Identification and characterisation of a novel RNA-binding partner for the US11 protein of HSV-1

By

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Always in my thoughts

Abstract

The US11 protein herpes simplex virus type-1 is a small, highly basic phosphoprotein, which localises to the nucleoli of infected cells. Although this non-essential protein has a number of activities attributed to it, its actual role during infection is unknown. The two major functions that have been associated with US11 are the antagonism of host-mediated translational shut-off and the ability to bind RNA, the latter of which is the focus of this thesis.

To date, US11 has been shown to bind five RNAs; the antisense transcript of the US11 5′ UTR, a truncated transcript of the UL34 gene, termed Δ34, the Rev-response element of human immunodeficiency virus type-1, the Rex-response element of human T-cell lymphotrophic virus type-1 and rRNA derived from the 60S ribosome subunit. The function of the RNA-binding activity of US11 during infection is as yet unclear. The RNAs bound by US11 appear, at least superficially to possess little in common, both in terms of their sequence and the biological influence of US11 on them. US11 may potentially interact with other RNAs in infected cells and the aim of this work was to attempt to test this hypothesis.

Firstly, methods in which US11-RNA complexes could be isolated from infected cell lysates were examined. Using a GST-US11 fusion protein to pull out interacting RNAs from lysates it proved possible to isolate the known binder, Δ34 RNA. Secondly, a reverse transcription-polymerase chain reaction (RT-PCR) method was developed to allow the amplification of sequences pulled out with GST-US11. This lead to the identification of a 585nt sequence that was present in three HSV-1 3′ co-terminal genes, UL12, UL13 and

UL14, which encode alkaline nuclease, a protein kinase and a nuclear protein of unknown function, respectively.

The interaction between this RNA, termed 12/14 RNA, and US11 was examined *in vitro*. The binding was found to be sequence-specific and mediated by the C-terminal domain of US11. US11 bound 12/14 RNA in a multimeric fashion, for which the N-terminal domain was not required. The affinity of the C-terminal domain of US11 for 12/14 RNA is less that that for Δ34 RNA, indicating that these RNAs may be bound in a slightly different fashion. The binding site for US11 was mapped to a 232nt region which encompasses 108nt of the UL12 5′ UTR, 225nt of the 3′ ORF and 7nt of the 3′ UTR of UL13 and lies within the 3′ UTR of UL14. The interaction between this shorter RNA and US11 was examined and the binding found to be dependent on secondary structure.

The influence of US11 on the expression of UL12, UL13 and UL14 was examined in infected cells. In the absence of US11 the levels of UL12 and UL14 remain unchanged, but the expression of UL13 is elevated at early times post-infection. It therefore appears that US11 can down-regulate the expression of the UL13 protein kinase at early times during infection.

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Abbreviations

A	adenine
A	amps
APS	ammonium persulphate
ASFV	African swine fever virus
ARM	arginine-rich RNA binding motif
ATP	adenosine triphosphate
BHK C13	baby hamster kidney cells clone 13
BLV	Bovine leukaemia virus
bp	base pairs
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
Ci	Curie
CAT	chloramphenicol acetyltransferase
CPE	cytopathic effect
cpm	counts per minute
CRV	cell released virus
°C	degrees Celsius
Da	dalton
dATP	2′-deoxyadenosine-5′-triphosphate
dCTP	2′-deoxycytodine-5′-triphosphate

DD	differential display
dGTP	2′-deoxyguanosine-5′-triphosphate
DFC	dense fibrillar component
dH_2O	deionized water
DMEM	Dulbecco's modified eagle madium
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dsRBD	double stranded ribonucleic acid binding domain
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
dTTP	2′-deoxythymodine-5′-triphosphate
E	early
EAP	Epstein-Barr virus encoded RNA-associated protein
EBER	Epstein-Barr virus encoded RNA
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetra-acetic acid
EF-1δ	eukaryotic elongation factor 1δ
EGTA	ethylene glycerol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
EIAV	Equine infectious anemia virus
EIF-2	eukaryotic initiation factor-2
EMSA	electrophoretic gel mobility shift assay

ERBB	electrophoretic gel mobility shift assay RNA-binding buffer
FC	fibrillar centre
FCS	foetal calf serum
FIV	Feline immunodeficiency virus
FMRP	fragile-X mental retardation protein
FMR-2P	fragile-X mental retardation protein related protein-2
G	guanine
g	gram
GADD	growth arrest and DNA damage
GC	granular component
GDP	guanosine diphosphate
GF	growth factor
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour
HeLa	Human epithelial cell line
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid
HIV	Human immunodeficiency virus
hnRNP	heterogeneous nuclear ribonucleoprotein
Hsp	heat shock protein

HSV	Herpes simplex virus
HTLV	Human T-cell lymphotrophic virus type 1
Hve A	herpesvirus entry mediator A
Hve C	herpesvirus entry mediator C
I	inosine
ICP	infected cell protein
ICVR	intron-containing viral RNA
IE	immediate early
Ig	immunoglobulin
IL	interleukin
INF	interferon
IPTG	isopropyl-β-D-thiogalactoside
IRL	internal long repeat
ISR	internal short repeat
k	kilo
kb	kilobase pairs
kd	dissociation constant
KH	hnRNP K homology domain
l	litre
L	late
LB	L-broth
LATs	latency associated transcripts
LPS	lipopolysaccharides

LTR	long terminal repeats
μ	micro
m	milli
M	molar
mAb	monoclonal antibody
MBP	myelin basic protein
MyD	myeloid differentiation primary response
Met	methionine
MI	mock infected
min	minute
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
n	nano
NBCS	new born calf serum
NES	nuclear export signal
NLS	nuclear localisation signal
NoLS	nucleolar localisation signal
NP40	nonidet P40
NPC	nuclear pore complex
nt	nucleotide
ORF	open reading frame
^{32}P	phosphorus-32 radioisotope
PAA	phosphonoacetic acid

pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PA RT-PCR	polyadenylation-dependent reverse transcription-polymerase chain reaction
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
pfu	plaque forming unit
pi	post-infection
Pi	inorganic phosphate
PKR	protein kinase RNA activated
PMSF	phenylmethylsuphonylfluoride
PNK	T4 polynucleotide kinase
poly $(A)^+$/ poly(A)	polyadenylated
PPII-helix	poly-L-proline type-II helix
PRRV	Porcine reproductive and respiratory syndrome virus
RACE	rapid amplification of cDNA ends
RGG	arginine glycine glycine repeats
RNA	ribonucleic acid
RNase	ribonuclease
RNAP II	RNA polymerase II
rpm	revolutions per minute
RRE	Rev-response element

rRNA	ribosomal ribonucleic acid
RSV	Respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcription-polynucleotide chain reaction
s	second
^{35}S	sulphur-35 radioisotope
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel
SI RT-PCR	sequence-independent reverse transcription-polymerase chain reaction
SL	stem-loop
snRNA	small nuclear RNA
snoRNA	small nucleolar ribonucleic acid
ss	single stranded
SV40	Simian virus-40
T	thymine
TEMED	N, N, N', N'-tetramethylene diamine
TdT	terminal deoxynucleotidyl transferase
TRBP	Tar binding protein
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA
TRL	long terminal repeat
TRS	short terminal repeat

U	unique or unit or uracil
UL	long unique
US	short unique
U snRNA	U-rich small nuclear RNA
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volts
vhs	virion host shutoff protein
VZV	Varicella Zoster virus
v/v	volume/volume
wt	wild type
w/v	weight/volume
XRE	Rex-response element

Amino acids

Name	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Aspargine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Proline	Pro	P
Phenylalaine	Phe	F
Serine	Ser	S
Threonine	Thr	T
Tyrosine	Tyr	Y

Tryptophan	Trp	W
Valine	Val	V

1.0 Introduction

1.1 The *Herpesviridae*

The *Herpesviridae* are a family of enveloped viruses with a double stranded, linear DNA genome that infect a wide range of vertebrates and at least one invertebrate (Comps & Cochennec, 1993; Roizman & Sears, 1993). Over 112 herpesviruses have been isolated to date. This family of highly successful viruses varies considerably in size, structure, pathology and biology and are highly host specific, although more than one herpesvirus can infect an individual organism. Herpesviruses share a set of 'core' genes in particular those involved in nucleotide metabolism, DNA replication, capsid structure and virion maturation, indicating that they share a common progenitor. The 'non-core' genes are more specific to the virus subfamilies and include genes involved in latency, immune evasion and pathogenesis.

A trait common to all herpesviruses is the ability to assume a non-replicative, latent state and persist within the host after primary infection (Whitley, 1996). This enables the virus to evade immune clearance and reactivation from latency can lead to recurrent rounds of disease.

1.1.1 Classification

The *Herpesviridae* can be divided in to three subclasses, *Alpha-* (α), *Beta-* (β) , and *Gammaherpesvirinea* (γ), based on their biological properties (Roizman *et al,* 1981) and more recently with sequencing of many herpesviruses, the genetic content, which in general, agrees with the original classification. *Alphaherpesviruses* have a wide host

range in cell culture, spread rapidly, have a short growth cycle, destroy the host cell and can establish a latent infection in sensory ganglia. *Betaherpesviruses* have a narrow host range, a slow growth cycle and slow viral spread in tissue culture and establish a latent infection in secretory glands and lymphoreticular cells. *Gammaherpesviruses* infect lymphocytes *in vivo*. In tissue culture they generally grow in lymphoblastoid cell lines, but some can also grow in epithelial and fibroblastoid cell lines (Roizman & Sears, 1996).

1.1.2 Morphology

The classification of viruses to the *Herpesviridae* family is primarily based on virion morphology (Dargan, 1986; Rixon, 1993). Virions are composed of four distinct structures: the DNA core, the capsid, the tegument and the envelope (Fig. 1.1). The DNA core consists of the linear virus genome packaged at high density without any proteinacious components, occupying the entire capsid volume. The capsid of HSV-1 is approximately 125nm in diameter and is composed of 162 capsomers, of which 150 are hexamers (hexons), 12 pentamers (pentons), and 320 triplexes (Wildy *et al.*, 1960; Schrag *et al.*, 1989; Zhou *et al.*, 1994) (Fig. 1.2). The tegument, an amorphous proteinaciuos layer, lies between the nucleocapsid and the envelope. The tegument is made up of at least 18 proteins, the functions of which are diverse and include viral gene transactivation, uncoating, modifying the intracellular environment and regulatory proteins. The envelope, derived from the host cell membranes, is studded with viral glycoproteins (Wildy & Watson, 1963).

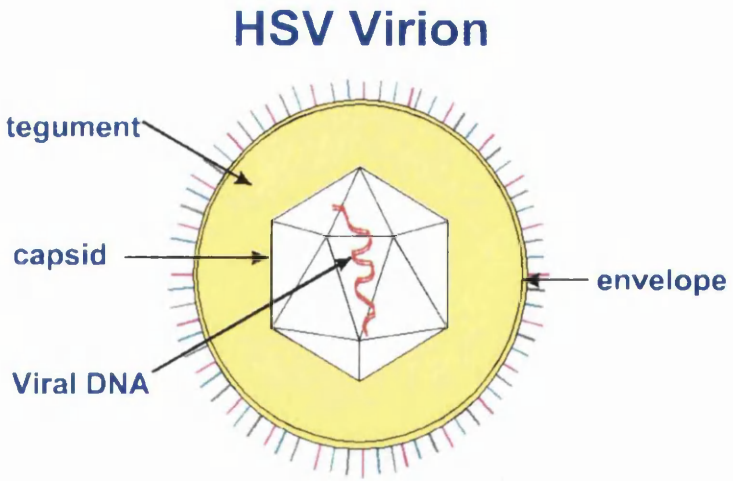


Fig. 1.1: Schematic representation of the HSV-1 virion.
Reproduced from Clements & Brown, 1997.

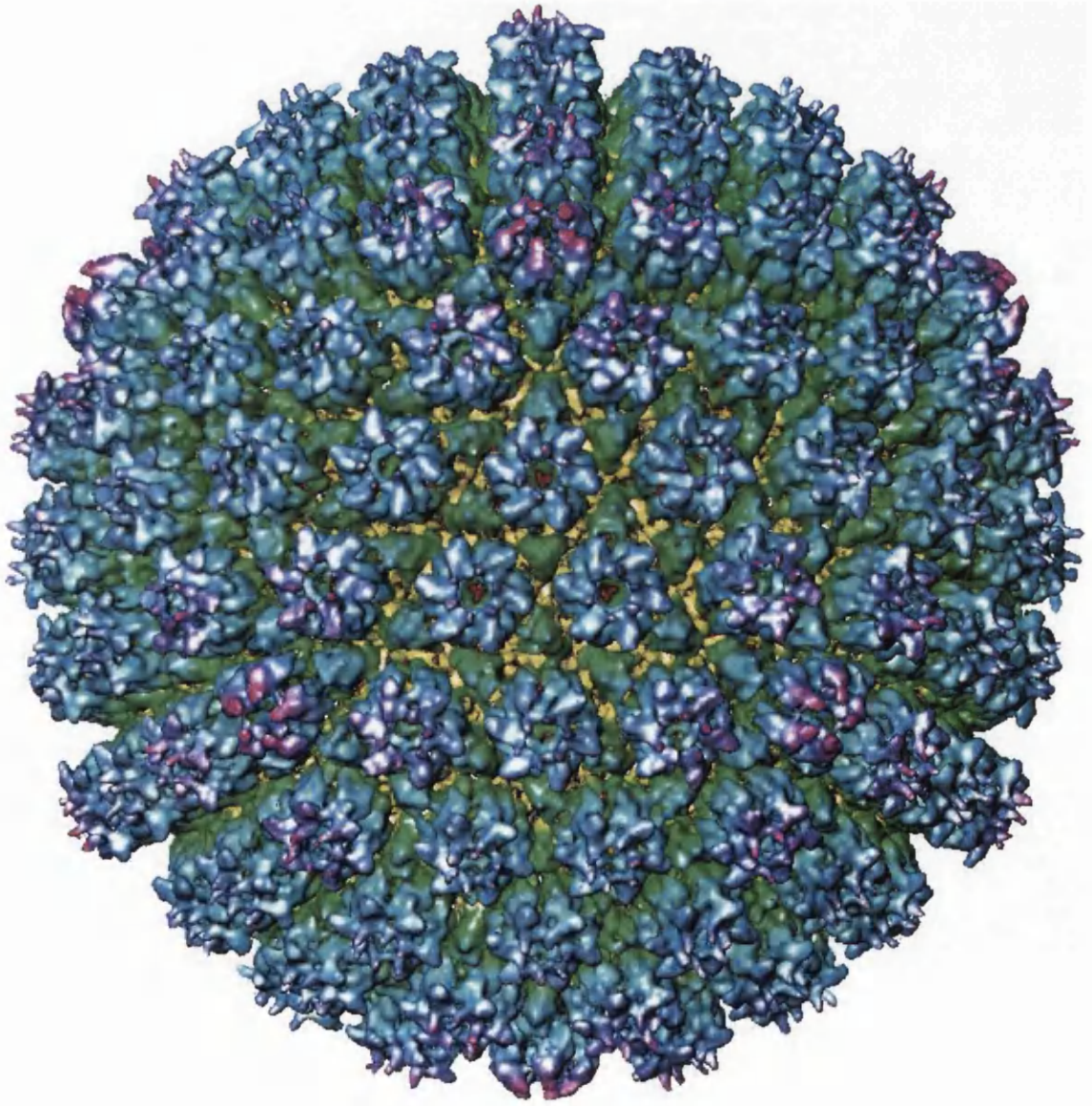


Fig. 1.2: Reconstruction of HSV-1 B capsid. Reproduced from Zhou *et al.*, 1995.

1.1.3 Human Herpesviruses

There are eight herpesviruses known to infect humans (Table 1.1). They cause a wide variety of disease states ranging from minor lesions to viral encephalitis (Whitley & Schlitt, 1991). As is the case with all herpesviruses, after primary infection a life long latent infection is established from which the virus can reactivate. Herpesvirus infection in immunocompromised individuals is of particular medical significance (Cotte *et al.*, 1993).

