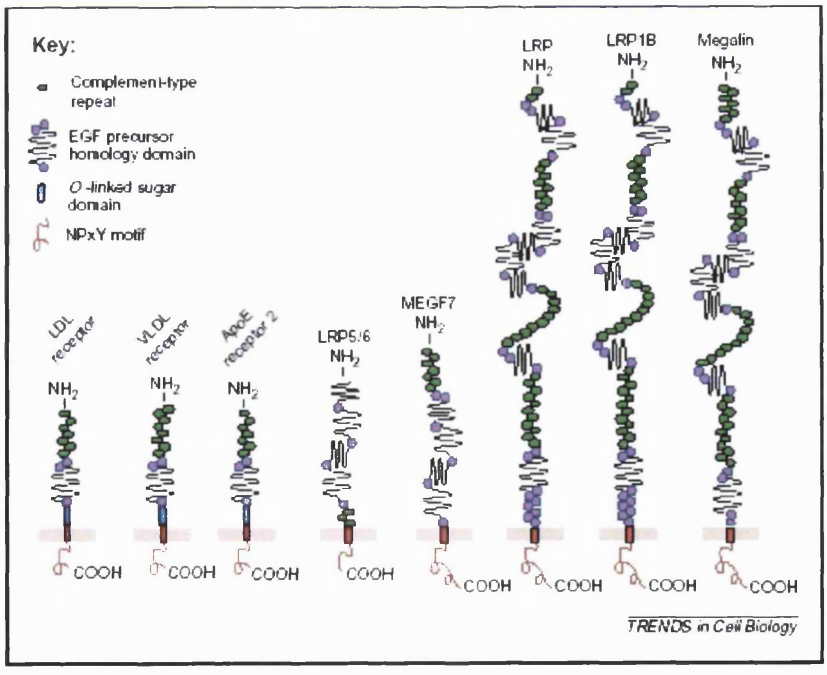


Figure 6.1 Schematic showing the known members of the LDL receptor gene family (Nykjaer and Willnow, 2002).

LRP was cloned in 1988 by Herz et al. (1988) and later shown to be the receptor for methylamine activated A2M (A2M*) (Kristensen et al., 1990; Strickland et al., 1990). The protein is composed of a heterodimer consisting of a 85 kDa subunit and a 515 kDa subunit. It is produced as a single polypeptide of 4525 amino acids and is subjected to cleavage by furin in the trans-Golgi compartment. The cleaved subunits remain noncovalently associated with one another. LRP is a multi-functional receptor highly expressed in hepatocytes (Gliemann, 1998; Herz and Strickland, 2001). The functions of LRP are extremely diverse and include roles in lipid metabolism, the homeostasis of proteinases and proteinase inhibitors, cellular entry of viruses and toxins, activation of lysosomal enzymes, cellular signal transduction and neurotransmission (Hussain et al., 1999). Ligands include serpins and A2M, however these are mainly bound following the conformational changes which result from cleavage of the RSL or reaction with small amines respectively (Herz and Strickland, 2001). Different ligands bind specifically to a defined ligand-binding cluster or clusters (Figure 6.2) (Willnow et al., 1994).

The possibility that LRP is up-regulated in SCC derived cells raised the question whether SCCA1 expression in cell lines (via transient transfection) results in enhanced expression of LRP. If this is the case, it is possible that LRP mediates enhanced binding of HBV to cells and internalisation via RME. There is a precedent for a role for LRP in virus binding and entry. LRP (and also the LDL-R) has been implicated as the receptor for the minor group of human rhinoviruses (Hofer et al., 1994). There also have been reports that other members of the LRF family act as receptors for subgroup A Rous sarcoma virus (Bates et al., 1993) and for members of the *Flaviviridae* (hepatitis C virus, GB virus C and bovine viral diarrhoea virus) (Agnello et al., 1999).

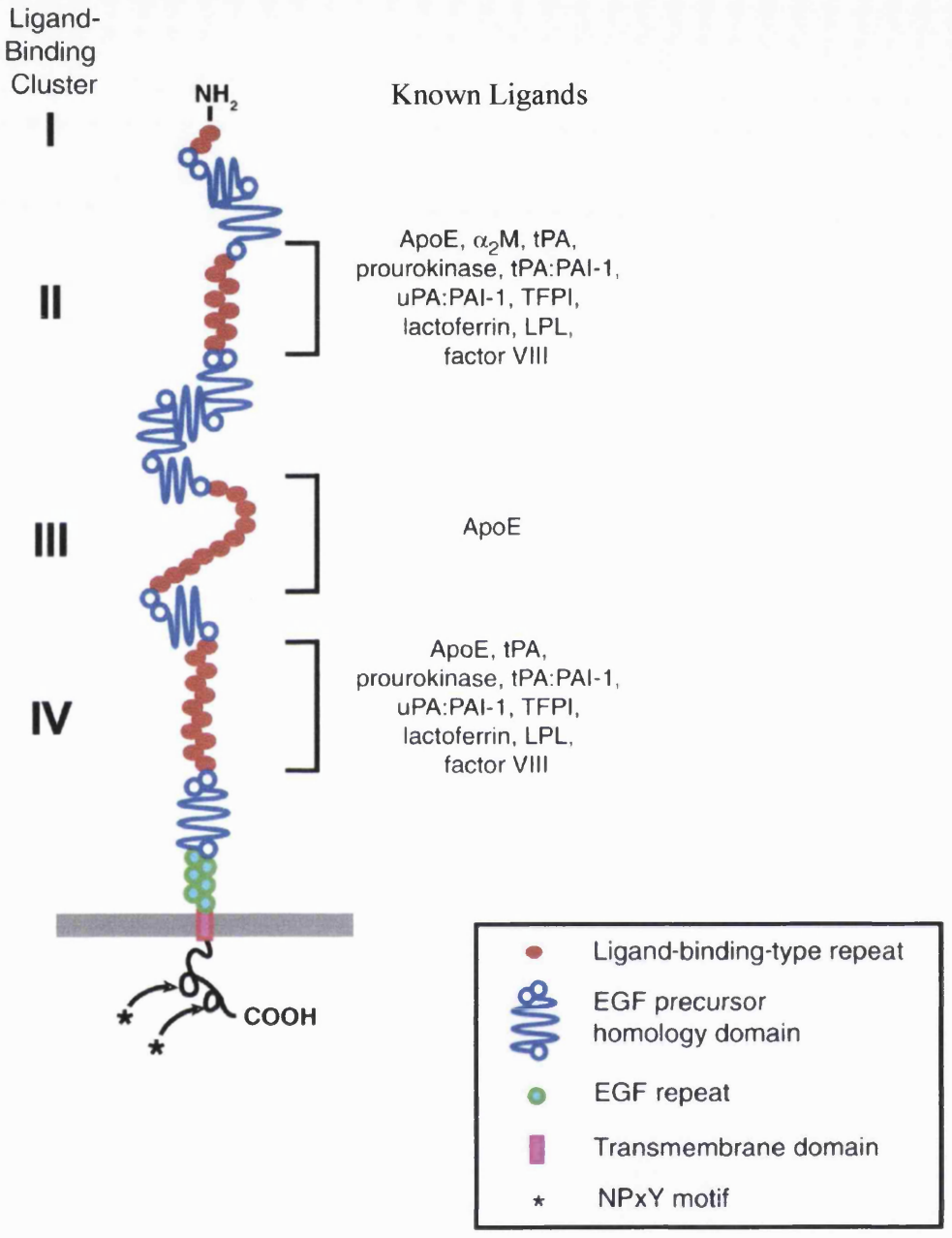


Figure 6.2 Schematic illustrating the structural domains of the LRP. Ligand binding repeats are arranged in clusters, with different ligands binding to specific and separate clusters. RAP (receptor associated protein) binds to all clusters and is a universal competitor for the above ligands (modified from Herz and Strickland, 2001).