1.2 HSV-1

1.2.1 Clinical features of HSV-1

The clinical manifestation of HSV-1 is predominantly associated with oral lesions (cold sores) that last 4-7 days. However, 30-50% of genital herpes lesions are caused by HSV-1 (Kinghorn, 1993). Rarely, HSV-1 infection can lead to more serious diseases such as rhinitis, Herpetic whitlow, primary herpes dermatitis, eczema, herpeticulum, keratoconjunctivitis and encephalitis (Whitley, 1996).

1.2.2 Genomic arrangement of HSV-1

The genome of HSV-1 is 152kb in size and encodes over 80 polypeptides (McGeoch *et al.*, 1985, 1986, 1988). It consists of two covalently linked unique regions, the unique long (U_L) and unique short (U_S) (Fig. 1.3). The U_L region is 107.9kb long and encodes

Herpesvirus	Class	Associated illness
Herpes simplex virus type-1 (HSV-1)	α	Causes 80-95% of oral lesions (cold sores). 30-50% of genital lesions are caused by HSV-1. Rarely: conjunctivitis, Herpetic whitlow, keratitis, and encephalitis
Herpes simplex virus type-2 (HSV-2)	α	Causes 5-20% of oral lesions (cold sores). 50-70% of genital lesions are caused by HSV-2. Rarely: conjunctivitis, Herpetic whitlow, keratitis, and encephalitis. Maternally transmitted neonatal infections are generally life threatening.
Varicella zoster virus (VZV)	α	Primary infection: chicken pox (rash) appears 14-15 days post-infection, accompanied by fever. Reactivation: shingles; lesions appear at the relevant dermatone often accompanied by extreme pain which can last for months after lesions have healed.
Epstein Barr virus (EBV)	γ	Primary infection in children is often asymptomatic. Primary infection in older children and adults associated with infectious mononucleosis (glandular fever). Associated with Burkitt's lymphoma and nasopharngeal carcinoma.
Human cytomegalo-virus (HCMV)	β	Primary infection: enlargement and fusion of macrophages. Often asymptomatic though can be fatal especially in new borns. Infection is problematic in immunocompromised individuals where symptoms include gastro-enteritis and retinitis.
Human herpesvirus 6 (HHV-6)	β	Infant rash, exanthem subitum.
Human herpesvirus 7 (HHV-7)	β	Febrile illness.
Kaposi's sarcoma associated herpesvirus (KSHV)	γ	Associated with Kaposi's sarcoma, a vasculated nodular skin lesion.

Table 1.1: Human herpesviruses.
The eight herpesviruses known to infect humans, classification and the diseases associated with them.

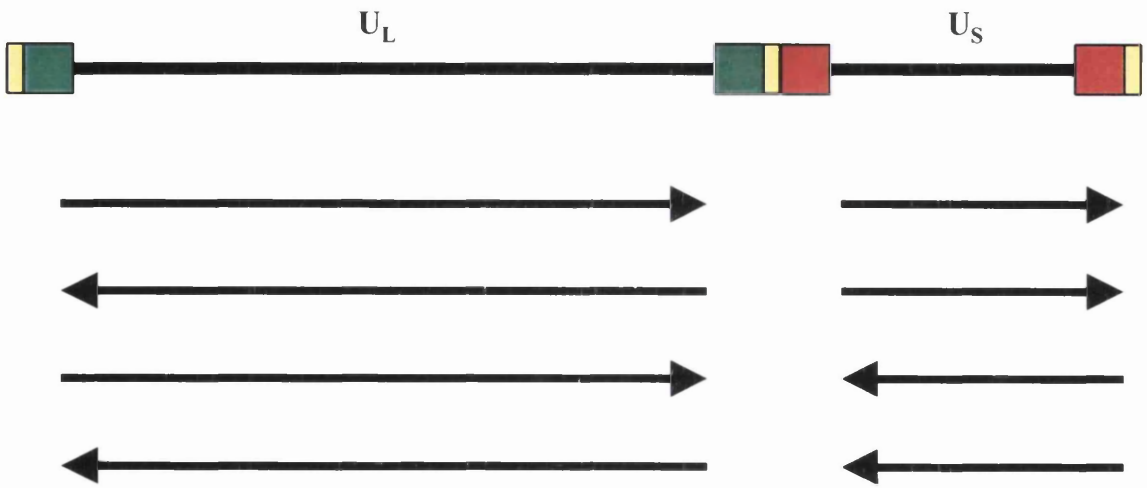


Fig. 1.3: Schematic diagram of the HSV-1 genome.
The unique DNA elements, U_L and U_S are covalently linked and flanked by inverted repeats (green and red rectangles). The *a* sequence is represented by a yellow rectangle and is present at each end of the genome and in an inverted orientation at the L-S junction. The L and S regions can invert relative to each other forming four isomers (represented by the arrows) (Hayward *et al.*, 1975).

at least 59 genes. The U_S region is 13kb and encodes approximately 13 genes. The unique sequences are flanked by inverted repeats, R_L (9kb) and R_S (6.5kb) (McGeoch *et al.*, 1988). The R_L and R_S are not related in sequence apart from the *a* sequence (400bp) located at the genome termini. One copy of the *a* sequence is located at the S terminus, whereas the L can terminate with more than one copy. The *a* sequence is also present at the L-S junction as an inverted repeat and again can be present at more than one copy (Wagner & Summers, 1978).

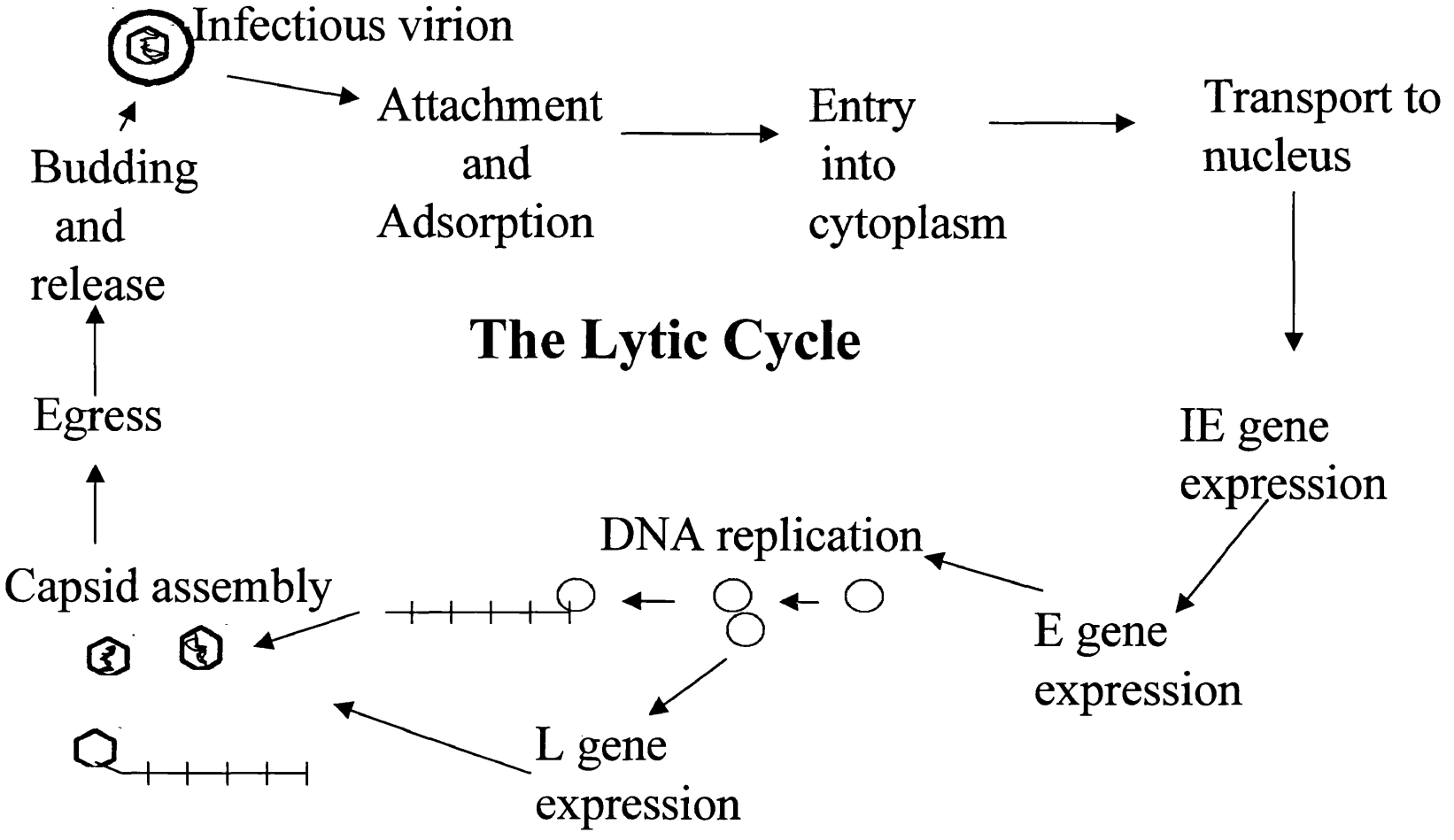
1.2.3 The lytic cycle

The lytic cycle is the process whereby the virus enters a cell, replicates and releases progeny virions into the surrounding medium (Roizman & Sears, 1996). In essence the lytic cycle consists of virus attachment, entry, gene transcription, DNA replication, capsid assembly, virion maturation and egress, resulting in the death of the host cell (Fig. 1.4).

1.2.3.1 Attachment and adsorption

The attachment of the HSV-1 virion to the host cell membrane is mediated by the interaction between the viral glycoproteins and cell surface receptors. HSV-1 virions have been shown to bind highly sulphated glycosamine glycans such as heparin sulphate and dextran sulphate via gC (and probably gB), mannose-6-phosphate receptors and the complement receptor C3b (Spear, 1993; Shieh & Spear, 1994). Fusion of the virion membrane with the cellular membrane is a pH-independent process that requires gB, gD, gH and gL (Spear, 1993).

Fig.1.4: A Schematic diagram of the lytic cycle.
Attachment and adsorption is mediated by interactions between the host cell membrane components and the virion glycoproteins. The capsid is transported to the NPC and the DNA released into the nucleoplasm.The viral genes are transcribed in a temporal cascade. Viral DNA is replicated initially through a theta replication mechanism, followed by the rolling circle mechanism. The viral DNA is packaged into the capsid in the nucleus. The virion is thought to mature and egress through the secretory pathway and is released into the surrounding medium.


Infectious virion
Attachment and Adsorption
Entry into cytoplasm
Transport to nucleus
IE gene expression
E gene expression
L gene expression
DNA replication
Capsid assembly
Egress
Budding and release
The Lytic Cycle

A number of cell surface receptors are involved in initiating fusion, tumour necrosis factor-like molecule (or herpesvirus entry mediator (HveA)) interacts with gD (Montgomery *et al.*, 1996). Poliovirus receptor-related protein (HveC) is thought to be a major mediator of entry via an interaction with gD in mucosal and neuronal infection (Gertaghty *et al.*, 1998). The abundance of virion-cell surface interactions indicates that HSV-1 is likely to utilize more than one attachment/entry pathway.

1.2.3.2 Nucleocytoplasmic transport

The nucleocapsid traverses the cytoplasm, shedding tegument components, transported via the microtubule network and a dynein-like motor (Penfold *et al.*, 1994; Sodeik *et al.*, 1997). The DNA is delivered to the nuclear pore complex (NPC). Empty capsids can be visualised closely associated with NPCs (Batterson *et al.*, 1983).

1.2.3.3 Gene expression

HSV-1 genes are transcribed by the host cell DNA-dependent RNA polymerase II (RNAP II) (Costanzo *et al.*, 1977). Viral mRNAs are post-transcriptionally modified (capping, cap methylation and polyadenylation) by existing cellular pathways. However, as only five of the HSV-1 genes contain introns, splicing is not generally required. HSV-1 genes are transcribed in a temporal cascade, with the induction of one class directly or indirectly leading to the induction of the next (Honess & Roizman, 1974; Honess & Roizman, 1975; Clements *et al.*, 1977). Three kinetic classes can be identified, the immediate early (IE) or α genes, the early (E) or β genes, and the late (L) or γ genes.

i. Immediate Early gene transcription

The expression of immediate early genes occurs without *de novo* viral protein synthesis. The virion-borne transactivator, α-TIF (VP16), binds to TAATGARAT sequences upstream of IE promoters, complexing with the host transcription factors Oct-1 and HCF and recruiting the RNAP II holoenzyme (Contanzo *et al.*, 1977; Batterson & Roizman, 1983; O'Hare *et al.* 1988; O'Hare, 1993). Four of the five IE gene products, ICP27, ICP22, ICP4 and ICP0 are responsible for the regulation and expression of the E and L genes (reviewed by Hayward, 1993; Subak-Sharp & Dargan, 1998). The fifth, ICP47, inhibits the presentation of antigenic peptides by MHC I molecules (Jugovic *et al.*, 1998).

ii. Early gene expression

In general, E genes encode proteins involved in nucleotide metabolism and DNA replication. The promoter regions of E genes are composed of a TATA box, a cap site and upstream sequences required for the binding sites for cellular factors such as SP1 (Jones & Tijan, 1985). No upstream sequences specific for viral proteins have been detected, though IE gene products may be involved in the stabilization of cellular transcription factor binding or aid their recruitment.

iii. Late gene expression

Late genes can be subdivided into two categories, leaky late or γ_1 genes and true late or γ_2 genes. Leaky late genes are expressed at low levels prior to DNA replication whereas, the expression of true late genes is dependent on DNA replication (Honess & Watson, 1977). In general, late promoters consist of a TATA box and cap site (Johnson & Everett, 1986b; Homa *et al.*, 1988). In some, notably the true late genes encoding gC and US11, a downstream activator sequence (DAS), located 20-40bp downstream of the TATA box is required for expression (Shapira *et al.*, 1987; Kibler, *et al.*, 1991; Guzowski *et al.,* 1994).

Two models for the requirement of DNA expression for late gene expression have been proposed. The first suggests factors that repress transcription are bound to the promoters of late genes and are displaced by the passage of the replication fork. The second hypothesis states that late genes possess weak promoters, and the amplification of the DNA template is required to produce significant levels of expression.

1.2.3.4 Rendering the host cell permissive to support the viral life cycle

HSV-1 infection leads to the redirection of cellular factors and metabolism to the massive task of producing large amounts of infectious virions. Upon infection, HSV-1 down-regulates host cell DNA synthesis, translation and splicing, in favour of the production of its own macromolecules. Numerous cellular components and pathways are modified during HSV-1 infection, and two of these events are discussed here.

The first phase of this assault is initiated by the virion host shut-off (*vhs*) protein which is delivered in the tegument. *vhs*, which displays a limited homology with the fen-1 nuclease family, causes the destabilization and degradation of mRNA (Kwong *et al.*, 1988).

The IE gene product ICP27 causes the redistribution of splicing factors and the inhibition of splicing (Hardwicke & Sandri-Goldin, 1994; Phelan *et al.*, 1996; Bryant *et al.*, 2001). Most cellular genes contain introns, compared with only five genes of HSV-1. The activity of ICP27, therefore, results in the nuclear retention of cellular genes.

1.2.3.5 HSV-1 DNA replication

Expression of the E gene products leads to the onset of DNA replication. HSV-1 encodes seven genes directly involved in DNA replication and several accessory enzymes required for nucleotide metabolism (reviewed by Lehman & Boehmer, 1999). Viral DNA is thought to be replicated initially by theta replication, followed by rolling circle replication to produced concatameric DNA molecules (Roizman & Sears, 1996). Replication begins with the binding of one of the three origins of replication (Ori) by the UL9 Ori binding protein (Olivo *et al.*, 1988). This leads to the recruitment of the helicase/primase complex (UL5, UL8, and UL52), to form a replication bubble and the binding of DNA polymerase (UL30 and UL42) (Crute *et al.*, 1989). The replication fork proceeds with the continuous synthesis of DNA on one strand and the discontinuous synthesis on the other, and is maintained by the single strand specific DNA binding protein, UL29 (Knopf, 2000).

1.2.3.6 Capsid assembly

With the onset of DNA replication and late gene expression, all the components necessary for the production of mature virions are in place. Capsid assembly begins in the cytoplasm and is completed within the nuclear compartment. VP5 interacts with pre-VP22a and VP26, and VP19C interacts with VP23 in the cytoplasm (Homa & Brown, 1997). It is thought that the nuclear localisation signals contained in pre-VP22a and VP19C mediate the nuclear import of these structural complexes. The spherical pro-capsid is assembled via protein-protein interactions between the viral structural and scaffold proteins (Newcomb *et al.*, 2001). The viral DNA is packaged and cleaved into genome length units directed by signals in the terminal *a* sequence (Stow *et al.*, 1983). VP22a is expelled and simultaneously the capsid undergoes a structural transition to an icosahedral shape (Rixon, 1993).

Three forms of capsid are found inside infected cells, A-, B- and C- capsids which can be resolved via sucrose density gradient ultracentrifugation (Gibson & Roizman, 1972). C-capsids contain viral DNA and are able to form mature infectious virions. B-capsids, the precursor of C-capsids, do not contain DNA. The core of B-capsids is occupied by VP22a and can package viral DNA leading to the extrusion of VP22a. A-capsids, however, do not contain DNA or VP22a and are thought to be the result of abortive packaging attempts. A-capsids cannot form infectious particles.

1.2.3.7 Virion maturation and viral egress

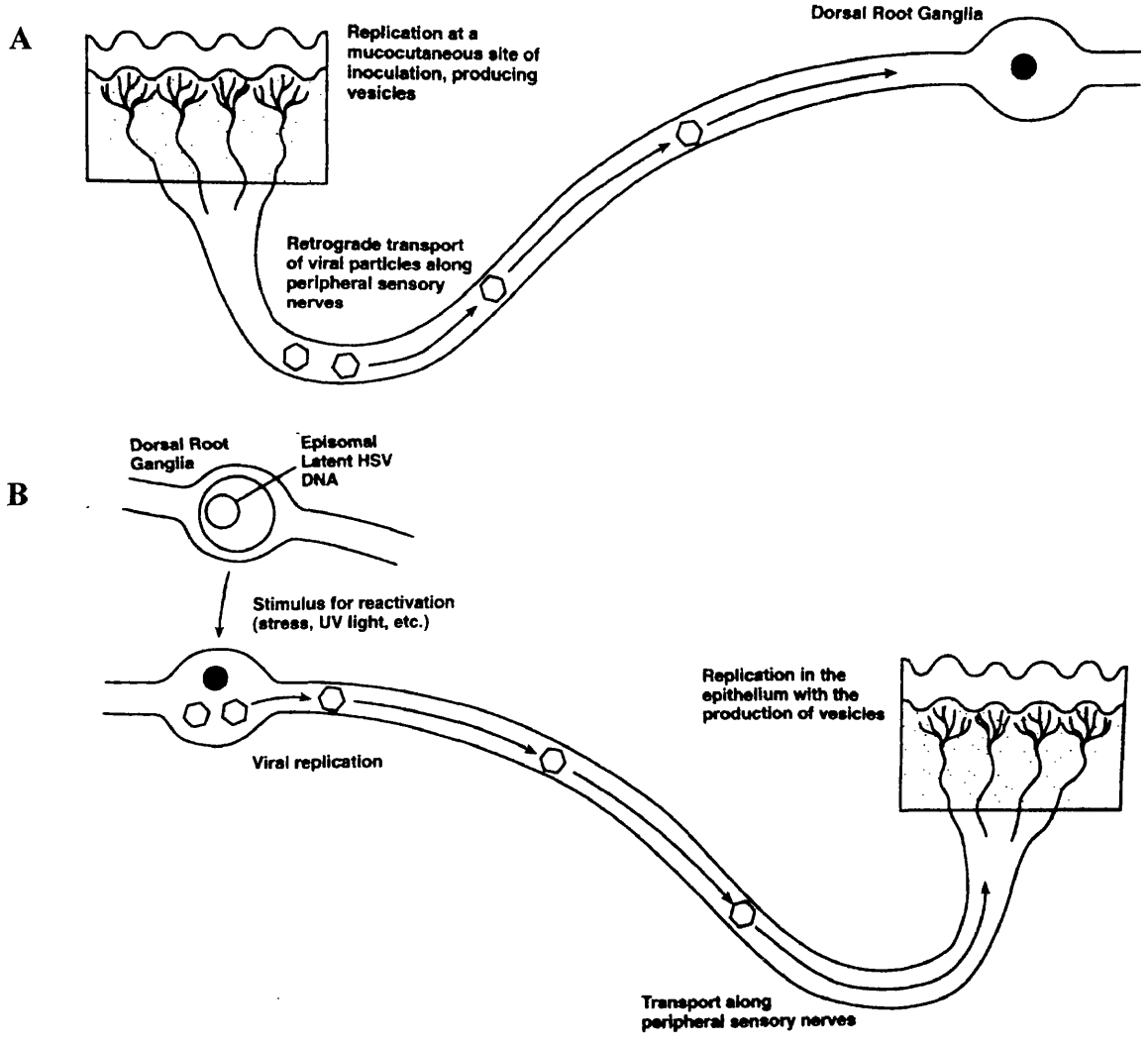
The capsid buds through the inner nuclear membrane and into the perinuclear space, acquiring an envelope (Granzow *et al.*, 2001). The next stage in virion maturation/egress is a matter of some debate. The most recent evidence suggests that de-envelopment occurs as the capsid buds through the outer nuclear membrane, followed by re-envelopment in a post-Golgi compartment (Browne *et al.*, 1996; Skepper *et al.*, 2001). The virion is thought to exit the cell via the secretory pathway (Rixon, 1993).

1.2.4 HSV-1 Latency

All herpesviruses can assume a biologically silent state in specific cell types. After initial virus amplification at a primary oral mucosal site of infection, HSV-1 enters sensory neurones innervating that site and are transported via retrograde axonal transport to the trigemminal and dorsal root ganglia (Kristensson *et al.*, 1986; recently reviewed by Preston, 2000) (Fig. 1.5). Here the virus assumes a latent state as an episome (Mellerick & Fraser, 1987).

During latency the only transcriptionally active region of the genome is a 10.4kb fragment within the R_L/R_S component, giving rise to the latency associated transcripts (LATs) (Spivack & Fraser, 1988; Stevens *et al.*, 1988). Although not required for the establishment and maintenance of latency, they may be involved in the reactivation process (Sedarati *et al.*, 1989; Javier *et al.*, 1988). Reactivation from latency is poorly

Fig. 1.5: Establishment, maintenance and reactivation from latency.
After replication at a mucosal site, HSV-1enters sensory neurons where it is transported to the dorsal root ganglia. Here it resides in a latent state (A). Upon reactivation the virus replicates and is transported to the mucosal site where it replicates to produce infectious virions (B). Reproduced from Whitley (1996).


A
Replication at a mucocutaneous site of inoculation, producing vesicles
Dorsal Root Ganglia
Retrograde transport of viral particles along peripheral sensory nerves
B
Dorsal Root Ganglia
Episomal Latent HSV DNA
Stimulus for reactivation (stress, UV light, etc.)
Viral replication
Replication in the epithelium with the production of vesicles
Transport along peripheral sensory nerves

understood. It can be triggered by external stress or stimuli, such as UV-irradiation and hormonal irregularities (Hill, 1985).

1.3 The US11 protein of HSV-1

The US11 protein of HSV-1 is a small basic phosphoprotein that localizes to the nucleolus of infected cells. The US11 gene is conserved only in HSV-1 and HSV-2. US11 is non-essential for virus growth *in vitro* and *in vivo* and the function of US11 during the HSV-1 lytic cycle is unclear. The major properties associated with US11 are RNA-binding and the antagonism of the interferon-induced, double-stranded RNA-dependent protein kinase (PKR) pathway.

1.3.1 US11: a non-essential gene

The US11 gene is located in the U_S region of HSV-1 the genome (Marsden *et al.*, 1978; Honess *et al.*, 1980). Deletion of US11 itself does not appear to have an impact on the growth, establishment of latency, or reactivation of the virus *in vivo* or *in vitro* models (Longnecker & Roizman, 1986; Brown & Harland, 1987; Longnecker & Roizman, 1987; Meignier *et al.*; 1988; Nishiyama *et al.* 1993).

1.3.2 The US11 gene and evolution

The US11 gene is a true late gene, its expression being dependent on DNA replication (Johnson *et al.*, 1986; Johnson & Everett, 1986a & b). US11 mRNA is transcribed from the leftward strand of the genome and its mRNA is 3′ co-terminal with US12 and US10,

sharing a common polyadenylation signal (Fig. 1.6). US11 is a 161 amino acid protein encoded by a 483bp ORF, lying between nucleotide positions 145246 and 144763 (Rixon & McGeoch, 1984, 1985; McGeoch *et al.*, 1986). The US11 TATA box and transcriptional start sites lie within the ORF of US12. Its first Met-ATG lies within the 3′ UTR of US12 and its stop codon within the ORF of US10. The TATA box, transcriptional start site and first Met-ATG of US10 lies within the ORF of US11. The overlap between the ORF of US10 and that of US11 spans 249bp, 83 amino acids. US10 and US11 are transcribed in different reading frames and therefore do not share a common amino acid sequence (Rixon & Clements, 1982; Rixon & McGeoch, 1984 & 1985).

Within the US11 and US10 ORFs lies a three and one-third 18bp tandemly reiterated repeat sequence, TCTCCCGGGAGACCGGGG, encoding a 6 amino acid repeat motif of SPREPR in US11 and LPGSPG in US10. The number of copies of this tandem reiteration varies between virus strains and is thought to account for the intrastrain difference in the size of the US11 protein and restriction fragments encompassing this region (Lonsdale *et al.*, 1979; Rixon & McGeoch, 1984; Umene & Yoshida, 1989).

US11 can be divided into two domains, the N-terminal (amino acid 1-83) and the C-terminal domain (amino acid 84-161). The C-terminal domain of US11 consists almost entirely (amino acid 84-156) of 24 repeated tripeptide units, XPR, where X represents any amino acid (Fig. 1.7). The 6 amino acid repeat encoded by the 18bp reiterated DNA sequence lies within this region, encoding 7 of the XPR repeats. The tripeptide repeat motif extends either side of the reiterations and all possible codons for P and R are utilized, therefore there is no equivalent extended repeat in US10 outside of that encoded by the tandem reiteration. Rixon & McGeoch (1984) suggested that the polypeptides are under selective pressure as opposed to the DNA sequence as the

A

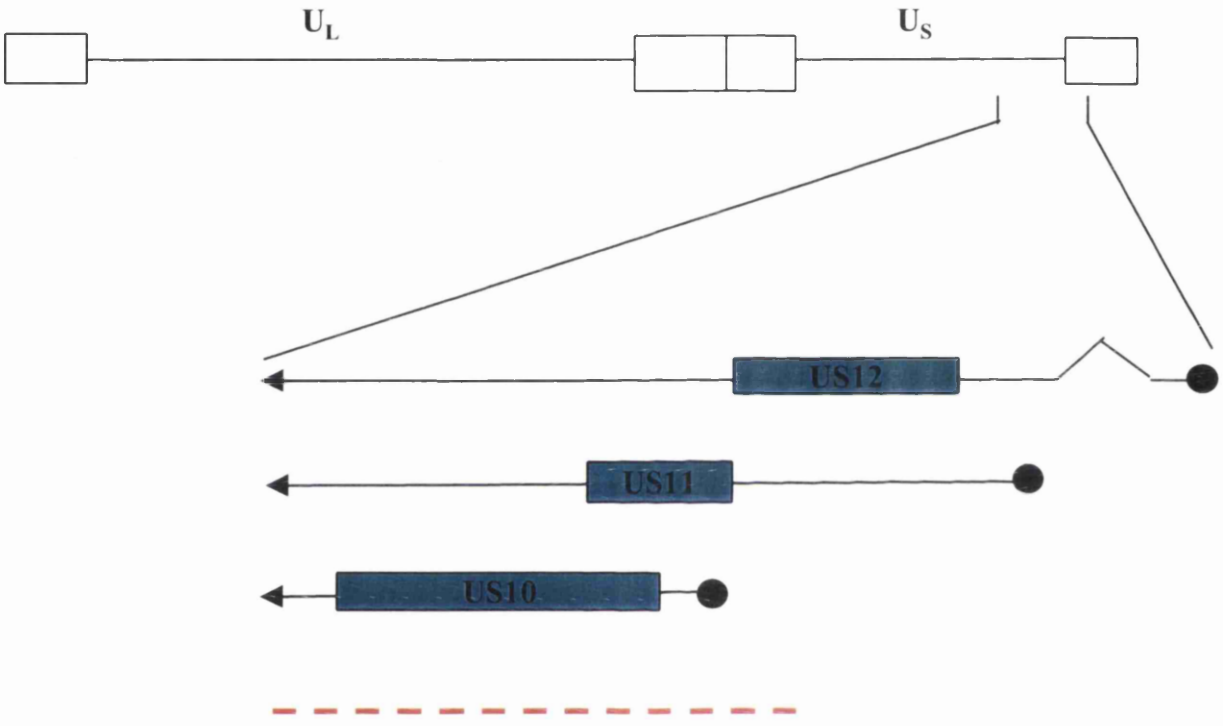


Fig. 1.6: The genomic location, sequence and structure of the US11 gene.
A: A schematic diagram of the HSV-1 genome. The unique regions are depicted as lines and the major repeat regions are represented by white boxes. An enlarged region of the US10, US11 and US12 genes. The untranslated regions are represented by a line, the 3′ ends by an arrow head, the 5′ ends by a circle and the ORFs by a green box. A red dashed line indicates the region of U_S component shown in panel B (not to scale). B: The sequence of the U_S region containing US11. TATA boxes are in bold and underlined, US11 downstream activation sequence (DAS) is in green (Kibler *et al.*, 1991; Guzowski *et al.*, 1994), first Met-ATG start codons are in red, translational stop codons are in blue, sequence reiterations are boxed and in bold, polyadenylation signal is in magenta, and the mRNA cleavage site is indicated by an asterisk. (Rixon & Clements, 1982; Rixon & McGeoch, 1984, 1985; McGeoch *et al.*, 1986).

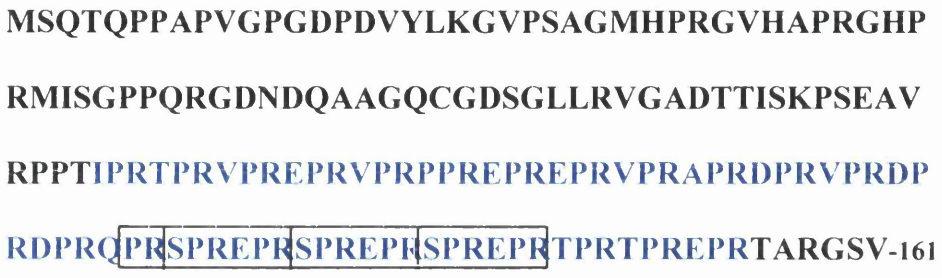


Fig. 1.7: The amino acid sequence of US11.

The XRP repeats are highlighted in blue and the reiterated sequence is boxed. (Rixon & McGeoch, 1984).

reiterated sequence does not encode a substantial proportion of the tripepide repeats. As all the potential codons for proline and arginine are used, it is likely that selection allowed US11 to gain and/or maintain its C-terminal tripeptide repeats whilst not constraining US10 to an extended repeat motif.

Rixon & McGeoch (1984) suggest two evolutionary pathways by which this phenomenon could arise. The tandem reiterations originally encoded the entire C-terminal domain of US11, and by either random mutations or through selective pressure on US10, acquired its present day sequence. Alternatively, a pre-existing reiterated amino acid sequence predisposed the DNA to the formation of tandem duplications which could arise without compromising the amino acid sequence.