6.2 MATERIALS AND METHODS

6.2.1 Quantitative real-time RT-PCR:

Total cellular RNA was purified using the RNAgents® Total RNA Isolation System (Promega) according to the manufacturer's protocol, and quantified by agarose gel electrophoresis and spectrophotometry at 260 nm. Random hexamers were used to prime cDNA synthesis as described in Chapter II.

Quantitative real-time PCR was performed using the Rotor-Gene amplification system (Biogene, Kimbolton, UK). Each 25 µl PCR reaction was prepared as follows:

Component	**Volume**
Water	17.75 µl
dNTPs (2 mM)	1 µl
Forward primer (10 pmol/µl)	0.5 µl
Reverse primer (10 pmol/µl)	0.5 µl
Buffer (10 x) supplied by manafacturer	2.5 µl
Taq polymerase (Qiagen)	0.5 µl
SYBR green (Biogene) 8 U/µl	1.25 µl
cDNA	1 µl
Total	**25 µl**

Primers used for the amplification of human glyceraldehyde 3 phosphate dehydrogenase (GAPDH), which served as a house-keeping gene control, were as follows: GAPDHfor (5' tga tga cat caa gaa ggt ggt gaa g 3') and GAPDHrev (5' tcc ttg gag gcc atg tgg cca t 3'). These primer sequences correspond to bases 840 – 864 (5') and bases 1057-1079 of the human GAPDH cDNA, and the product of PCR is 240 base pairs in length. LRP amplification was performed using the following primers: LRPfor (5' acc gac tgg gaa aca aag tcc 3') and LRPrev (5' gaa gat gcc ggg aat aca gc 3') which results in a PCR product of 555 base pairs. Cycling was

performed as follows: 95°C for 2 min, followed by 45 cycles of 95°C for 25 sec, 60°C for 25 sec and 72°C for 25 sec. A final extension step was performed at 72°C for 1 min. Melt curve analysis was performed by ramping products from 55°C to 99°C acquiring fluorescence readings for each degree change. Relative levels of GAPDH and LRP expression were calculated according the threshold cycle (C_T) value, normalised using the value of the sample with the lowest level of each product, and the data expressed as the ratio of LRP to GAPDH. Specificity of the desired PCR products was determined by melting curve analysis and confirmed by agarose gel electrophoresis and ethidium bromide staining.

6.2.2 Bacterial expression and functional analysis of recombinant RAP

6.2.2.1 Transformation of *E. coli* BL21 with pGEX-KG-RAP

A plasmid encoding a GST-RAP fusion protein, pGEX-KG-RAP, was kindly provided by Prof. J. Herz (University of Texas Southwestern Medical School). This plasmid and a control plasmid, pGEX 4T 3 (Amersham Pharmacia), which encodes GST, were transformed into *E. coli* BL21 as described in Chapter IV. Pilot studies were performed to confirm bacterial expression of the GST-RAP fusion protein and GST control protein in *E. coli* BL21. Transformed *E. coli* colonies were used to inoculate 5 ml cultures of LB containing 100 μg/ml ampicillin (controls comprised untransformed *E. coli* BL21 grown in antibiotic deficient LB). Cultures were incubated at 37°C, 225 rpm for four hours (to ensure that cells were in the exponential phase of growth). Recombinant protein expression was induced by the addition of 100 mM IPTG to a final concentration of 100 nM and induction continued at 30°C, 225 rpm for 90 min. Analysis was performed using pelleted cells (1.5 ml) resuspended in 200 μl SDS-PAGE loading buffer (appendix 1) and resolved on a 12% polyacrylamide gel. Coomassie Blue staining and western blotting (using an anti-V5 antibody) was used to confirm expression of recombinant protein.

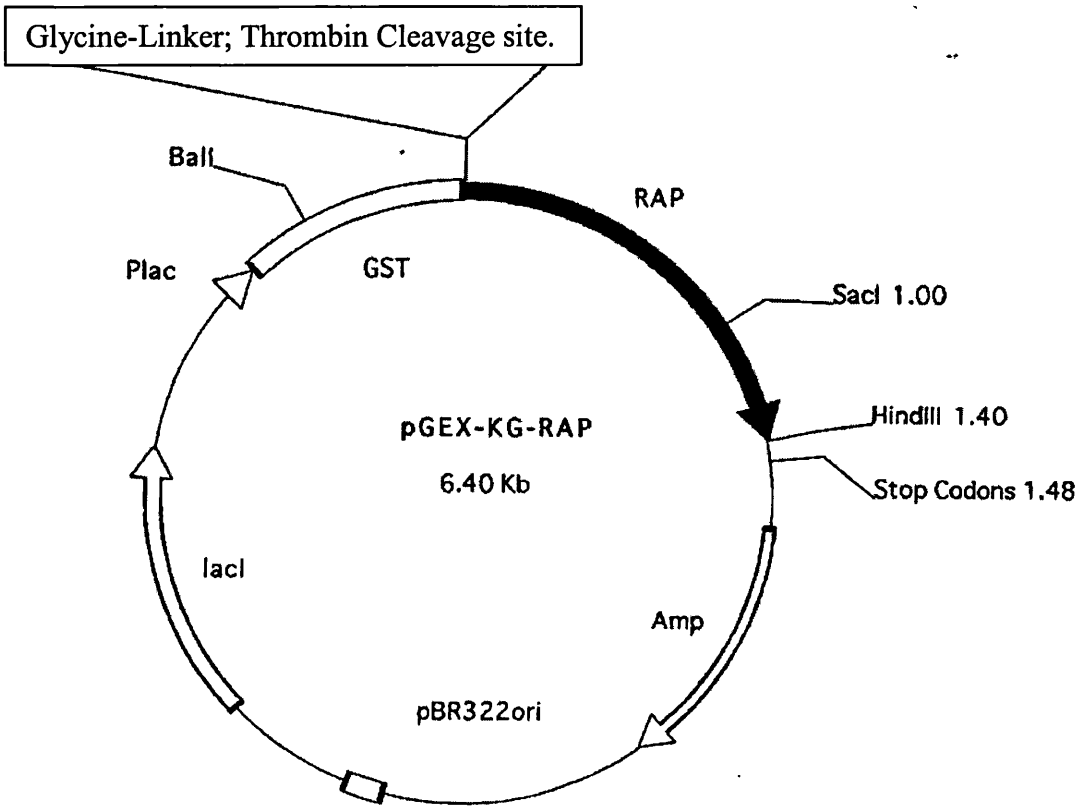


Figure 6.3 pGEX-KG-RAP plasmid circle map (modified from Herz et al., 1991).

6.2.2.2 Batch expression of GST and GST-RAP

A single colony of transformed *E. coli* BL21 cells was inoculated into LB containing ampicillin (100 µg/ml) and incubated overnight at 37°C, 225 rpm. This culture (500 µl) was used to inoculate 100 ml LB containing ampicillin and incubated at 37°C, 225 rpm until the culture reached an OD_{600} of 0.6. Cells were induced to express the fusion protein by the addition of 100 µl of 100 mM IPTG, followed by incubation at 30°C for 3 hours. Bacteria were pelleted at 3000 rpm for 10 min and the pellet stored overnight at -20°C.

6.2.2.3 Purification of GST and GST-RAP

Cells were resuspended in 1 ml of lysis buffer (15 % sucrose, 50 mM Tris HCl, 50 mM EDTA containing 1 mg/ml lysozyme). Samples were incubated on ice for 30 min, then forcibly added to 2 ml water containing 0.2 % triton X-100, 0.5 mM phenylmethylsulfonyl fluoride using a syringe. Tubes were mixed vigorously, then the bacterial lysate was passed sequentially through 23 and 25 guage needles. Bacterial debris was pelleted by centrifugation at 10,000 *g* for 5 min. The supernatant was added to glutathione-agarose (Amersham Pharmacia) which had been equilibrated in PBS. Samples were incubated overnight on a rotator at 4°C. The resin was washed six times with ice-cold PBS, using centrifugation at 3,500 *g* for 5 min. After washing, recombinant proteins were eluted by the addition of 100 µl glutathione elution buffer (50 mM Tris HCl, 10 mM reduced glutathione, pH 8.0) and incubated at room temperature for 5 min before centrifugation at 3,500 *g* for 5 min. The protein-containing supernatant was removed and stored on ice. Contaminating reduced glutathione was removed by washing the protein three times with PBS using Nanosep 10K filters. Recombinant protein was analysed for homogeneity using SDS-PAGE and Coomassie Blue staining. Protein was quantified using the BCA protein quantification system, diluted to 1 mg/ml and stored at -20°C.

6.2.2.4 Activation of alpha 2 macroglobulin with methylamine

Alpha 2 macroglobulin (A2M) was obtained from Sigma, resuspended in PBS to a final concentration of 1 mg/ml, and stored in aliquots at -20°C. A2M was activated, forming A2M*, by incubation with 200 mM methylamine for three hours at room temperature. Activation was confirmed by comparison of activated and non-activated A2M using 4% agarose gel electrophoresis in Tris-glycine buffer (0.25 M Tris HCl pH 8.3, 1.92 M glycine). Protein samples were applied to the gel by mixing with 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water). Electrophoresis was performed at 100 V for 6 hours. The gel was stained with Coomassie Blue as described above for SDS-PAGE. The use of analytical SDS-PAGE was precluded due to the extremely large size of the protein (~ 750,000 Da), and the relatively small size difference between A2M and A2M*.

6.2.2.5 Radiolabelling of A2M* (producing ^{14}C-A2M*)

Alpha-2-macroglobulin (Sigma) was dissolved in PBS (pH 7.4) and stored in aliquots at -20°C. ^{14}C-methylamine hydrochloride (50 mCi/mmol) was obtained from Amersham Pharmacia and dissolved in distilled water at a final concentration of 0.05 μmol/μl. A gluteraldehyde-^{14}C-methylamine hydrochloride conjugate (glut-^{14}C-MA) was formed by incubating equimolar quantities (0.25 μmol) of gluteraldehyde and ^{14}C-methylamine hydrochloride together in PBS (pH 7.4) for 1 hour at room temperature. The glut-^{14}C-MA was then incubated with an excess of A2M (100 μg at a concentration of 1 μg/μl) in PBS (pH 7.4), thereby allowing the free aldehyde group of glut-^{14}C-MA to react with amine residues present on the A2M, resulting in glut-^{14}C-MA-A2M. Reductive amination using sodium cyanoborohydride (1 M in PBS, pH 7.4, freshly prepared, was added to the protein to a final concentration of 0.1 M) was then carried out for 2 hours at room temperature to stabilise the glut-^{14}C-MA-A2M conjugate. The radiolabelled protein (glut-^{14}C-MA-A2M) was washed eight times with 50 mM phosphate buffer (pH 8.0, containing 150 mM NaCl) using a Nanosep 10K centrifugal filter (Pall Filtron). Centrifugation was performed at 5,000 *g* for 4 min. The association of ^{14}C with the retained protein was monitored during the labelling process by performing scintillation counting on the flow-through and material retained by the centrifugal filter during the washing procedure. The A2M was activated by incubation with 200 mM "cold" methylamine as described above, and washed six times to remove unbound methylamine. The final product, glut-^{14}C-MA-A2M* was diluted to 1 mg/ml and analysed using scintillation counting and gel filtration chromatography (described below).

6.2.2.6 Gel filtration chromatography

Gel filtration chromatography was performed using Bio-gel A-1.5M which was equilibrated in PBS. The resin was packed into a 14 cm x 1 cm column. The radiolabelled protein (100 μg at a concentration of 1 μg/μl) was loaded onto the column in 3% glycerol and thirty factions of 500 μl were collected for analysis using scintillation counting and BCA protein assays.

6.2.2.7 Scintillation counting

Scintillation counting was performed by adding 150 µl aliquots of fractions to 2 ml of scintillant (OptiScint "HiSafe", Wallac). Counting was performed for 5 min per sample using a Beckman LS 6500 Multipurpose Scintillation Counter.

6.2.2.8 Confirmation of functional integrity of RAP using ^{14}C-A2M* in cell-binding assays

Competitive binding assays were performed to confirm that recombinant GST-RAP competed with A2M* for cellular binding sites (i.e. cell surface LRP molecules). Hepatocyte-derived Huh7 cells, which are known to express LRP, were seeded into 24 well plates at a density of 4 x 10^5 cells/well. Cultures were incubated overnight at 37°C in 5% CO_2. Cell culture medium was removed and replaced with 1 ml/well DMEM (no serum) containing varying amounts of either rGST-RAP or rGST (negative control). Cells were incubated for 20 min before the addition of 10 µg ^{14}C-A2M*. Cells were incubated for a further 4 hours at 37°C in 5% CO_2, then monolayers were washed three times with PBS and solubilised in 200 µl per well of 0.1 M NaOH. Scintillation counting was performed as described above.

6.3 RESULTS

6.3.1 LRP expression in SCCA1 transfected cells compared with control cells

Total RNA was purified from resected liver tissue (kindly provided by Dr. Clare Selden, RFUCMS), from HepG2 cells, mock transfected Huh7 cells, and Huh7 cells transfected with pCDNA SCCA1. Reverse transcription was performed for each sample and followed by real time PCR using primers specific for GAPDH. The relative amount of GAPDH in each sample was determined using comparative means. Samples were normalised using GAPDH values for subsequent experiments. Expression of LRP in each sample was then analysed using real-time PCR with normalised samples and primers specific for the LRP cDNA.

LRP expression was shown to be reduced in hepatocyte-derived cell lines, both HepG2 and Huh7 cells, compared with resected liver tissue (Figure 6.4). This suggested that the limited capacity of both cell lines for HBV binding and entry may be related to down-regulation of LRP in those cell lines. Huh7 cells transfected with pCDNA SCCA1 (which results in increased binding of HBV to cells) showed substantially higher levels of LRP expression, with an increase of approximately 60% in relative copy numbers from 1.14 ± 0.11 in Huh7 cells to 1.83 ± 0.09 in pCDNA SCCA1 transfected Huh7 cells. The level of LRP expression in transfected Huh7 cells slightly exceeds that seen in total liver tissue. This may be due to the presence in total liver of non-hepatocyte cells which do not produce LRP at levels reported in hepatocytes.