1.3.3 The structure of the US11 protein

The unusual highly repetitive nature of the C-terminal domain, with the appearance of a bulky proline residue every 3 amino acids, suggests that the domain forms a secondary structure with frequent turns, as the amido acid, proline, is often associated with changes in the direction of the polypeptide. Schaerer-Uthurralt *et al.* (1998) used computer aided modelling to predict the secondary structure of this domain. Two structures were predicted to arise from the repeated sequence, a right-handed 3_{10}-helix and a poly-L-proline type II helix (PPII-helix) (Fig. 1.8). These helical structures have a repeat unit of 3 amino acids per turn, the 3_{10}-helix forms a more extended structure, whilst the PPII-helix is considerably more compact and more stable, and is thought to be the structure adopted by the C-terminal domain of US11 (Roller *et al.*, 1996; Schaerer-Uthurralt *et al.*, 1998). The PPII-helix is a flexible structure and has been found to exist for short stretches in a number of globular proteins (Adzhubei *et al.*, 1993). Examples include avian pancreatic polypeptide which possesses an 8 residue

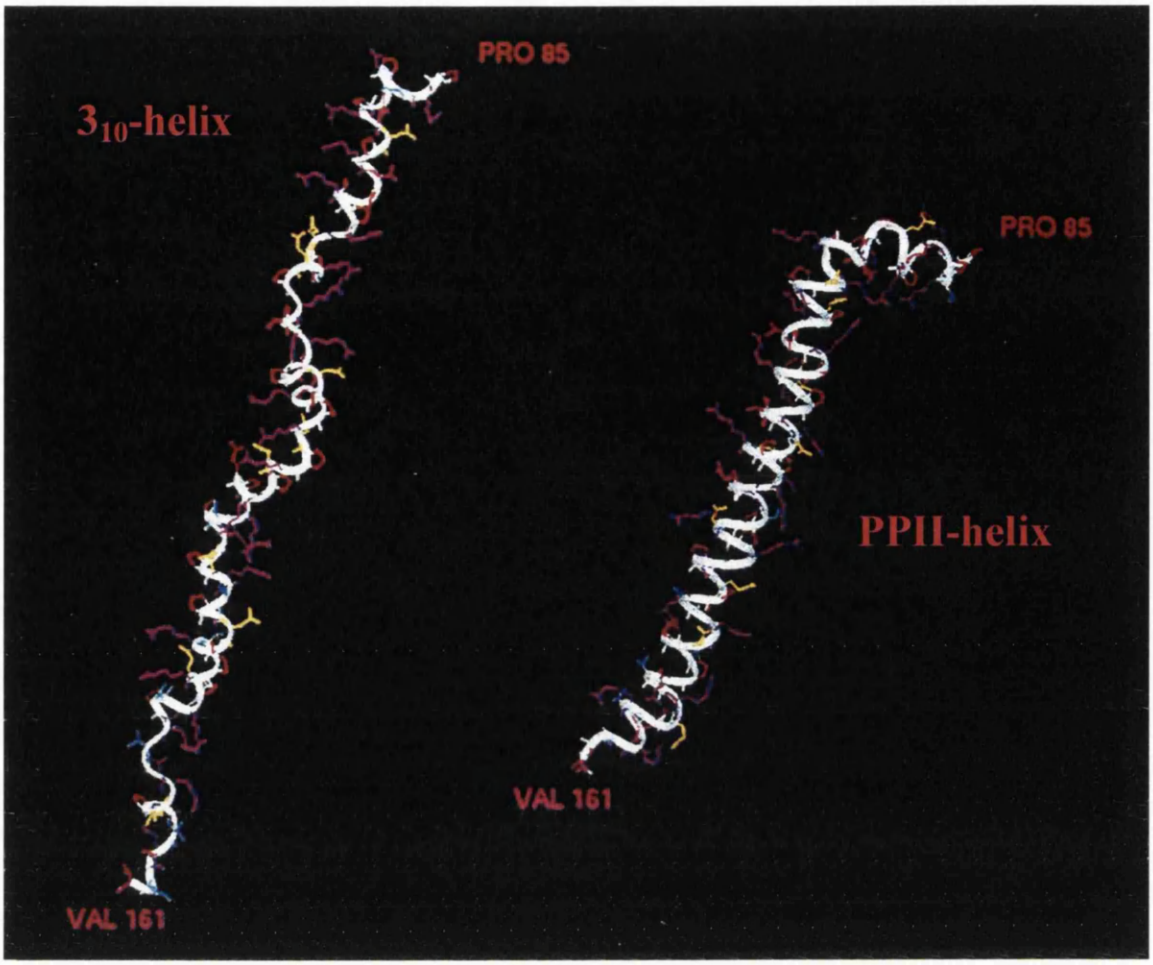


Fig. 1.8: The predicted structure of the C-terminal domain of US11.
Computer aided modelling of the C-terminal domain of US11 predicts a helical structure of 3 amino acids per turns. Two structures are predicted, the 3_{10}-helix (left-hand side) and the poly-L-proline type II helix (right-hand side). Reproduced from Schaerer-Uthurralt, *et al.*, 1998.

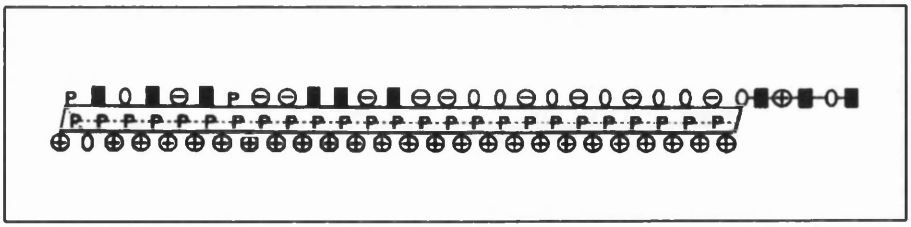


Fig. 1.9: Schematic diagram of the structure of the C-terminal domain of US11.
Reproduced from Roller *et al.*, 1996. Residues 76-161 of US11. Arg represented by circled plus signs, Pro with a P, the hydrophobic residues, Ile, Val and Ala, with shaded rectangles, the polar residues, Ser, Thr and Gln with ovals and the acidic residues, Asp and Glu, with circled minus signs.

PPII-helix, GPSQPTYP (Blundell *et al.*, 1981) and carbonic anhydrase B, NRPTQPL. The EB2 protein of EBV, an RNA-binding protein, contains 7 XRP repeats spanning 21 amino acids which lies between amino acids 151-172 RAPRAPRAPRVPRAPRSPRAP in a 479 amino acid protein. The PPII-helical structure of US11 is the longest found in nature thus far and represents a substantial structural domain as opposed to the other examples where this structure only encompasses several amino acids and at best, a short region. Roller *et al.* (1996) suggested that the PPII-helix, bearing 3 residues per turn, would present 3 faces, an uncharged relatively hydrophobic face comprised of proline residues, a positively charged, basic face consisting of arginine residues and the third face of varying nature, consisting mainly of hydrophobic, uncharged polar and acidic amino acids (Fig. 1.9) (Roller *et al.*, 1996).

1.3.4 Localization

The US11 gene product is incorporated into the virion tegument at an estimated 600-1000 molecules per virion and is therefore delivered to the host cell upon infection (Roller & Roizman, 1992). Ultrastructure studies have revealed that early in infection US11 co-localizes with viral DNA in the nucleus and perinuclear regions rich in ribosomes (Puvion-Dutilleul, 1987). At approximately 6h pi US11 begins to accumulate in the nucleolus, and levels increase in the cytoplasmic compartment, correlating with late gene expression (MacLean *et al.*, 1987; Puvion-Dutilleul, 1987; Besse, *et al.*, 1996). US11 does not require any viral products for its localization within the nucleolar compartment. In cells expressing US11 the morphology of the nucleoli are altered, being more compact and with fewer large fibrillar centres (Besse, *et al.*, 1996). At the

ultrastructure level, US11 localizes to the dense fibrillar component (DFC) and the granular component (GC) of the nucleolus (Besse, *et al.*, 1996).

1.3.4.1 The nucleolus

The nucleolus is a non-membrane bound, sub-nuclear structure involved in ribosome biogenesis. It is the site of the transcription of ribosomal DNA (rDNA) encoding 18S, 5.8S and 28S rRNAs by RNA polymerase I, rRNA processing and base modification, and the assembly of pre-ribosomal subunits (Schwarzacher & Mosgoeller, 2000). The nucleolus can be divided into three morphological, concentric domains, the inner fibrillar center(s) (FC), the dense fibrillar component (DFC) and the outer granular component (GC). Transcription of rRNA is thought to occur at the FC/ DFC interface. Early rRNA processing occurs in the DFC and assembly of pre-ribosomes in the GC (Maxwell & Fournier, 1995).

The nucleolus is a dynamic compartment, and in actively dividing cells rRNA represents an estimated 60% of total cellular RNA. As the site of ribosome biogenesis the nucleolus is a key target for regulation during events such as cell proliferation, senescence and stress response. In addition the nucleolus has been implicated in other cellular processes such as 5′ end trimming of certain tRNAs, assembly of the signal recognition particle, U6 small nuclear (sn) RNA modification, the sequestration of cell cycle regulation proteins and has even been implicated in ageing (Srivastava & Pollard, 1999; Carmo-Fonseca *et al.*, 2000).

1.3.4.2 Virus interaction with the nucleolus

The nucleolus is the site of localization of certain viral proteins and is often modified during viral infection. The effect of the viral infection on ribosome biogenesis is, in general, specific to the particular virus. A few viruses replicate in the nucleolus, such as Bourna disease virus and Minute virus of mice (Walton *et al.*, 1989; Pyper *et al.*, 1998). The mRNAs of HIV-1 and HTLV-1 may traffic through the nucleolus (Michienzi *et al.*, 2000) and it has been suggested that the nucleolar localization of the HTLV-1 Rex protein is required for its function in the nucleocytoplasmic transport of intron-containing viral transcripts (Nosaka *et al.*, 1989). Adenoviruses and herpesviruses cause disruption of the nucleolus. Adenovirus infection inhibits the synthesis and processing of rRNA and causes the redistribution of nucleolar antigens. The adenovirus protein V can cause the redistribution of the nucleolar proteins, B23 and nucleolin (Matthews *et al.*, 2000). Herpesvirus infection is associated with a decrease in the number and size of nucleoli, diminished rRNA processing, and rRNA transcription (Kyriakidis & Stevely, 1982; Besse *et al.*, 1996). Other viruses up-regulate nucleolar activity. The Semliki Forest virus protein capsid protein localises to the nucleolus and at low concentrations can stimulate protein synthesis, but at high concentrations has an inhibitory effect (Elgizoli *et al.*, 1989). Purified preparations of the large T-antigen of SV40 can stimulate rRNA transcription *in vitro* (Whelly *et al.*, 1978).

1.3.4.3 Nucleolar localization

As the nucleolus is not separated from the surrounding nuclear milieu by a membrane, nuclear proteins could conceivably traffic into and out of this compartment by simple

diffusion. However, as the nucleolus is distinctive in its composition there must be a mechanism allowing the exclusion and inclusion of factors.

It has been argued that proteins are targeted to the nucleolus by their association with nucleolar components. However, there are examples of proteins which interact with nucleolar components but do not enter the nucleolus such as p53, which can interact with rRNA (Fontoura, *et al.*, 1992), or the HSV-1 transcription factor, ICP4, which can interact with the nucleolar/ ribosomal protein L22 (EAP) (Leopardi & Roizman, 1996). Specific nucleolar localization sequences (NoLS) have been identified in cellular and viral proteins which can direct nucleolar accumulation (Hatanaka, 1990) (Table 1.2). In general, the NoLS consists of a stretch of basic amino acids, particularly rich in arginine residues which flanks the NLS. Experimentally, it is difficult to separate the NoLS from the NLS, as nuclear accumulation is required for access to the nucleolus. An NoLS is usually defined by mutations that allow targeting to the nucleoplasm, but exclusion from the nucleolus. The nucleolar shuttling protein, B23, has been implicated as a NoLS receptor which can direct the nucleolar accumulation of several proteins, notably the HIV-1 Tat and Rev proteins, and the cellular rRNA maturation and ribosome assembly factor, nucleolin (Dundr *et al.*, 1995; Li, 1996, 1997).

1.3.4.4 US11: Nuclear localization and nucleolar targeting

US11 does not appear to contain a consensus NLS. NLSs are short, modular stretches of amino acids which can broadly be divided into three categories. (1) A short stretch of basic amino acids, as typified by SV40 large T antigen, PKKKRKV (Kalderon *et al.*, 1984); (2) a bi-partite NLS, comprising two stretches of basic residues separated by 10-12 amino acids, such as the NLS of nucleolin, KRPAATKKAGQAKKKK; (Robbins *et*

Protein	Function	NoLS	Ref
MDV MEQ	Transcriptional transactivator, may bind RNA	RRRKRNDAARRRRRKQ	Liu *et al.*, 1997.
HIV-1 Rev	Nucleocytoplasmic targeting of intron-containing viral transcripts	RRNRRRRWRERQRQ	Cochrane *et al.*, 1990
HIV-1 Tat	Transcriptional transactivator	GRKKRRQRRRAHQN	Dang & Lee, 1989
HTLV-1 Rex	Nucleocytoplasmic targeting of intron-containing viral transcripts	PKTRRRPRRSQRKRPPTP	Nosaka *et al.*, 1989
PRRV N protein	Nucleocapsid protein	RGKGPGKKNKKKNPEK	Rowland *et al.*, 1999
Hsp70	Stress response protein, chaperone	FKRKHKKDISQNKRAVRR	Dang & Lee, 1989
Topoisomerase I	Unwinding supercoiled DNA	Residues 157-199. K rich.	Mo *et al.*, 2000
FXR2P	Associates with FMRP, ribosomes and RNA	RPQRRNRSRRRRFR	Tamanini *et al.*, 2000

Table 1.2: Nucleolar localization sequences.

This table lists several examples of nucleolar localisation sequences (NoLS). Abbreviations: MDV (Marek's disease virus), MEQ (MDV Eco Q), HIV-1 (Human immunodeficiency virus type-1), HTLV-1(Human T-cell lyphotropic virus type-1), PRRV (Porcine reproductive and respiratory syndrome virus), Hsp (Heat shock protein), FMRP (Fragile X mental retardation protein), FXR2P (FMRP related protein 2).

12 amino acids, such as the NLS of nucleolin, KRPAATKKAGQAKKKK; (Robbins *et al.*, 1991); and (3) NLSs such as that of *c-myc*, with charged/polar residues interspersed with a central basic cluster, PAAKRVKLD (Makkerh *et al.*, 1996). Molecules with a molecular weight less than 60kDa can diffuse passively through the nuclear pore, and it is therefore reasonable to suggest that US11 (21kDa) accesses the nuclear compartment in this fashion.

The C-terminal of US11 is highly basic, with an arginine residue every three amino acids, and given the broad consensus for NoLS it is easy to envision that this domain could function as such. However, as the NoLS is thought to be an extended NLS, and US11 does not appear to possess a consensus NLS, the presence of specific nucleolar targeting is questionable. US11 may be directed to localize to the nucleolus by non-specific associations with nucleolar components. US11 can associate with ribosomes, RNA and possibly DNA; and its affinity for either of these may concentrate it in the nucleolus, a site rich in all three.

The domain responsible for the nucleolar accumulation of US11 was mapped by Roller *et al.* (1996). Deletion of almost the entire C-terminal domain (amino acids 88-161) abolished nucleolar localisation of US11. Deletion of amino acids 91-121 resulted in the diminished accumulation of US11 in the nucleolar compartment, whereas deletion of amino acids 123-161 does not effect localisation. Deletions throughout the US11 protein do not result in exclusion from the nucleoplasm, indicating that there is no specific NLS. The loss of nucleolar association does not strictly correlate with an inability to bind RNA or to associate with ribosomes. US11Δ123-161, which localises to the nucleolus, does not co-sediment with the 60S ribosomal subunit and is unable to bind RNA.

1.3.5 Association with ribosomes

US11 was first detected in ribosomal preparations as a viral protein which migrated as several species in 2D gel electrophoresis by Masse *et al.* (1990). These species were termed v1 (the unphosphorylated form) v1a, v1b, v1c and v1d (the phosphorylated forms), plus other less abundant phosphorylated derivatives migrating above and below these species (Masse *et al.*, 1990; Diaz *et al.*, 1993). The identity of these species was revealed by CNBr cleavage and N-terminal sequencing, and confirmed by Western blotting with a polyclonal antibody raised against US11 (Diaz *et al.*, 1993). Fractionation of the ribosome subunits showed that the US11 derivatives were associated with different ribosomal fractions. Species v1, and v1a co-sedimented with the 40S fraction; v1, v1a and v1b with the 80S; and v1, v1a, v1b and v1c with the polysomal fraction (Masse *et al.*, 1990). Whether the phosphorylation state of US11 directs its association with a particular ribosomal complex or whether the differential phosphorylation is a consequence of the localisation of US11 is unclear.