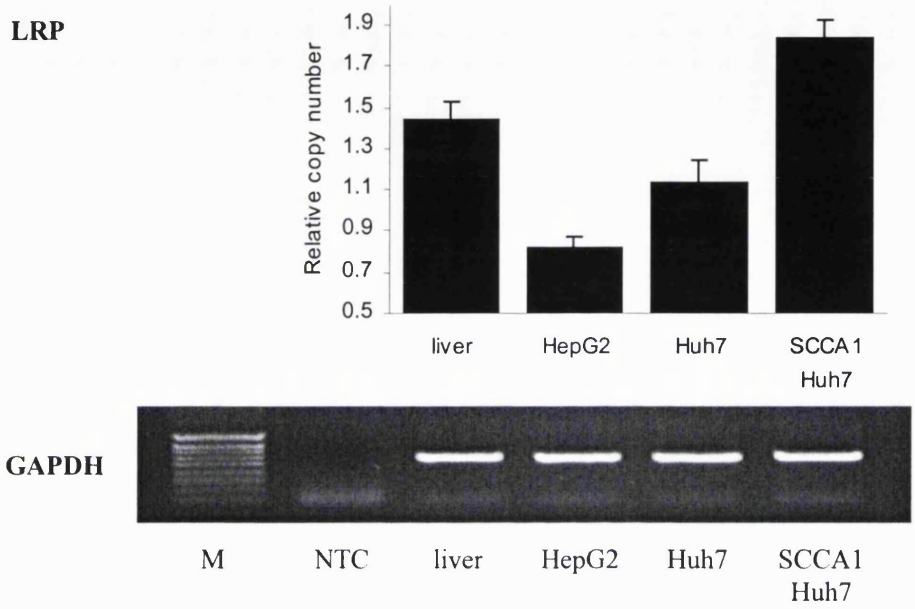


Figure 6.4 Relative expression of LRP in hepatocyte derived cells (HepG2 and Huh7) cells compared with primary liver tissue total RNA and with SCCA1 transfected cells. Samples were normalised using GAPDH (n=5, mean ± standard deviation). Upper panel: Relative copy number for LRP. Lower panel: PCR products for GAPDH.

6.3.2 Receptor Associated Protein

6.3.2.1 Bacterial expression of recombinant GST-RAP and GST

Receptor Associated Protein (RAP) is a 39 kDa protein which was identified initially by the fact that it consistently co-purified with LRP (Ashcom et al., 1990). Herz *et al.* showed that recombinant RAP (as a GST fusion protein) reversibly binds to the large subunit of LRP and also inhibits the binding and uptake of LRP ligands, including VLDL and activated A2M (Herz et al., 1991). RAP is required for the proper folding and export of LRP from the endoplasmic reticulum by preventing the premature binding of co-expressed ligands (Willnow et al., 1996; Li et al., 2002). RAP has been used as a competitive inhibitor in ligand-binding studies involving the expression of LRP (Herz and Strickland, 2001; Willnow et al., 1994) or as a means of reducing the expression of LRP in cells exposed to low levels of recombinant RAP (Webb et al., 1999; Ma et al., 2002).

Recombinant GST-RAP fusion protein (rGST-RAP) and recombinant GST (rGST), which served as a negative control, were produced in a bacterial expression system. Protein purification was performed using affinity chromatography with gluthathione-agarose. Purified protein was analysed for homogeneity using SDS-PAGE and Coomassie Blue staining. Figure 6.5 shows that both rGST-RAP and rGST were highly expressed in transformed induced bacterial cells by comparison with uninduced controls. Purification was effective producing homogenous proteins of the expected sizes of 68 kDa and 31 kDa respectively.

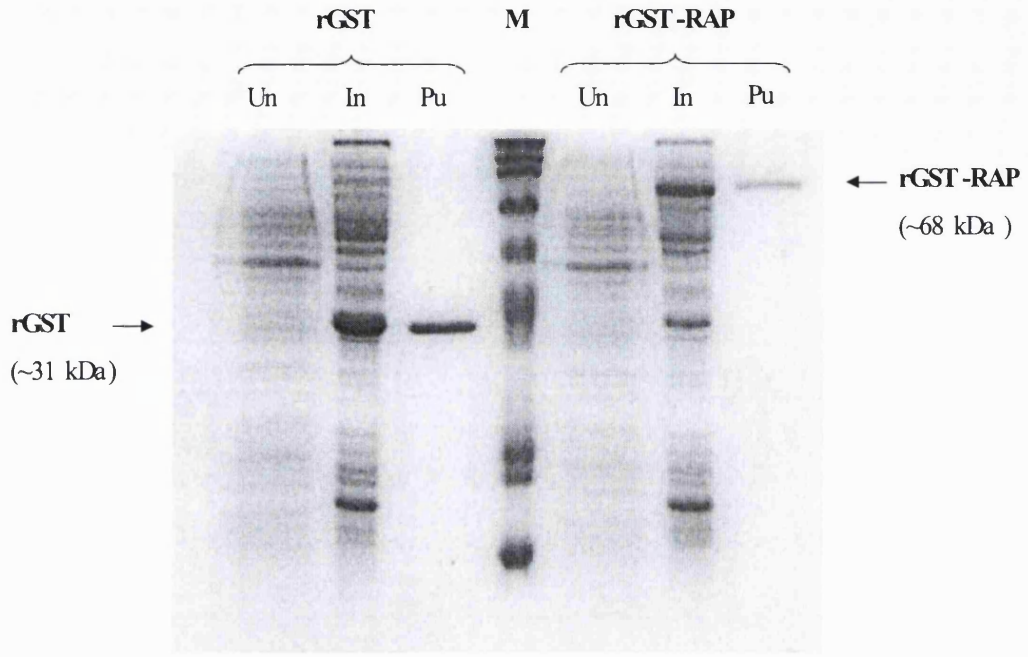


Figure 6.5 Coomassie blue staining of rGST-RAP and rGST following 12% SDS-PAGE. For both rGST and rGST-RAP induced (In) bacterial lysates show high levels of expression of both proteins compared with uninduced cells (Un). Purified (Pu) protein is shown to be a homogenous single protein band of 31 kDa (rGST) or 68 kDa (rGST-RAP).

6.3.2.2 Functional analysis of recombinant RAP

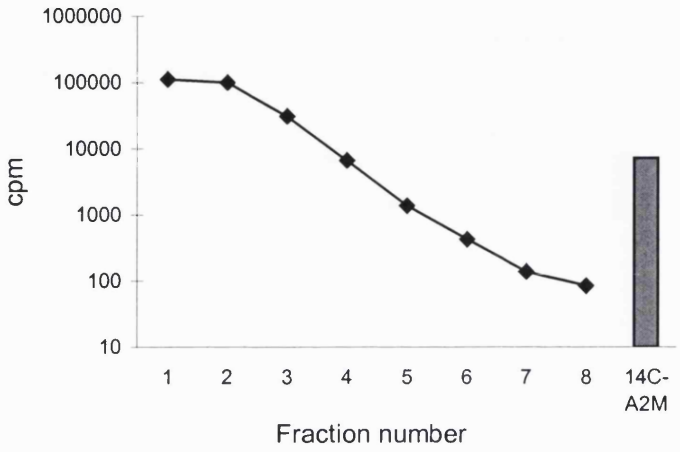
The expression of proteins in bacterial systems is fraught with difficulties largely because of the lack of post-translational modifications which are characteristic of eukaryotic systems (Ramage et al., 2002). Therefore, in order to confirm the functional integrity of the recombinant GST-RAP, competitive binding assays were performed to ensure that rGST-RAP competed efficiently with a ligand known to bind to LRP. Alpha 2 macroglobulin, upon activation, interacts with cluster II of the LRP. A2M exists in two forms described originally as electrophoretically "slow" and "fast" forms (Barrett et al., 1979). The "fast" form corresponds to what is now known as the activated conformation and results from reaction of native A2M with either trypsin (or related proteinases) or with small amines such as methylamine (Herz and Strickland, 2001). This interaction results in the formation of a thiol-ester (-SH) (Sottrup-Jensen et al., 1980; Sottrup-Jensen et al., 1981) formed between a cysteine and glutamic acid in the RSL (Howard, 1981) and a significant change in conformation. The activated A2M, referred to as A2M*, binds effectively to the LRP and is preferentially removed from plasma (Gonias et al., 1983).

Figure 6.6 Agarose gel electrophoresis (4%) followed by Coomassie Blue staining of A2M in the native and activated (A2M*) form. The activated form, A2M* migrates faster through the agarose due to altered conformation resulting from reaction with methylamine (Barrett et al., 1979).

A2M was radiolabelled using a novel procedure based on ^{14}C-methylamine-glutaraldehyde conjugation. Gluteraldehyde was conjugated to ^{14}C-methylamine by making use of the reactive aldehyde groups. This conjugate was then linked to the A2M via the single remaining aldehyde group of the gluteraldehyde. Sodium cyanoborohydride (final concentration of 0.1M), a mild reducing agent, was used to stabilise the glut-^{14}C-MA-A2M conjugate by reducing the imines to secondary amines. The radiolabelled protein was washed thoroughly using a centrifugal filter to remove residual unbound ^{14}C-methylamine. During washing, fractions were assessed for ^{14}C using scintillation counting. A decrease in ^{14}C was observed in the flow-through of successive washes, whereas the protein-containing fraction retained by the filter showed a continued association with ^{14}C after 8 washes (Figure 6.7 A). The labelled protein was reacted with methylamine resulting in conversion of ^{14}C-A2M to ^{14}C-A2M* (Figure 6.6) resulting in increased electrophoretic mobility. The ^{14}C-A2M* was further analysed by gel filtration chromatography.

Fractions were collected and assessed for protein content and the presence of ^{14}C using the BCA protein assay and scintillation counting. Figure 6.7 B shows that both protein and ^{14}C were co-eluted in a single tight peak, indicating a physical association between the two and therefore successful radiolabelling. The ^{14}C-A2M* was used to confirm that the rGST-RAP was functionally active in blocking the binding of A2M* to LRP. Binding assays were performed using Huh7 cells known to express LRP (Figure 6.8). Cells were incubated in the presence of increasing amounts of either rGST-RAP or rGST (negative control). Cells were incubated for 20 min before the addition of ^{14}C-A2M*, then incubated for a further 4 hours before monolayers were washed and solubilised. Cell-associated radioactivity was determined by performing scintillation counting on washed and solubilised cells. Figure 6.8 shows binding of ^{14}C-A2M* to cells and competitive inhibition of that binding in the presence of rGST-RAP (no competition was observed in the presence of increasing amounts of rGST). Therefore, the functional integrity of rGST-RAP was confirmed.

A)



B)

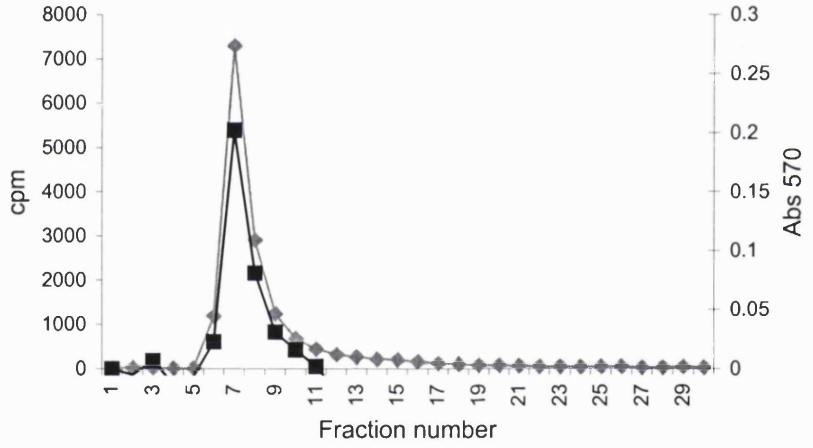


Figure 6.7 (A) Association of radioisotope with protein during sequential washes (wash number versus cpm). Flow-through from sequential washes numbered 1 to 8 (line graph) and the material retained by the centrifugal filter (bar graph), were analysed by scintillation counting. (B) Gel filtration chromatography of radiolabelled A2M* (fraction number versus cpm / Abs 570). Fractions were assayed for protein content (using the BCA protein assay, black line) and ^{14}C by scintillation counting (cpm, grey line).

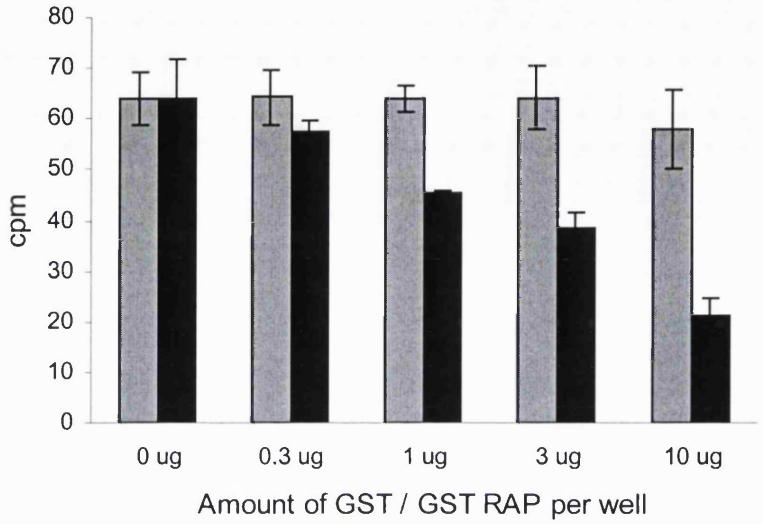


Figure 6.8 Competitive binding assay measuring the cell-associated ^{14}C-A2M* (cpm). Binding assays were performed in the presence of increasing amounts of rGST-RAP (black) or rGST (grey, negative control) ranging from 0.3 μg to 10 μg.

6.3.2.2 Effect of rGST-RAP on SCCA1-mediated virus cell binding

Binding of HBV to SCCA1-transfected cells was investigated as described previously using two approaches. Firstly rGST-RAP was utilised in competitive binding assays in which rGST-RAP (or rGST in control experiments) was added to transiently transfected cells 15 minutes prior to the addition of the virus suspension. Cells were maintained continuously in the presence of the inhibitor until they were harvested and subjected to PCR analysis, Southern blotting and densitometry. The presence of either rGST or rGST-RAP did not affect the level of virus-cell binding in transfected cells compared with transfected cells to which no recombinant protein had been added (Figure 6.9).