Diaz *et al.* (1993) found that US11 could be detected throughout a sucrose gradient fractionation of ribosome preparations from infected cells. This suggests that US11 associates weakly with ribosomes in a non-specific fashion, and this interaction is disrupted upon sucrose gradient centrifugation, leading to the distribution of US11 throughout the gradient as either aggregates or multimers. However, Roller & Roizman (1992) detected US11 specifically and differentially associated with ribosomal subunits. Separation of ribosomal subunits on a sucrose gradient and Western blotting for the presence of US11 in these fractions showed that US11 was present in small amounts at the top of the gradient, and co-sediments with the 60S, 80S and polysomal fractions,

with the 60S fraction containing the most US11. The fractions containing the 40S subunit did not contain detectable amounts of US11 suggesting that US11 specifically associates with the 60S subunit. Furthermore, they were able to demonstrate that viral mutants in which US11 had been truncated in the C-terminal domain US11Δ88-161, Δ91-121 and Δ121-161 were unable to co-sediment with ribosomes indicating that this interaction may be specific (Roller *et al.*, 1996).

In the presence of actinomycin D (Diaz *et al.*, 1993) and in the presence of PAA at 2h pi, (Roller & Roizman., 1992) US11 associates with ribosomes and it accumulates in ribosomal preparations in a moi-dependent fashion, indicating that virion-borne US11 is capable of this interaction. Furthermore, in a stable cell line constitutively expressing US11, it was found to associate with ribosomal fractions demonstrating that US11 does not require any other viral factors for this interaction (Roller & Roizman, 1992).

1.3.6 US11 and the dsRNA-activated PKR pathway

1.3.6.1 PKR: A host defence mechanism

In order to establish an infection a virus must first overcome the anti-viral responses of the host organism. One of the first antiviral defences a virus must face is the innate immune interferon (INF) response system. INF are cytokines produced in response to viral infection of a cell or other cellular stresses. INF is secreted into the extracellular medium and through the binding to cell surface receptors, triggers an intracellular signalling cascade and the induction of over 30 genes. This primes the cell to fight viral infection or overcome other cellular stresses (Williams, 1999).

i. PKR activation and translational shut-off

Protein kinase R (PKR, Protein kinase RNA-activated) (also termed DAI, PI-eIF2 or p68 kinase) is ubiquitously expressed at low levels in most tissues and is a major anti-viral effector of the INF system. Its expression is induced by INF and its antiviral action results in the global shut-off of translation, preventing viral gene expression, subsequent replication and spread, at the expense of the cell. PKR expression alone is not sufficient to cause this severe action, the kinase activity of PKR is activated by the presence of transcriptionally active virus. Therefore, PKR requires a means of detecting viral infection.

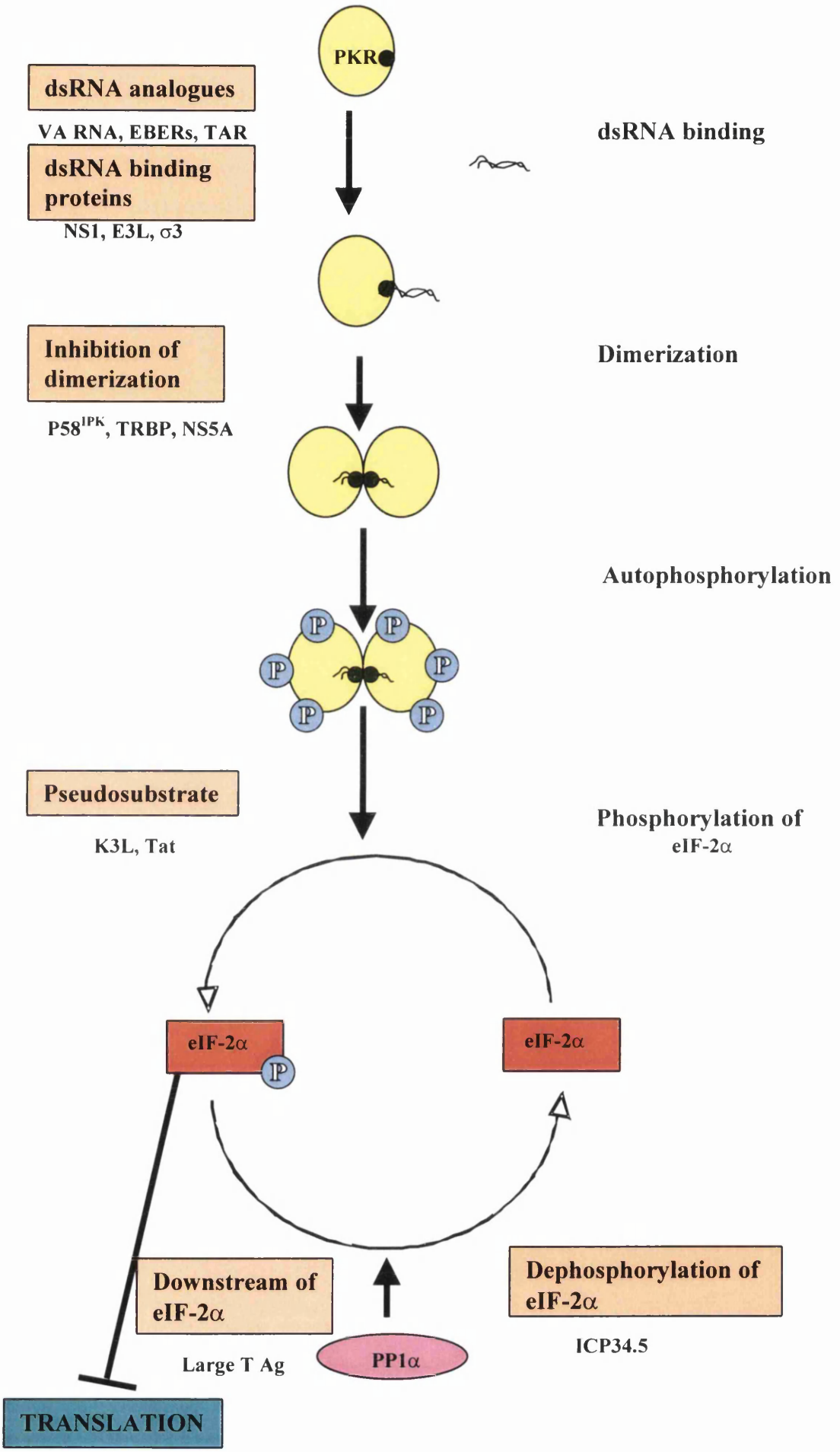
PKR can be activated by low concentrations of dsRNA, some highly structured ssRNAs (henceforth termed as activator dsRNAs) and polyanions. The kinase activity of PKR is stimulated by the binding of activator dsRNAs via its two dsRNA binding motifs (dsRBM). It is thought that two molecules of PKR bind to one molecule of activator dsRNA promoting dimerization (Consentino *et al.*, 1995). PKR then autophophorylates on several Ser and Thr residues, becoming activated (Taylor *et al.*, 1996). Activated PKR phosphorylates eukaryotic initiation factor 2 (eIF-2) α subunit on Ser-51. Phosphorylated eIF-2α forms a tight complex with the eIF-2 guaninosine-exchange factor, eIF-2β, and inhibits the exchange of GDP for GTP in the eIF-2 complex (Rowlands *et al.*, 1988; Ramaiah *et al.*, 1992). The recycling of the eIF-2 complex to form the eIF-2-GTP-Met-initiator tRNA ternary initiation complex is therefore prevented and the translation of mRNA is subject to global shut-off and growth arrest/cell death occurs.

ii. PKR and viral infection

During viral infection dsRNAs are synthesised due to transcription from both strands of dsDNA viruses or the highly structured RNA elements of RNA viruses, allowing PKR to "detect" a viral infection. Almost all viruses have therefore evolved ways in which to subvert the PKR response to allow effective viral gene expression, replication and dissemination (Fig. 1.10 and Table 1.3).

Some viruses produce highly structured RNAs at very high levels which mimic the activator dsRNAs. Adenovirus provided the first direct evidence that PKR was involved in the anti-viral response of INF. Cells infected with the adenovirus mutant dl331, which lacked the VAI gene, were blocked in protein synthesis (Thimmappaya *et al.*, 1982) and subsequent work showed that this was due to the phosphorylation of eIF-2α by activated PKR (Schneider *et al.*, 1985). The VAI RNA transcribed from this region was shown to have extensive secondary structure and bind to PKR and inhibit its activation. The EBV encoded RNAs (EBERs) are able to functionally substitute for the VAI RNAs of adenovirus and act in a similar manner to these RNAs. Another mechanism by which viruses can overcome the PKR pathway is to produce dsRNA binding proteins such as the vaccinia virus E3L protein or the reovirus σ3 protein, which bind to dsRNA activators and mask them from PKR. Influenza utilizes a pre-existing cellular inhibitory pathway to prevent the activation of PKR. Infection results in the release of the 58kDa protein inhibitor of PKR ($P58^{IPK}$) from an inhibitory complex with a member of the heat shock protein (Hsp) 90 family, termed inhibitor of $P58^{IPK}$ ($I\text{-}P58^{IPK}$). This allows $P58^{IPK}$ to interact with the dimerization domain of PKR

Fig. 1.10: The INF-induced, dsRNA-activated PKR pathway and viral antagonists.
The unboxed text on the right-hand side of the diagram lists the pathway leading to PKR activation and the inhibition of translation, which is diagrammatically depicted in the centre. The peach boxes indicate the stages at which this pathway may be antagonised by viral proteins, examples of which are listed below the boxes. The proposed mechanism of action of these viral proteins are given in Table 1.3.


PKR
dsRNA analogues
VA RNA, EBERs, TAR
dsRNA binding proteins
NS1, E3L, σ3
dsRNA binding
Inhibition of dimerization
P58IPK, TRBP, NS5A
Dimerization
Autophosphorylation
P
Pseudosubstrate
K3L, Tat
Phosphorylation of eIF-2α
eIF-2α
eIF-2α
Downstream of eIF-2α
Large T Ag
PP1α
Dephosphorylation of eIF-2α
ICP34.5
TRANSLATION

Mechanism	Virus/ Factor	Notes	Reference
dsRNA analogues	Adenovirus/ VA RNA	Abundant, highly structured RNAs which compete with activator dsRNA for PKR binding and inhibit activation.	Mathews & Shenk, 1991.
	EBV/ EBERs	Highly structured RNAs thought to act on PKR by competitive inhibition. Can substitute for VA RNAs. Non-essential for EBV growth in tissue culture.	Bhat & Thimmappayes, 1983; Clarke *et al.*,1990; Swaminathan *et al.*, 1991.
	HIV-1/TAR	Highly structured RNA at the 5'end of all HIV RNAs. Can bind to PKR *in vitro*. May act by competitive inhibition. May act by recruitment of PKR inhibiting protein.	Gunnery *et al.*, 1990.
dsRNA binding proteins	Influenza/ NS1	Thought to bind to and sequester activator dsRNAs in the nucleus.	Lu *et al.*, 1995; Bergmann *et al.*, 2000.
	Vaccinia/ E3L (p20 /25)	Binds to and sequesters activator dsRNAs.	Whitaker-Dowling & Youngner, 1984; Beatie *et al.*, 1995.
	Reovirus/ σ3	Binds to and sequesters activator dsRNAs. Expression of σ3 protein can rescue vaccinia ΔE3L virus.	Beattie *et al.*, 1995; Yue & Shatkin, 1997; Samuel, 1998.
Formation of heteromeric complexes with PKR	Influenza/ $P58^{IPK}$	Ubiquitious cellular protein released from complex with inhibitory factors upon influenza infection. Binds to PKR dimerization domain and prevents activation.	Lee *et al.*, 1992, 1994; Gale *et al.*, 1997.
	HIV-1/ TRBP	Cellular dsRNA binding protein. Binds HIV-1 TAR. Prevents autophosphorylation of PKR. Forms heterodimers with PKR.	Costentino *et al.*, 1995; Benkirane *et al.*, 1997.
	HHV-8/ vIRF-2	Interacts with PKR and inhibits autophosphorylation. Mechanism unknown.	Burysek & Pitha, 2001
	Vaccinia Virus/ EL3	Binds to the catalytic and dsRBD of PKR and inhibits autophosphorylation. May inhibit substrate binding or dimerization.	Romano *et al.*, 1998; Sharp *et al.*, 1998.
	HCV/ NS5A	Binds to PKR and prevents activation. Thought to prevent dimerization. NS5A sequence may influence intra-strain INF sensitivity.	Gale *et al.*, 1998; Pawlotsky & Grmanidis, 1999.
Pseudo-substrate for PKR	Vaccinia/ K3L	Shares homology with eIF-2α N-terminal. Probably competes with eIF-2α for binding the catalytic domain of PKR.	Beatie *et al.*, 1991, 1995; Kawagishi-Kobayashi *et al.*, 1997.
	HIV-1/Tat	Competes with eIF-2α for phosphorylation by PKR and inhibits PKR autophosphorylation *in vitro*. Some sequence similarity to eIF-2α.	Brand *et al.*, 1997; Cai *et al.*, 2000.
	Swinepox/ C8L	Inhibits phosphorylation of eIF-2α. Sequence similarity to eIF-2α. 43% amino acid identity to Vaccinia virus K3L.	Kawagishi-Kobayashi *et al.*, 2000.
	HCV/ E2	Binds to PKR and inhibits autophoshorylation *in vitro*. Sequence similarity to autophoshorylated residues in PKR. Inhibition does not appear to be solely due to competitive inhibition.	Taylor *et al.*, 1999, 2001.
Down regulation of PKR levels	HIV-1/Tat	PKR levels reduced in Tat expressing cell lines and HIV-1 infected cells.	Roy *et al.*, 1990; McMillan *et al.*, 1995; Cai *et al.*, 2000.
	Poliovirus	PKR down regulated in Poliovirus infected cells. Mechanism unknown.	Black *et al.*, 1993.
Down-stream of eIF-2α phosphory-lation	HSV-1/ ICP34.5	Redirects the phosphatase activity of PP1α to dephosphorylate eIF-2α via its *Gadd34* homology domain.	McGeoch & Barnett, 1991; Chou & Roizman, 1994; Brown *et al.*, 1997; He *et al.*, 1997a; Lieb *et al.*, 2000.
	SV40/ Large T-Antigen	Restores translation levels, but does not inhibit PKR activation or eIF-2α phosphorylation. Mechanism and point of action unknown.	Rajan *et al.*, 1995; Swaminathan *et al.*, 1996.

Table 1.3: Examples of viral antagonists of the INF-induced, dsRNA-activated PKR pathway.

and prevent activation. Other inhibitors act downstream of PKR activation acting as pseudosubstrates for PKR and hence are phosphorylated in preference to eIF-2α such as the K3L protein of vaccinia virus. The ICP34.5 protein of HSV-1/-2 acts downstream of PKR activation and eIF-2α phosphorylation by redirecting the activity of protein phosphatase 1α (PP1α) to dephosphorylate eIF-2α (He *et al.*, 1997a).

1.3.6.2 The ICP34.5 protein of HSV-1, *Gadd34/MyD116* and the PKR pathway

ICP34.5 possesses at least two functions. It is a neurovirulence factor, allowing the replication and spread of virus in the CNS and it is an antagonist of the INF-induced dsRNA activated PKR pathway. ICP34.5 ($\gamma_1$34.5, RL1, or neurovirulence factor) is located within the inverted repeats of the U_L sequence and is therefore present as two copies per genome (Thompson *et al.*, 1983; Thompson *et al.*, 1989; Dolan *et al.*, 1992, Mckay *et al.*, 1993). ICP34.5 consists of three domains, a large N-terminal domain, a central tripeptide tandem repeat region of Pro Ala Thr (PAT), repeated 5-10 times (strain-dependent) and a C-terminal domain with homology to the C-terminal domain of the murine *MyD116* and its hamster homologue, the *Gadd34* protein (McGeoch & Barnett, 1991).

The entire coding region of ICP34.5 is required for its function as a neurovirulence factor and mutants in this gene are severely impaired in replication and the establishment of latency in the CNS following intracranial inoculation of mice (Chou *et al.*, 1990; MacLean *et al.*, 1991; Bolovan *et al.*, 1994; Spivack *et al.*, 1995).

The C-terminal domain of ICP34.5 is responsible for its inhibition of host mediated translational shut-off. In tissue culture, deletion of the ICP34.5 gene or its C-terminus restricts viral replication in a cell type-dependent manner. Cell lines such as BHK and

Vero are permissive for ICP34.5 mutant virus growth, whereas, in non-permissive cell lines, such as the neuroblastoma cell line SK-N-SH, virus growth is severely attenuated (Chou *et al.*, 1990; Chou & Roizman, 1992). In non-permissive cell lines virus growth is almost completely inhibited by host mediated translational shutoff via the PKR pathway (He & Roizman, 1994). The high degree of conservation between the C-terminal domain of ICP34.5 and that of *MyD116/Gadd34* proteins suggests that ICP34.5 acquired this domain from this cellular gene during evolution in order to counteract the PKR pathway.

The MyD116 gene, is a member of the MyD (Myeloid differentiation primary response) gene family. It is expressed early in differentiation in response to differentiation inducers, such as IL-6, and in terminally differentiated myeloid cells (Lord *et al.*, 1990). It is thought to act as both a negative regulator of cell growth and of apoptosis depending on temporal expression (Leibermann & Hoffman, 1998). It is structurally similar to ICP34.5, consisting of a large N-terminal domain, a Pro, Glu, Ser, Thr (PEST) rich 38 amino acid sequence repeated 4.5 times, and a C-terminal domain which shares homology with that of ICP34.5 (Lord *et al.*, 1990; McGeoch & Barnett., 1991).

The GADD34 gene belongs to a family of genes, the GADD (growth arrest and DNA damage) family, induced in response to certain growth factors, genotoxic stress and apoptotic signals (Zhan *et al.*, 1994, Hollander, *et al.*, 2001). Over-expression of *Gadd34* results in growth arrest and apoptosis. In cells over-expressing the anti-apoptotic proteins *Bcl*-2 or cowpox virus CRM-1 there is a decrease in the induction of *Gadd34* and apoptosis (Hollander *et al.*, 2001). Interestingly, co-expression of the ICP34.5 protein and *Gadd34* results in the suppression of *Gadd34*-mediated growth arrest (Hollander *et al.*, 2001).

He *et al.* (1996) demonstrated that the C-terminal domain of ICP34.5 could be replaced by the homologous domain of the MyD116 gene to promote virus growth in non-permissive cells lines by restoring translation levels. Using the MyD116 C-terminal domain as bait in a two-hybrid screen, He *et al.* (1997a) detected an interaction with the catalytic subunit of protein phosphatase 1α (PP1α). Furthermore, in GST-pull down assays they were able to show that the C-terminal domains of ICP34.5 and *MyD116* could interact with PP1α. Previously, it had been observed that whilst PKR was activated in cells infected with ICP34.5 virus mutants and *wt* virus, eIF-2α was only phoshorylated in the absence of the ICP34.5 C-terminal domain (Chou *et al.*, 1995). *In vitro* eIF-2α dephosphorylation assays showed that there was a dramatic increase in eIF-2α dephosphorylation in the presence of the C-terminal domain of ICP34.5 or *MyD116*. ICP34.5 can be purified from infected cells in a large molecular weight complex containing the catalytic subunit of PP1α. Mapping of the site of interaction between ICP34.5 and PP1α revealed that the binding site for PP1α was within the *MyD116/Gadd34* homology domain. In addition the sequence bound by PP1α is present in all the subunits, accessory or regulatory proteins that can bind directly to the catalytic subunit of PP1α (He *et al.*, 1998). Substitution of amino acids within the PP1α binding consensus sequence in ICP34.5 results in the loss of virus growth and PKR pathway inhibition in non-permissive cell lines (He *et al.*, 1998).

The ICP34.5 gene is conserved in HSV-2, where it appears to performs a similar role (Taha *et al.*, 1989; MacLean *et al.*, 1991; Brown *et al.*, 1994). The only other viral homologue found to date is the LMW23-NL protein of the arbovirus African Swine Fever Virus (ASFV), which possesses a C-terminal domain with a high degree of homology with that of ICP34.5, and the *MyD116/Gadd34* (Sussman *et al.*, 1992; Zsak *et al.*, 1996). Therefore, it would appear that ASFV and HSV-1 and -2 acquired the

MyD116/GADD34 gene homology domains independently as a means of inhibiting host mediated translational shutoff. The MyD/GADD genes generally have a negative effect on cell growth whilst ICP34.5 prevents the inhibition of translation. It would therefore appear that the MyD/GADD homology domain is context dependent, with the N-terminal domains of these proteins defining the action.

1.3.6.3 US11 and the PKR pathway

i. Mapping of the suppressor locus of a ICP34.5 mutant virus

US11 was first implicated in the antagonism of the PKR pathway when isolated as a suppressor mutant of the ICP34.5 virus phenotype in a non-permissive cell line. A list and description of the viruses mentioned in this text that were used in the study of the interaction of US11 with the PKR pathway is given in Table 1.4.

Mohr & Gluzman (1996) constructed a HSV-1 virus in which both copies of the ICP34.5 gene had been replaced by β-glucuronidase (SPBg5e virus). SPBg5e was subjected to serial passage in the non-permissive neuroblastoma cell line, SK-N-SH. After 4-5 passages the viruses which displayed a CPE were grown in permissive Vero cells. The suppressor isolates grew 50-100 fold better than the parent virus in SK-N-SH cells. The protein profiles from SK-N-SH cells or from the non-permissive glioblastoma cell line, U373, infected with suppressor mutant isolates, wild type HSV-1 or SPBg5e were examined by *in vivo* labelling with [^{35}S]-methionine/-cysteine for 1h at 12h pi. This revealed that the suppressor mutants were able to synthesis viral proteins to nearly wild type levels and selectively shut-off host protein synthesis, whereas the SPBg5e infected cells were almost totally deficient in the synthesis of cellular or viral proteins.

Virus Name	Strain	Description of genotype	Ref.
R3616	F	LacZ insertion into both copies of the ICP34.5 gene.	Chou *et al.*, 1990.
R5104	F	LacZ insertion into both copies of the ICP34.5 gene and deletion of US8-US12. Insertion of the US11 ORF fused to the ICP47 promoter and the US10 ORF into the *tk* locus.	Cassady *et al.*, 1998b.
SBPg5e	Patton	Both copies of the ICP34.5 gene replaced by the β-glucuronidase gene.	Mohr & Gluzman, 1996.
Sup-1	Patton	Suppressor mutant isolate of SBPg5e. Deletion in US component resulting in the disruption of the US12 ORF and juxtaposition of the US12 promoter to the US11 ORF.	Mohr & Gluzman, 1996

Table 1.4: Recombinant viruses used in the investigation of the interaction between US11 and the PKR pathway.
This table gives a description of the viruses used in the study of the interaction between US11 and PKR mentioned in the text.

This indicated that the cellular PKR response to viral infection had been effectively opposed in the suppressor mutant isolates.

The *Bam*HI genomic fragments of the suppressor isolates were subcloned into a vector and subsequent restriction enzyme analysis revealed that the suppressor mutations of all the isolates were within the *Bam*HI Z fragment. Further restriction analysis and sequencing showed that all the suppressor mutants displayed a common trait. They all possessed deletions in the U_S/TR_S region resulting in the disruption of the US12 ORF (encoding ICP47, or α47), removal of the *cis*-acting sequences that direct the transcription of US11, and juxtaposition of the US12 promoter upstream of the US11 ORF (Fig. 1.11).

Mulvey *et al.* (1999) mapped the minimal deletion required in the U_S region to bring about the suppressor phenotype in the SPBg5e virus by a marker rescue assay. The smallest deletion tolerated for the suppression phenotype was a 140bp deletion into the U_S component and a 51bp deletion into the TR_S component (Fig. 1.11).

Examination of the kinetics of US11 expression in the Sup-1 virus revealed that the US11 gene was under the control of the US12 promoter causing US11 to be expressed as an immediate early gene (He *et al.*, 1997b). The immediate early expression of US11 was found to be responsible for the suppression of host-mediated translational shut-off in ICP34.5 mutant viruses (Cassady *et al.*, 1998a; Mulvey *et al.*, 1999). Additionally, Poppers *et al.* (2000) mapped the domain of US11 responsible for the suppression of the ΔICP34.5 phenotype to the C-terminus.

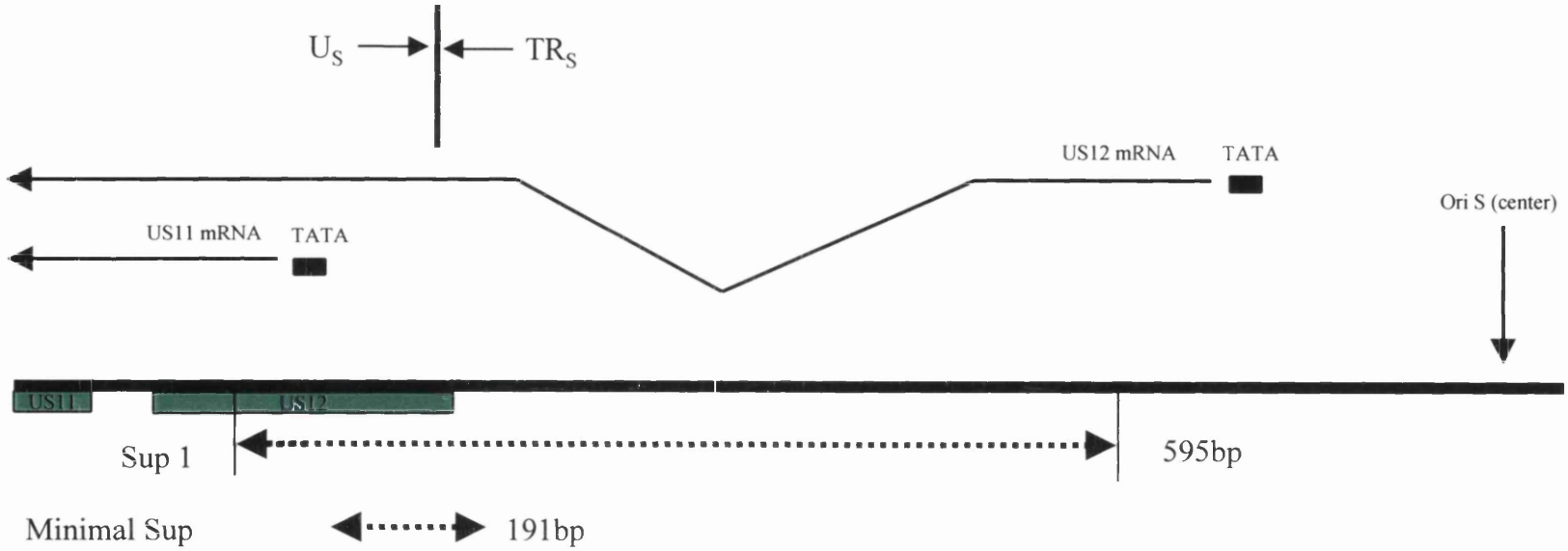


Fig. 1.11: Diagram illustrating the suppressor mutation of ΔICP34.5 phenotype.
The region deleted in the suppressor virus, Sup-1, is indicated, as mapped by Mohr & Gluzman (1996). The minimal deletion required to bring about the suppressor phenotype is indicated, as mapped by marker rescue assay (Mulvey *et al.*, 1999). Both deletions bring US11 under the control of the UL12 promoter. Not to scale.

ii. IE expression of US11 results in a decrease in PKR activation and eIF-2α phosphorylation.

The *in vivo* PKR pathway target of US11 has yet to be deduced. Both the inhibition of PKR autophoshorylation/activation and the block of eIF-2α phosphorylation have been suggested to be potential candidates for the mode of US11 action (Cassady *et al.*, 1998a & b; Mulvey *et al*, 1999; Poppers *et al.*, 2000).

Cassady *et al.* (1998a & b) suggested that US11 prevents the phosphorylation of eIF-2α by activated PKR. Using *in vivo* [^{32}P] orthophosphate labeling of HeLa cells infected with R5104, Cassady *et al.* (1998a) demonstrated that PKR was still activated in the presence of ectopically expressed US11. Furthermore, purified eIF-2α incubated in the presence of [γ-^{32}P] ATP and lysates from cells infected with the R5104 virus showed decreased labelling compared to eIF-2α incubated with lysates from cells infected with R3616. Using R3616 infected cell lysates as a source of activated PKR, Cassady *et al.* (1998b) demonstrated that the addition of GST-US11 could inhibit eIF-2α phosphorylation in a dose-dependent manner. In addition, US11 became phosphorylated itself, indicating that US11 may act as a pseudosubstrate for PKR.

However, Mulvey *et al.* (1999) demonstrated that PKR phosphorylation was reduced by over 50% in the presence of Sup-1 infected cell lysates in an *in vitro* PKR labelling assay. In addition, Cassady *et al.* (1998b) using purified PKR, eIF-2α, [γ-^{32}P]ATP and the addition of polyI.C. (a dsRNA mimic which activates PKR), demonstrated that the phosphorylation of eIF-2α and autophosphorylation of PKR was inhibited by US11 in a dose-dependent manner. The C-terminal domain of US11 was shown to be necessary and sufficient to reduce the autophoshorylation of PKR *in vitro* in a dose-dependent manner (Poppers *et al.*, 2000).

The potency of GST-US11 mediated inhibition of eIF-2α phosphorylation and PKR autophosphorylation *in vitro* is greatly increased if it was added prior to the activation of PKR, suggesting that PKR activation is the main target of US11. In addition, GST-US11 phosphorylation was only detected when higher concentrations of GST-US11 were added to the reaction in the presence of pre-activated PKR. An interaction between US11 and PKR was detected in a GST-PKR pull-down assay with HSV-1 infected HeLa cell lysates. This interaction was not abolished by RNase treatment of the lysates (Cassady *et al.,* 1998b).

iii. The target of US11 in the PKR pathway

Several hypotheses can be made with respect to the mechanism by which US11 inhibits PKR activation and eIF-2α phosphorylation:

(1) US11 is phosphorylated in the presence of activated PKR *in vitro* (Cassady *et al.*, 1998b), and it can be inferred that US11 acts as a pseudosubstrate for PKR phosphorylation, competing with the authentic cellular substrate, eIF-2α. Similarly, the HIV-1 transactivitor protein, Tat, and the vaccinia virus K3L protein are thought to act as psuedosubstrates for PKR (Beatie *et al.*, 1991, 1995; Brand *et al.*, 1997; Kawagishi-Kobayashi *et al.*, 1997). However, phosphorylation of US11 observed does not appear to directly correlate with the decrease in eIF-2α phosphorylation. In addition, the level of US11 phoshorylation varies between experiments. When US11 was added prior to PKR activation no phosphorylation of US11 was observed (Cassady *et al.*, 1998b). US11 does not share any obvious homology to eIF-2α and as the level of PKR autophosphorylation is decreased, this is more likely to be the primary reason for the decrease in eIF-2α phosphorylation. The phosphorylation of US11 may be an artifact of

the *in vitro* phosphorylation assay conditions rather than a true reflection of the *in vivo* situation. However, HIV-1 Tat can both inhibit eIF-2α phosphorylation and PKR autophosphorylation (Brand *et al.*, 1997), so this possibility cannot be ruled out.

(2) Cassady *et al.*(1998b) have suggested that US11 may bind to the active site of PKR and inhibit access to eIF-2α. This would be reflected in a decrease of autophosphorylation, which is consistent with the available data. The E3L protein of vaccinia virus has been shown to interact with the dsRBD and catalytic domain of PKR (Sharp *et al.*, 1998). US11 may mask the substrate binding site, and hence prevent eIF-2α phosphorylation.

(3) US11 may form a heterotrimeric complex with PKR and dsRNA. US11 has been shown to bind to several RNAs *in vivo* and *in vitro* (Roller & Roizman, 1990; Roller & Roizman, 1991; Diaz *et al.*, 1996), and the exact details of what constitutes actual *in vivo* activator dsRNA substrates for PKR are unclear (Gunnery & Mathews, 1998). Poppers *et al.* (2000) suggest that US11 may prevent PKR dimerization by both interacting with PKR directly and by interacting with the same dsRNA activator. A mechanism similar to this has been proposed for the vaccinia virus E3L protein (Romano *et al.*, 1998; Sharp *et al.*, 1998). The E3L protein, which possesses a dsRBM and can bind to dsRNA, is thought to act by competing with PKR for the binding of dsRNA (Davies *et al.*, 1993). However, Romano *et al.* (1998) found that E3L can interact with PKR directly and suggested that this interaction may be stabilized via the mutual binding of an activator dsRNA molecule, resulting in the formation of a heterotrimeric complex. TRBP contains a dsRBM and is thought to utilize a similar mechanism, forming a heteromeric complex with PKR, an activity which is not dependent on dsRNA binding (Consentino *et al.*, 1995; Benkirane *et al.*, 1997).