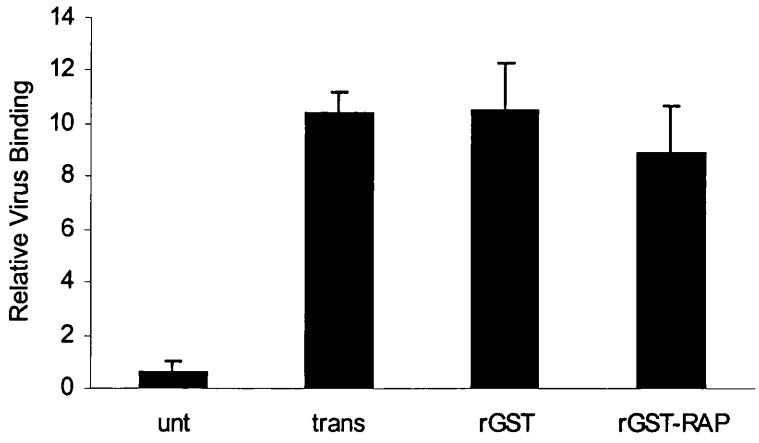


Figure 6.9 Results of competitive binding assay in which virus inoculum was added to transiently transfected cells in the presence of rGST or rGST-RAP (3 μg / well) (n=3, mean ± standard deviation).

An alternative strategy made use of the observation that cells exposed to low levels of recombinant RAP (200 nM) showed reduced expression of LRP (Webb et al., 1999; Ma et al., 2002). Therefore, experiments were performed in which cells were transfected as previously and then maintained in the presence of 200 nM rGST-RAP (or 200 nM rGST) for 48 hours prior to performing cell-virus binding assays. Results of these experiments showed no reduction in virus DNA bound to cells maintained in the presence of rGST-RAP (Figure 6.10).

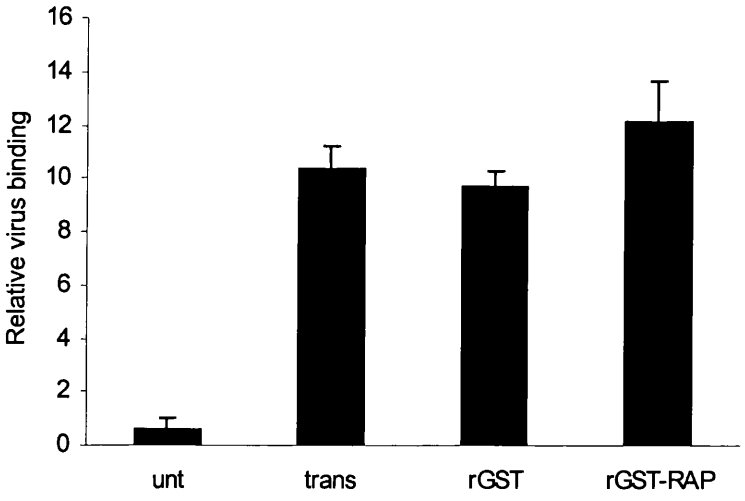


Figure 6.10 The effect of pre-incubation of transfected cells with 200 nM rGST or rGST-RAP on cell-associated virus DNA (n=3, mean ± standard deviation).

6.4 DISCUSSION

The theoretical possibility that LRP plays a role in enhanced binding of HBV to cells is supported by the observations that:

1) LRP is highly expressed by hepatocytes (Moestrup et al., 1992) which are the primary host cells for HBV infection.

2) The hepatic clearance of SECS and, to a lesser extent, unmodified serpins is mediated by LRP (Crisp et al., 2000; Knauer et al., 1997; Kounnas et al., 1996; Orth et al., 1992; Poller et al., 1995).

3) SCC-derived cells which express SCCA1, also express the A2M receptor (LRP) at increased levels compared with a variety of other cell lines and primary tissue (Basu et al., 2001).

The upregulation of LRP reported in SCC derived cell lines was shown to be induced in Huh7 cells which transiently express SCCA1. Transfected Huh7 cells were shown to up-regulate LRP expression by a factor of more than 50%, reaching levels which approximate those observed in total liver. This observation raised questions regarding a possible role for LRP in SCCA1-mediated binding of HBV to cells. Therefore Huh7 cells transiently transfected with SCCA1 were utilised in virus-cell binding assays to assess the effect, if any, of rGST-RAP, which is a well-characterised ligand for LRP, on enhanced virus binding. The use of rGST-RAP, either directly as a competitive ligand or indirectly as a means of suppressing expression of the LRP, resulted consistently in no reduction in levels of cell-associated virus DNA. Therefore, although transient expression of SCCA1 does indeed result in enhanced expression of LRP *in vitro*, this up-regulation is not responsible for enhanced binding of HBV to SCCA1-expressing cells.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

This investigation was initiated by the recent report by De Falco *et al.* (2001b) that SCCA1 may play a role in the infection of host cells by HBV. The purpose of this study was to confirm the findings described in that paper, and subsequently to investigate the mechanism whereby SCCA1 mediates the binding of HBV to host cells. A cDNA for SCCA1 was therefore amplified from HepG2 cells and cloned into expression vectors. Transient transfection of SCCA1 in mammalian cells, whether hepatocyte-derived or not, resulted in enhanced binding of HBV to cells (compared with mock transfected controls). Furthermore, cell-associated virus DNA was shown to have been internalised to a greater extent in SCCA1-transfected cells compared with untransfected cells, indicating internalisation of bound virus particles. Sequence analysis, at the nucleotide and amino acid level, confirmed the identity of the cloned cDNA (and HBV-BP) as SCCA1. HBV-BP was reported to differ slightly from the known sequence of SCCA1, and of particular interest was an amino acid change in the catalytic reactive site loop, Gly351Ala. The cDNA amplified for use in our experiments does not share that change. De Falco *et al.* (2001b) proposed the possibility that the Gly351Ala mutation may be significant in the effect of SCCA1 on virus-cell binding. The results presented here show that this is not the case.

These finding serve as independent corroboration of the data presented by De Falco *et al.* (2001b) suggesting that SCCA1 may be important in the binding and entry of HBV into cells. Therefore, the possible role of SCCA1 as a candidate receptor was further investigated. Although SCCA1 expression certainly results in increased binding of HBV to cells, it was not known whether enhanced binding and entry correlated with further virus replication in transfected cells. In these studies, there has been no direct evidence of virus replicative intermediates in cells transfected with SCCA1. Therefore, it seems that SCCA1 expression confers on transfected cells an enhanced capacity for virus binding and internalisation, but there remains a downstream impediment preventing the virus from undergoing a complete cycle of replication. The observation that SCCA1 expression resulted in greater enhancement of virus binding to hepatocyte-derived Huh7 cells than was observed with Cos7 cells (an effect that was independent of SCCA1 expression levels) supports the hypothesis that more than one receptor is required for virus entry, with the second component of the putative receptor complex being expressed in hepatocyte-derived

cell lines. Future studies should address the question of whether a second receptor (co-receptor) can be identified using non-hepatocyte-derived cell lines which express SCCA1.

The proposed role of SCCA1 as the cellular receptor for HBV is supported by the fact that there is direct binding of the HBV preS1 domain to SCCA1 (De Falco *et al.*, 2001b) and also by the observation that SCCA1 expression in transfected cells results in cell surface localisation of a proportion of the expressed protein. It is therefore theoretically possible that the virus could bind to cell surface SCCA1 and subsequently gain entry via receptor mediated endocytosis. However, a significant argument against that model is the fact, reported in the literature and confirmed in this study, that SCCA1 is not highly expressed in the liver. Although the cDNA clone used in these experiments was derived from a hepatocyte cell line, the level of expression in both the HepG2 and Huh7 cell lines is extremely low (undetectable using northern blots and real-time PCR). By comparison, the levels of expression required for enhanced binding of HBV to transfected cells are relatively high, being easily detectable using single-round RT-PCR amplification.