US11 can interact with GST-PKR in a pull-down assay, an interaction which is not dependent on RNA (Cassady *et al.*, 1998b). Whether this interaction is direct has yet to be established.

(4) As US11 appears to act upstream of PKR activation and is an RNA-binding protein, Poppers *et al.* (2000) have also suggested that US11 may act as a dsRNA binding protein, competing with PKR for activator dsRNA.

(5) PKR is localized to the ER and the nucleolus (Jeffrey *et al.*, 1995). It associates with ribosomes via its dsRBM in an RNA-independent manner (Wu *et al.*, 1998). It is thought that PKR associates with ribosomes as inactive monomers, and that cytosolic PKR is dimeric. The 60S ribosomal protein, L18, can inhibit PKR activation by competing with activator dsRNA for the binding of the PKR dsRBM I, essentially masking this site. L18 may mediate PKRs association with ribosomes and sequester it as an inactive form at these sites (Raine *et al.*, 1998; Kumar *et al.*, 1999).

US11 is associated with ribosomes (Masse *et al.*, 1990; Roller & Roizman, 1992; Diaz *et al.*, 1993) and may interact with PKR at this site and sequester PKR. Poppers *et al.* (2000) suggested that US11 may tether PKR to ribosomes, and prevent PKR activation by soluble activator dsRNA.

The mapping of the PKR domain(s) bound by US11 will give a greater insight into the mechanism of its action. From an evolutionary perspective it is interesting to note that HSV-1 apparently encodes two proteins which oppose the PKR pathway, utilizing different mechanisms. US11 may have originally been expressed at early times during infection and acted as the primary PKR antagonist in the HSV-1/-2 progenitor. Upon the acquisition of the MyD116/GADD34 homology domain by ICP34.5, US11 may have become redundant in this pathway, allowing it to evolve other functions and a late promoter (Cassady *et al.*, 1998a & b; Mulvey *et al.*, 1999; Roizman, 1999).

1.3.7 US11 and heat shock

After hyperthermic stress cells respond by shutting off protein synthesis and general transcription and elevate the transcription of the genes encoding the heat shock proteins (Hsps). The Hsps are molecular chaperones which can interact with denatured proteins to prevent aggregation and aid refolding. In addition, Hsps have other less well defined functions in the stress response pathway. They can prevent the transcription of proinflammatory genes, aid vesicle trafficking, and may be involved in nucleolar maintenance (Morcillo *et al.*, 1997). Indeed many Hsp genes encode small nucleolar RNAs (snoRNAs), RNAs involved in rRNA processing, within their introns.

PKR is a target and effector for many stress response pathways, such as tumour necrosis factor, bacterial lipopolysaccharides and heat shock, leading to the inhibition of translation and in some cases apoptosis (Fig. 1.12). During heat shock PKR is activated, probably by another cellular kinase, leading to the phosphorylation of eIF-2α and the inhibition of protein synthesis.

US11 can increase the rate of recovery of cells subjected to heat shock. HeLa cells transiently or constitutively expressing US11 restore protein synthesis more quickly and are less likely to undergo apoptosis (Diaz-Latoud *et al.*, 1997). This is not due to an increase in the levels of Hsps. US11 most probably exerts its effect by decreasing the levels of activated PKR and/or phosphorylated eIF-2α.

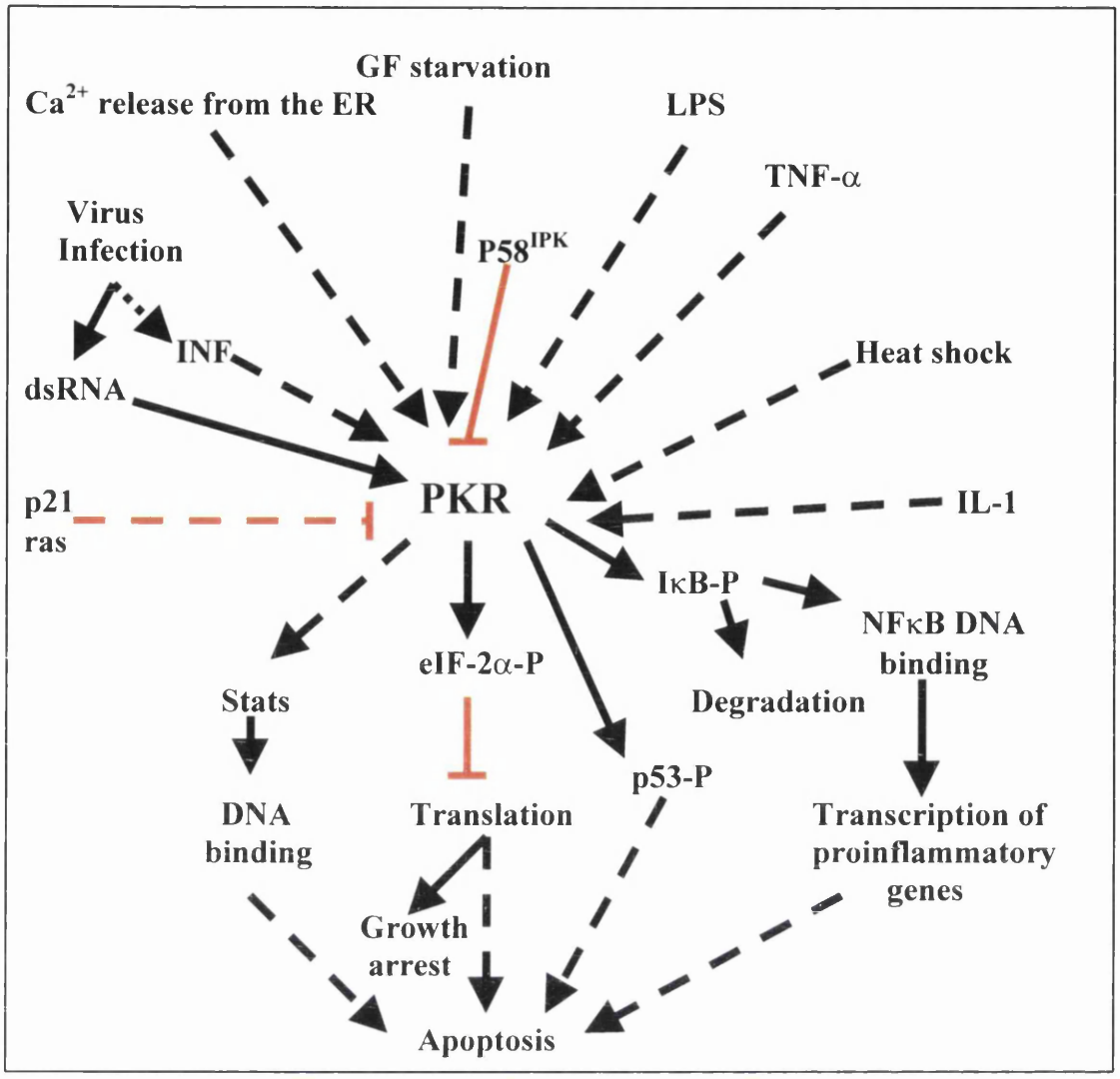


Fig. 1.12: The PKR stress response pathway.
Examples of stress pathways which impinge upon PKR. Dashed lines indicate indirect interactions, solid lines direct interactions, black lines stimulation, red lines repression. LPS (bacterial lipopolysaccharides), IL (interleukins), INF (interferons), ER (endoplasmic reticulum) and GF (growth factor). Recent reviewed by Williams (1999).

1.3.8 The DNA-binding activity of US11

Several studies have implicated US11 as a DNA binding protein. Firstly, Bayliss *et al.* (1975), isolated 16 virus-specific polypeptides by the chromatography of [^{35}S]-methionine labelled protein extracts of HSV-1 infected cells on a native calf thymus DNA-cellulose column. One of these species migrated at 21kDa, and may therefore correspond to US11. Using ^{125}I labelling of protein bound to purified HSV-1 DNA, Hyman (1980) demonstrated that 4 proteins were bound to HSV-1 DNA, one of which migrated at approximately 20kDa. Dalzeil & Marsden (1984) also isolated a HSV-1 21kDa protein by chromatography of infected cell proteins on a DNA-cellulose column. Furthermore, this 21kDa protein could be specifically competed off the column by a 400bp *a* sequence fragment of HSV-1. Conclusive proof that this 21kDa DNA-binding protein was US11 was provided by MacLean *et al.* (1987), by Western blot analysis of proteins which bound to DNA-cellulose with an anti-US11 polyclonal antibody.

However, further studies on the DNA-binding proteins of HSV-1 have not shown US11 to bind DNA and US11 does not appear to have a role in DNA cleavage or packaging. In addition, the localisation of US11 to the nucleolar compartment, from which HSV-1 DNA is excluded, would suggest an alternative role (MacLean *et al*, 1987; Puvion-Dutilleul, 1987). Roller & Roizman (1990) suggested that the DNA-binding activity of US11 detected in the above mentioned experiments was a result of its high basicity and not a specific interaction.

1.3.9 The RNA-binding activity of US11

To date US11 has been shown to bind five RNA substrates, the antisense transcript of the 5′ UTR of the US11 gene, a truncated transcript of UL34 RNA, the retroviral intronic export elements, the Rev-Response Element (RRE) of HIV-1 and the Rex-Response element (XRE) of HTLV-1, and the rRNA of the 60S ribosome subunit. How the interactions between these RNAs and US11 were elucidated and their interaction with US11 are discussed in detail below.

1.3.9.1 The antisense transcript of the 5′ UTR of the US11 gene

The interaction between US11 and the antisense transcript of its own 5′ UTR was described by Roller & Roizman (1990). It was discovered while looking for ICP4-RNA interactions and was the first time that US11 was found to display RNA-binding activity. Roller & Roizman (1990) used partially purified ICP4 from a crude nuclear extract of HSV-1 infected HeLa cells in an electrophoretic mobility gel shift assay (EMSA) with a radiolabelled transcript derived from the plasmid pRB3881, which includes the antisense transcript of the 5' UTR of US11. This transcript was retarded in EMSA with the crude ICP4 extract. However, the mobility shift generated was not as large as would be expected from ICP4 and could not be super-shifted when incubated with an anti-ICP4 antibody. Using the pRB3881 RNA probe with a crude nuclear extract of infected HeLa cells, the probe could be protected from RNase T1 digestion. It was noted that on native gels the pRB3881 RNA probe ran as two species, about 30% of the probe migrated as a slower species and 70% as the faster migrating species. Of the

two, only the slower migrating species shifted in EMSA with infected cell nuclear extract demonstrating that the binding was dependent on the secondary structure adopted by the probe. Deletions of the pRB3881 plasmid were made and the minimal binding element found to be 87nt, mapping within the 5' UTR of US11 on the rightward strand. The interaction between the component in the nuclear extract and the RNA was shown to be sequence-specific in competition assays. Infected cell nuclear extracts of HSV-1 x HSV-2 intertypic recombinants were used in RNase T1 protection assays to map the location of the gene encoding the RNA-binding protein, using the observed difference in mobility of the protected complex the two virus types as the basis for mapping. Furthermore, two viruses R7023 and R3630, which lack the US12 and US11 ORFs, did not possess the RNA binding activity. Using PAA it was shown that this activity was dependent on the expression of a late gene, and therefore, most likely to be US11. Using *in vitro* transcribed and translated US11 and an epitope tagged US11 virus construct the RNA-binding activity was shown to be due to US11.

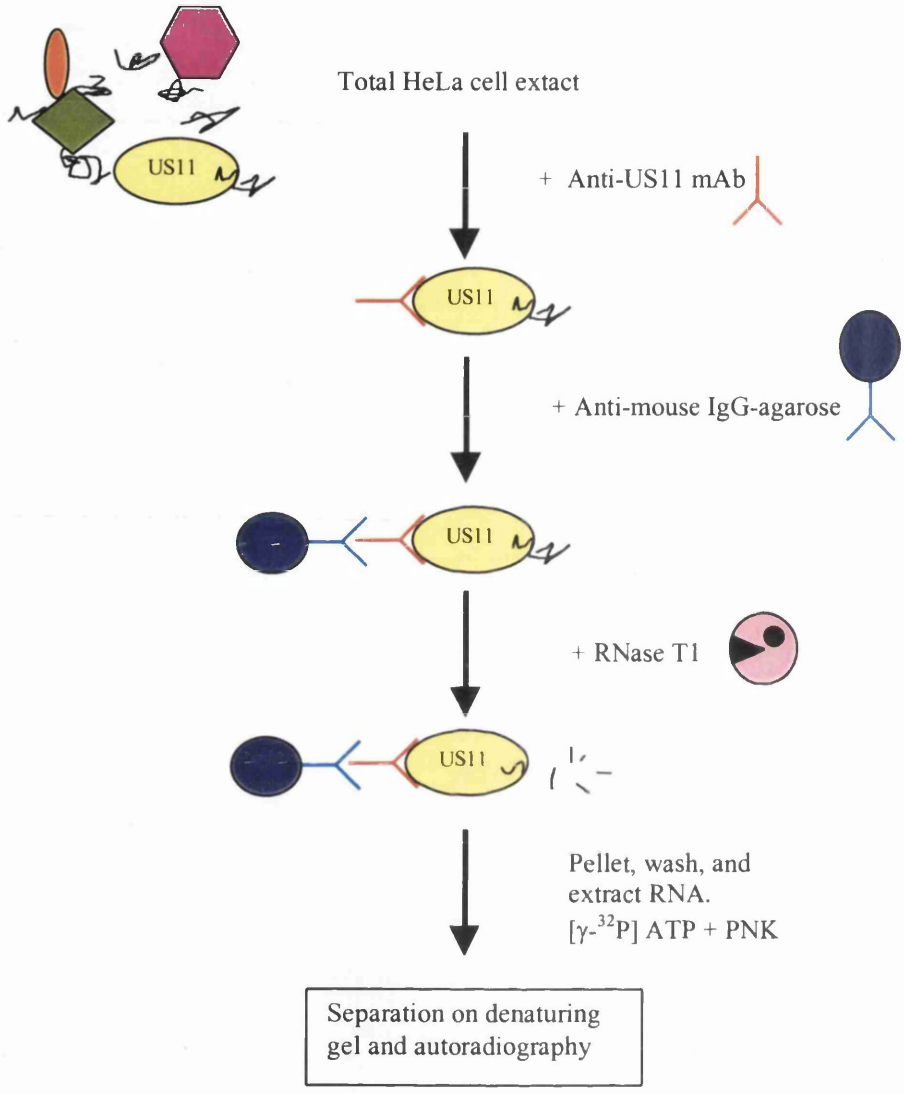
The transcript of the antisense 5' UTR of the US11 could not be detected in infected cells. Roller & Roizman (1990) suggested that this RNA is either a substrate analogue or it is down-regulated by US11 to an extent that it is undetectable in infected cells. However, recently, transcripts which are anti-sense to the US11 have been detected in infected cells: a 7.4kb polyadenylated transcript, and three non-polyadenylated species of 7, 1.3, and 1kb (McCormick *et al.*, 1999). Furthermore, in a virus in which 222bp of OriS is deleted, US11 accumulation is diminished and an approximately 16kb polyadenylated transcript which is antisense to the US11 ORF is expressed. This raises the possibility that transcripts antisense to the US11 ORF may regulate its expression.