Further characterisation of the interaction between SCCA1 and HBV made use of recombinant SCCA1. Contrary to the previous report (de Falco *et al.*, 2001b), recombinant SCCA1 which was functionally active (as shown using proteolysis assays) was unable to compete with HBV for binding to cells. Intriguingly, pre-incubation of the virus with recombinant SCCA1 resulted in slight enhancement of virus binding. This finding suggests that SCCA1 may function as a bridge between the virus and the cellular receptor, as discussed in chapter IV. Therefore, although it is clear that SCCA1 mediates enhancement of HBV binding to cells, the mechanism whereby this occurs is not known. The lack of expression of SCCA1 in the liver suggests an indirect role for SCCA1 in virus-cell binding, as do the observations that functional recombinant SCCA1 does not prevent binding of HBV to cells, but instead may even enhance virus-cell binding. A possible explanation for these data is that SCCA1 functions to enhance virus-cell binding indirectly by one of two mechanisms, (1) by acting as a bridge between the virus and the unknown cellular receptor or (2) by secondarily affecting the expression of another molecule which acts as the cellular receptor for the virus.

In developing these models, the biological function and mechanism of action of serpins stimulated an interest in the possibility that the protease inhibitory activity of SCCA1 was important in the mediation of virus-cell binding. The hepatic clearance of serpin-enzyme complexes was intriguing and led to investigations into the possibility that HBV subverts this clearance system in order to gain access to hepatocytes. This hypothesis was investigated firstly by examining the role of the reactive site loop of SCCA1. Therefore, extensive use was made of site-directed mutagenesis to generate variants of SCCA1 that were functionally unable to inhibit proteinases. These variants were tested for their ability to enhance binding of HBV to cells. In no case was abrogation of function of SCCA1 shown to result in decreased virus-cell binding compared with wild type SCCA1.

A second approach to the potential role of the hepatic serpin clearance mechanism in HBV binding and entry investigated the role of the LDL receptor related protein (LRP) in enhanced binding of virus to cells. It was hypothesised that expression of SCCA1 in cells may result in upregulation of LRP expression, and that LRP could act alone, or in concert with SCCA1, to bind to HBV and facilitate entry via receptor mediated endocytosis. Although LRP expression was indeed shown to be enhanced by the transient expression of SCCA1, the use of a known ligand for LRP, the receptor associated protein, in competitive binding assays suggested that the enhanced expression of LRP was not responsible for increased binding of virus to cells.

Although beyond the scope of this project, it may be interesting to identify the region of SCCA1 which interacts with the preS1 domain of the virus. Random mutagenesis of SCCA1 may be useful in addressing this question. It may also be valuable to use SCCA1-expressing cells in order to identify the putative secondary or co-receptor. Preliminary experiments performed during this study attempted to make use of pull-down assays in which HBV and/or SCCA1 were incubated with metabolically labelled cell membranes. The technique was not found to be suitably sensitive to detect labelled proteins bound to HBV and/or SCCA1. The use of more sensitive techniques may be more successful.

Although these results support the possible role of SCCA1 in virus binding and entry, the significance of this interaction *in vivo* is not known. There are certainly examples where mechanisms of cell entry operating *in vitro* are not those utilised for virus entry *in vivo* (Marsh and Bron, 1997; Miller and Hutt-Fletcher, 1992). The *in vivo* significance of SCCA1 can only be addressed by the development of relevant animal models.

It remains possible that virus entry into transfected cells (but the absence of replicative intermediates) is an artificial result of the over-expression of a protein which is not normally highly expressed in the host cells, with virus entry possibly occurring via the endosomal degradation pathway. As discussed in chapter I, there is evidence suggesting that HBV entry into host cells, like that of Epstein Barr virus, is pH-independent but requires endocytosis. In a dual receptor model it is possible that SCCA1 transfection enhances endocytosis and, therefore, virus entry. If the second component of the receptor complex is responsible for modification of the virus resulting in exposure of e.g. a fusion peptide required for escape from endosomes (Marsh and Pelchen-Matthews, 2000), then in the absence of that second receptor the virus may be internalised, but would be unable to escape the endosome and therefore destined for endosomal degradation.

Although the hypothesis that HBV could gain entry to hepatocytes via the serpin clearance system proved to be incorrect, we have confirmed that SCCA1 does indeed play a role in the binding and entry of HBV into hepatocyte-derived cell lines. These findings may be of use in the long-term goal of establishing a cell-culture system in which the HBV can replicate. The development of a cell culture system for HBV and the identification of the cellular receptor remain, therefore, ongoing problems in the investigation of the early events of the HBV replicative cycle.

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APPENDIX I

COMPONENTS OF BUFFERS

Appendix I

GENERAL SOLUTIONS

TE buffer

10 mm	Tris-Cl, pH 7.5
1 mM	EDTA

TNE buffer

10 mm	Tris-Cl, pH 7.5
1 mM	EDTA
150 mM	NaCl

BACTERIAL CULTURES

SOC medium

2g	tryptone
0.55	yeast extract
1 ml	1 M NaCl
1 ml	1 M KCl

Make up to 100 ml with distilled water, and autoclave. Add (sterile) 1ml 2M Mg^{++} (1M $MgCl_2$, 1M $MgSO_4$) and 1ml 2M glucose
pH should be 7.0

Luria-Bertani (LB) Broth

5 g	Bacto-yeast extract
10 g	NaCl
10 g	Bacto-tryptone

Add distilled water to 900 ml, pH to 7.0, then make up to 1000 ml, and autoclave for 20 min.

Luria-Bertani (LB) Agar

Prepare LB broth (above). Add 15 g/l agar before autoclaving. Allow LB agar to cool to 50°C before addition of antibiotics.

Terrific Broth

12 g	Bacto-tryptone
24 g	Bacto-yeast extract
4 ml	glycerol

Make up to 900 ml with distilled water and autoclave. After autoclaving, add 100 ml sterile solution of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4. Store at 4°C until required.

Ampicillin stock solution

Dissolve Ampicillin (sodium salt, Sigma) in distilled water to a final concentration of 100 mg/ml. Filter sterilise and store at -20°C in aliquots of 500 µl.

Appendix I

AGAROSE GEL ELECTROPHORESIS

50X TAE

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA (pH 8.0)

Add distilled water to 1000 ml. Dilute to 1X TAE for use in agarose gel electrophoresis.

SOUTHERN/NORTHERN BLOTTING

20X SSC

175.3 g sodium chloride
88.2 g sodium citrate

Add distilled water to 800 ml, adjust pH to 7.0. Adjust volume to 1000 ml.

50X Denhardt's Solution

5 g Ficoll (Pharmacia)
5 g polyvinylpyrrolidone
5 g bovine serum albumin (faction V) (Sigma)

Add distilled water to 500 ml, and store aliquots of 50 ml at -20°C

Oligonucleotide Labelling Buffer (-dCTP)

TE buffer:

10 mM Tris HCl
1 mM EDTA
pH 7.5

Solution O:

1.25 M Tris HCl, pH 8.0
1.25 M $MgCl_2$

Solution A:

2 ml solution O
36 µl 2-mercaptoethanol
10 µl 100 mM dATP
10 µl 100 mM dGTP
10 µl 100 mM dTTP

Solution B:

Random Hexamers resuspended in TE buffer at 90 OD units/ml.

For 1 ml OLB, add:

0.2 ml solution A
0.5 ml 2 M Hepes, pH 6.6
0.3 ml solution B

Store in aliquots of 10.5 µl at -20°C

Appendix I

Hybridisation buffer
For 50 ml:
24.5 ml distilled water
15 ml 20X SSC (see above)
5 ml Denhardt's solution (see above)
2.5 ml 10% SDS
0.5 ml boiled salmon sperm DNA (10 mg/ml)
Hybridisation solution was heated to 65°C before being placed on membranes.

SDS-PAGE

Acrylamide stock solution
Acrylamide-bisacrylamide (29:1) solution was prepared by adding 29g of acrylamide and 1g bis-acrylamide to 100ml of distilled water, and stirred for 60 minutes. The solution was filtered, and stored at 4°C in the dark.

Stacking gel buffer
1M Tris-Cl pH 6.8. Autoclave and store at 4°C.

Resolving gel buffer
1.5M Tris-Cl pH 8.8. Autoclave and store at 4°C.

10% ammonium persulphate
Made up fresh in distilled water.

10% sodium dodecyl sulphate (SDS)
Dissolve 10g of SDS in 100 ml distilled water

Reservoir buffer
0.25M Tris-Cl pH 8.3, 1.92M glycine, 1%(w/v) sodium dodecyl sulphate (SDS), in distilled water.

PAGE sample buffer
10% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue in 1.25M Tris-Cl pH 6.8.

Gel fixative
30% (v/v) methanol, 10% (v/v) acetic acid in distilled water.

APPENDIX II

SEQUENCE OF THE cDNA FOR SCCA1
GENBANK ACCESSION NUMBER AJ515707

Appendix II

1: AJ515706. Homo sapiens mRNA...[gi:25005271]

```
LOCUS       HSA515706               1203 bp    mRNA    linear   PRI 12-NOV-2002
DEFINITION  Homo sapiens mRNA for squamous cell carcinoma antigen 1 (SERPINB3
            gene).
ACCESSION   AJ515706
VERSION     AJ515706.1  GI:25005271
KEYWORDS    SCCA1 gene; SERPINB3 gene; squamous cell carcinoma antigen 1.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1
  AUTHORS   Moore,P.L., Ong,S. and Harrison,T.J.
  TITLE     Binding of HBV to cells is mediated by SCCA1 but does not require
            the reactive site loop
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1203)
  AUTHORS   Harrison,T.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (12-NOV-2002) Harrison T.J., Medicine, University College
            London, Royal Free Campus, Rowland Hill Street, London, NW3 3PF,
            UNITED KINGDOM
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:9606"
                     /cell_line="HepG2 (hepatocellular carcinoma)"
     gene            1..1203
                     /gene="SERPINB3"
     CDS             31..1203
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                     /product="squamous cell carcinoma antigen 1"
                     /protein_id="CAD56658.1"
                     /db_xref="GI:25005272"
                     /translation="MNSLSEANTKFMFDLSQQFRKSKENNIFYSPISITSALGMVLLG
                     AKNNTAQQIKKVLHFDQVTENTTGKAATYHVDRSGNVHHQFQKLLTEFNKSTDAYELK
                     IATKLFGEKTYLFLQEYLDAIKKFYQTSVESVDFANAPEESRKKINSWVESQTNEKIK
                     NLIPEGNIGSNTTLVLVNAIYFKGQWEKKFNKEDTKEEKFWPNKNTYKSIQMMRQYTS
                     FHFASLEDVQAKVLEIPYKGKDLSMIVLLPNEIDGLQKLEEKLTAEKLMEWTSLQNMR
                     ETRVDLHLPRFKVEESYDLKDTLRTMGMVDIFNGDADLSGMTGSRGLVLSGVLHKAFV
                     EVTEEGAEAAAATAVVAFGSSPTSTNEEFHCNHPFLFFIRQNKTNSILFYGRFSSP"
BASE COUNT      402 a    257 c    258 g    286 t
ORIGIN
        1 ccagcacagt ggcggccgct cgagttcacc atgaattcac tcagtgaagc caacaccaag
       61 ttcatgttcg acctgtccca acagttcaga aaatcaaaag agaacaacat cttctattcc
      121 cctatcagca tcacatcagc attagggatg gtcctcttag gagccaaaaa caacactgca
      181 caacagatta agaaggttct tcactttgat caagtcacag agaacaccac aggaaaagct
      241 gcaacatatc atgttgatag gtcaggaaat gttcatcacc agtttcaaaa gcttctgact
      301 gaattcaaca aatccactga tgcatatgag ctgaagatcg ccaccaagct cttcggagaa
      361 aaaacgtatc tatttttaca ggaatattta gatgccatca agaaatttta ccagaccagt
      421 gtggaatctg ttgattttgc aaatgctcca gaagaaagtc gaaagaagat taactcctgg
      481 gtggaaagtc aaacgaatga aaaaattaaa aacctaattc ctgaaggtaa tattggcagc
      541 aataccacat tggttcttgt gaacgcaatc tatttcaaag ggcagtggga gaagaaattt
      601 aataaagaag atactaaaga ggaaaaattt tggccaaaca agaatacata caagtccata
      661 cagatgatga ggcaatacac atcttttcat tttgcctcgc tggaggatgt acaggccaag
      721 gtcctggaaa taccatacaa aggcaaagat ctaagcatga ttgtgttgct gccaaatgaa
      781 atcgatggtc tccagaagct tgaagagaaa ctcactgctg agaaattgat ggaatggaca
      841 agtttgcaga atatgagaga gacacgtgtc gatttacact tacctcggtt caaagtggaa
      901 gagagctatg acctcaagga cacgttgaga accatgggaa tggtggatat cttcaatggg
      961 gatgcagacc tctcaggcat gaccgggagc cgcggtctcg tgctatctgg agtcctacac
     1021 aaggcctttg tggaggttac agaggaggga gcagaagctg cagctgccac cgctgtagta
     1081 gcattcggat catcacctac ttcaactaat gaagagttcc attgtaatca ccctttccta
     1141 ttcttcataa ggcaaaataa gaccaacagc atcctcttct atggcagatt ctcatccccg
     1201 tag
//
```

Revised: July 5, 2002.

APPENDIX III

PUBLICATIONS AND CONFERENCE SUBMISSIONS ARISING FROM THIS WORK

Appendix III

Papers

1. Moore, P.L., Damelin, L.H. and Harrison, T.J. ^{14}C-Methylamine-Gluteraldehyde Conjugation as an Alternative to Iodination for Protein Labelling. *Biotechniques.* In press.

2. Moore, P.L., Ong, S. and Harrison, T.J. SCCA1-Mediated Binding of Hepatitis B Virus to Hepatocytes Does Not Involve the Hepatic Serpin Clearance System. *Journal of Biological Chemistry.* Submitted.

Conferences

1. Moore, P.L. and Harrison, T. J. SCCA1-mediated HBV binding does not require the reactive site loop. Presented at the Hepatitis Virus Workshop. Society For General Microbiology, 150th Ordinary Meeting, Warwick, UK, April 8-12, 2002.

2. Moore, P.L., Ong, S. and Harrison, T.J. SCCA1-mediated binding of HBV to cells does not require the Reactive Site Loop. Presented at the 2002 Meeting on the Molecular Biology of Hepatitis B Viruses, Pacific Grove, California, USA. September 29 to October 3, 2002.