1.3.9.2 Δ34 RNA

Roller & Roizman (1991) demonstrated that US11 could bind a truncated viral transcript in HSV-1 infected cells using co-immunoprecipitation of US11 and RNA. Total infected cell extracts of HeLa cells were incubated with a mouse anti-US11 mAb followed by the addition of goat anti-mouse antibody conjugated to agarose. To remove non-specific binding of RNA the reaction mixture was treated with RNase T1 and the US11/RNA/antibody complexes washed extensively. When RNAs were eluted and 5' end labelled using T4 polynucleotide kinase (PNK) and [γ–^{32}P]ATP as the phosphate donor and separated by denaturing gel electrophoresis. With a US11 null virus with or without the addition of an anti-US11 antibody or with *wt* virus without the anti-US11 antibody, no labelled RNA species were pulled out, but, using a anti-US11 antibody to pull out US11/RNA complexes from *wt* infected cells many RNA species were obtained which varied greatly in size (Fig. 1.13) The end labelled RNAs were used to probe a Southern blot of *Bam*HI digested HSV-1 DNA, a weak hybridization was seen with the 1.3kb *Bam*HI C' fragment.

Using the *Bam*HI C' region as a probe in a Northern blot of cytoplasmic RNAs, a small, non-polyadenylated RNA was shown to be expressed at 6-8 fold higher levels in *wt* virus infected cells compared to a US11$^-$ virus. Mapping of the transcript found that this 485nt RNA was derived from the UL34 region and that it has the same 5' end as UL34 but was truncated by 1015nt at the 3' end, terminating within the ORF. This RNA was termed Δ34 RNA.

A


US11
Total HeLa cell extact
+ Anti-US11 mAb
US11
+ Anti-mouse IgG-agarose
US11
+ RNase T1
US11
Pellet, wash, and
extract RNA.
[γ-32P] ATP + PNK
Separation on denaturing
gel and autoradiography

B

Fig. 1.13: Co-immunoprecipiation of US11-RNA complexes.
A: Diagrammatic representation of the protocol devised by Roller & Roizman (1991) for the isolation of RNAs that are bound by US11. Abbreviations: Antibody (Ab), monoclonal (m), immunoglobulin G (IgG), polynucleotydlyl kinase (PNK). B: The result from the experiment is shown and is reproduced from Roller & Roizman (1991).

Using an RNase T1 protection assay and native gel electrophoresis to resolve the protected species it was demonstrated that a protected complex formed when HSV-1(F) infected cell extracts were used, which could be supershifted with the inclusion of an anti-US11 mAb. Deletion mutants of the transcript were constructed and used to map the site of interaction with US11. As much as 257nt could be deleted from the 5' end of the transcript and still retain binding activity, whereas deletion of 36nt from the 3' end resulted in loss of binding. The binding site therefore resides within the terminal 264nt of Δ34.

Roller & Roizman (1991) suggest that the Δ34 RNA is derived either by endonucleolytic cleavage or premature termination of the full length UL34 mRNA . The sequence at and around the 3' end of the RNA consists of a run of T residues embedded in a G+C-rich sequence, and is capable of forming a secondary structure similar to that of termination sites of attenuated products of the c-*myc*, adenovirus major late, SV40 late and mouse minute virus P4 promoters. They therefore suggested that US11 may act as an anti-terminator for UL34 RNA or enhance its degradation. However, expression of full-length UL34 is not affected by the presence of US11, which would be expected if US11 were acting as an anti-terminator.

From the data presented by Roller & Roizman (1991) it can be concluded that US11 down-regulates the level of Δ34 RNA in infected cells. US11 may achieve this by preventing the truncation of UL34 mRNA either by acting as an anti-terminator within the nucleus or preventing endonucleolytic cleavage in the nuclear or cytoplasmic compartments. US11 may act within the nuclear compartment to retain the Δ34 RNA or target it for degradation. Alternatively, US11 may act in the cytoplasmic compartment to target the RNA for degradation.

1.3.9.3 Ribosomal RNA

US11 is strongly associated with the ribosomes which is thought to be at least in part responsible for its nucleolar localization. Roller & Roizman (1992) have shown by sucrose gradient fractionation that US11 co-sediments with the 60S, 80S and polysomal fractions, whereas, Masse et al. (1990) and Daiz et al. (1993), have also detected it in 40S fractions.

Using rRNA, in vivo labelled with [^{32}Pi], Roller & Roizman (1992) purified rRNA from the 40S (18S rRNA) and the 60S (28S rRNA, 5.8S rRNA and 5S rRNA) ribosomal subunits. The ability of a recombinant β-galactosidase-US11 fusion protein to bind rRNA in vitro was examined. A fragment of rRNA derived from the 60S rRNA was protected in an RNase T1 protection assay and the RNA/protein complex could be supershifted with the inclusion of an anti-US11 mAb in native gel electrophoresis. However, with the 18S rRNA derived from the 40S subunit was not protected from RNase T1 digestion by β-galactosidase-US11. It was therefore concluded that US11 was associated with the 60S subunit of ribosomes by virtue of its rRNA binding activity. Indeed the ability to associate with ribosomes and bind RNA do reside in the C-terminal domain of US11 which supports this data.

1.3.9.4 Rex- and Rev-response elements

i. Nuclear export of intron-containing viral transcripts by HIV-1 Rev

In eukaryotic cells mRNAs undergo a variety of post-transcriptional processing events such as polyadenylation and splicing, prior to their export to the cytoplasm as mature mRNAs. Indeed, transcription, processing and export are very tightly linked processes (reviewed by Cullen, 2000b; Hirose & Manley, 2000; Proudfoot, 2000). Nascent RNAs are retained in the nucleus until they are processed or degraded. Such a mechanism allows, not only another level of regulation on gene expression, but also prevents immature mRNAs from entering the cytoplasm where the proteins that they encode may be non-functional or deleterious to the cell.

Retroviruses express some of their proteins from incompletely spliced transcripts, these intron-containing transcripts would therefore be seen as immature mRNAs by the host cell machinery and hence retained in the nucleus. However, retroviruses have evolved ways of promoting the export of these mRNAs, and HIV-1 is one such example (Cullen, 1998). During its life cycle HIV-1 produces fully spliced transcripts encoding Nef, Rev and Tat, unspliced transcripts Gag, and Gag-Pol, and a singly spliced transcript encoding Env (Cullen & Greene, 1990). HIV-1 overcomes the nuclear retention of these intron-containing mRNA by encoding the Rev protein (Malim, *et al.*, 1988). Rev is essential for the expression of Gag, Pol and Env proteins and the cytoplasmic accumulation of their mRNAs. Rev acts by binding to a *cis*-acting intronic sequence in these mRNAs, termed the Rev-Response Element (RRE) via an arginine-rich RNA-binding motif (ARM) (Zapp & Green, 1989; Malim *et al.*, 1990). Upon binding, Rev

multimerizes via hydrophobic domains that flank its ARM, creating a ribonucleoprotein complex consisting of one molecule of RRE-containing RNA and over 10 Rev molecules (Malim & Cullen, 1991). Rev possesses an NLS (the ARM of Rev also functions a NES) and a leucine-rich NES, allowing it to shuttle in and out of the nucleus. The inactivation of the NES, by mutation of critical residues, results in a dominant-negative phenotype, in that, when co-expressed with *wt* Rev, both the mutant and *wt* Rev and intron-containing transcripts are retained in the nucleus (Venkatesh & Chinnadurai, 1990). This is because the dominant negative mutant can both bind the RRE and multimerise with functional Rev molecules, forming a complex which cannot be exported.

The NES of REV interacts with Crm-1, a member of the importin family of nucleocytoplasmic translocation factors which interact with nucleoporins of the nuclear pore complex (NPC) (Fornerod *et al.*, 1997; Neville *et al.*, 1997; Stade *et al.*, 1997). The Crm-1 dependent pathway is essential for the export of the cellular U-rich small nuclear RNAs (U snRNAs) and 5S rRNA (Cullen, 2000a). The export of intron-containing viral mRNAs is therefore achieved by Rev binding to the RRE and accessing a cellular export pathway.

Since the discovery of the ability of Rev to transactivate the cytoplasmic accumulation of intron-containing mRNAs, other viral and cellular proteins have been shown to be able to functionally substitute for Rev in this activity, among them are the Rev-homologues from other lentiviruses, the Rex protein of HTLV-1, and the US11 protein of HSV-1 (Table 1.5).

US11 and the HIV-1 protein Rev are both small, basic phosphoproteins which bind RNA and localize to the nucleolus. It was these similarities which prompted Diaz *et al.*

Table 1.5: Proteins that can functionally substitute for the complex retroviral proteins, Rex and Rev.

This table documents proteins which can functionally substitute for complex retrovirus Rev/Rex-related export proteins in promoting the export of their respective intron-containing RNA. The biological properties of these proteins are compared. A list of the column headings and abbreviations used are listed below in alphabetical order.

Atypical hydrophobic NES: A stretch of hydrophobic amino acids in which Leu residues are important but there is no core tetramer. BLV: Bovine leukaemia virus. Co-operation with Rev: Refers to the ability of a protein to synergise with Rev when co-transfected, resulting in higher levels of export of intron-containing RNA than either protein transfected alone or the sum of their individual transactivation. EIAV: Equine infectious anaemia virus. FIV-1: Feline immunodeficiency virus type-1. HERV-K: Human endogenous retrovirus-K family. HIV-1: Human immunodeficiency virus type-1. HIV-1 rev^- virus rescue: The ability of a protein restore replication/late gene expression of a rev^- provirus in a transfection assay. HTLV-1/-2: Human T-lymphoma virus type-1/-2. Homo-oligomers: The ability of a protein to interact with itself forming homo-oligomers. ICVR: Intron-containing viral RNA. KH domain: HnRNP K homology RNA-binding sequence. Leu-rich NES: A stretch of hydrophobic amino acids which possess a critcal core tetramer, LXLX. ND: Not done. NES: Nuclear export signal. NLS: Nuclear localization signal. RGG box: Arg Gly Gly repeat RNA-binding motif. RPX repeats: Arg Pro, X = any amino acid. RRM: Arginine-rich motif. SIV-1: Simian immunodeficiency virus. Shuttling: Active transport of the protein between nuclear and cytoplasmic compartments in both directions. Substitution: Refers to the ability of a wild type protein to functionally substitute for the wild type Rev/Rex proteins of complex retroviruses in promoting the cytoplasmic accumulation of RNA derived from reporter constructs containing the complex retroviral RNA response elements. TD negative: A trans-dominant negative phenotypic mutant. The ability of a mutant in a given protein to inhibit the function of a wild type protein when co-expressed and cause the nuclear retention on intron-containing viral mRNA.

Protein	Function	Substituion	Co-operation with Rev	HIV-1 rev⁻ virus rescue	Localization	RNA binding domain	NLS/ NES	Shuttling	Export pathway	Homo-oligomer	TD negat-ive	Reference.
HIV-1 Rev	Export of ICVR	HERV-K c-ORF, HIV-2 & SIV Rev	N/A	N/A	Nucleus & cytoplasm. Nucleolar	RRM	Leu-rich NES Arg-rich NLS	Yes	Crm-1	Yes	Yes	Malim *et al.*, 1988; Lewis *et al.*, 1990; Berchtold *et al.*, 1994; Kalland *et al.*, 1994; Madore *et al.*, 1994; Magin *et al.*, 1999.
FIV-1 Rev	Export of ICVR	HIV-1 Rev	N/D	N/D	Nucleolar	RRM	Atypical hydro-phobic NES	Yes	Crm-1	N/D	N/D	Phillips *et al.*, 1992; Zou *et al.*, 1997; Otero *et al.*, 1998.
HTLV-1 Rex	Export of ICVR	HIV-1 & -2 Rev, BLV + HTLV-2 Rex	N/D	Yes	Nucleus & cytoplasm. Nucleolus.	RRM	Leu-rich NES Arg-rich NLS	Yes	Crm-1	Yes	Yes	Hidaka *et al.*, 1988; Rimsky *et al.*, 1988; Felber *et al.*, 1989; Rimsky *et al.*, 1989; Lewis *et al.*, 1990; Kim *et al.*, 1996;.
HLTV-2 Rex	Export of ICVR	HIV-1 & 2 Rev	No	N/D	Nucleus & nucleolus	RRM	Leu-rich NES Arg-rich NLS	Yes	Crm-1	Yes	Yes	Rosenblatt *et al.*, 1988; Lewis *et al.*, 1990; Kim *et al.*, 1991; Bakker *et al.*, 1996; Ciminale *et al.*, 1997.
Human Sam68	G1/S phase progression	HIV-1 & -2 & EIAV Rev, HTLV-1 & -2 Rex	Yes	Yes	Nucleus excluded from nucleolus	KH domain RGG box	Pro/Tyr-rich NLS	Yes	Tap/ RHA	Yes	Yes	Reddy *et al.*, 1999; & 2000a & b.
HSV-1 US11	RNA binding protein. Suppression of PKR activation.	HIV-1 Rev, HTLV-1 Rex	Yes	No	Nucleus & cytoplasm, Nucleolus.	RXP repeats	N/D	N/D	N/D	Yes	Yes	Diaz *et al.*, 1996; Schaerer-Uthurraly; 1998; Duc Dodon *et al.*, 2000.

(1996) to examine whether US11 could functionally substitute for the Rev protein in promoting the export of intron-containing retroviral mRNA.

ii. US11 interacts with the XRE of HTLV-1

In transfection assays Diaz *et al.* (1996) demonstrated that US11 could promote the export of a non-spliceable and spliceable primary transcript of the HTLV-1 *env* gene and hence, transactivate glycoprotein expression. *In vitro* binding assays were performed with the XRE and recombinant GST-US11, demonstrating that US11 is capable of binding this element. The XRE and Δ34 RNAs were equally good substrates for US11 *in vitro* , with a Kd of 100-130nM. However, US11 has a greater affinity for the XRE than the RRE (Schaerer-Uthurralt *et* al., 1998).

iii. US11 interacts with the RRE of HIV-1

US11 can promote the cytoplasmic accumulation of a non-spliceable primary transcript of HIV-1 *env* mRNA and hence, glycoprotein expression. US11 and the Rex protein can transactivate the expression of HIV-1 *env* to a similar degree, about 5-6 fold less than that of Rev itself. *In vitro* binding assays have demonstrated that US11 can bind to the HIV-1 intronic export element in the RRE (Diaz *et al.*, 1996). However, the binding of US11 does not exactly mirror that of Rev. While Rev binds to the Stem-Loop (SL) II of the RRE, US11 cannot bind this element either because it requires additional sequences or as it binds elsewhere on the RRE (Duc Dodon *et al.*, 2000). This is not surprising as US11 and Rev share no real sequence homology.

As with Δ34 RNA, deletion of the C-terminal domain of US11 prevents it from binding the XRE and RRE. Deletion of the first 40 amino acids of US11 do not effect its ability to bind the XRE/RRE. However, US11Δ1-40 is unable to transactivate *env* expression and when co-transfected with the full-length protein acts in a *trans*-dominant negative fashion (Diaz *et al.*, 1996). This indicates that US11Δ1-40 may be multimerizing with full length US11, forming non-functional complexes and preventing transactivation.

Unlike the transactivation of HTLV-1 *env* expression, US11 is unable to support the cytoplasmic accumulation of a spliceable HIV-1 *env* transcript. It is also unable to rescue a rev$^-$ infectious pro-virus (Diaz *et al.*, 1996; Duc Dodon *et al.*, 2000), but can co-operate with sub-optimal amounts of Rev protein to promote rev$^-$ HIV-1 replication by promoting cytoplasmic accumulation of singly spliced and unspliced mRNAs (Duc Dodon *et al.*, 2000). The transactivation of glycoprotein expression is dependent on the presence of the Rev-NES and the first 40 amino acids of US11 as constructs with either domain deleted are unable to rescue rev$^-$ HIV-1.

Co-transfection of a US11 expression construct with a construct containing the RRE fused to an intron-containing CAT gene and suboptimal amounts of a Rev-expressing vector also allowed the export of unspliced CAT mRNA. This export was shown to be mediated by the Crm-1 pathway as it was inhibited by leptomycin B to a similar extent with optimal amounts of Rev alone and with US11 and sub-optimal amounts of Rev (Duc Dodon *et al.*, 2000).

iv. US11 can interact with Rev

Using the mammalian monohybrid assay Duc Dodon *et al.* (2000) demonstrated that US11 can interact with Rev bound to the SLII of the RRE. Additionally, in

immunoprecipitation and GST-pull-down assays US11 could be co-precipitated with Rev. A mutant of Rev (GST-m10Rev) with the NES removed could still interact with US11.

v. The functional significance of US11 and the RRE/XRE

Although many retroviral proteins have been shown to be able to substitute for Rev in the export of intron-containing HIV-1 mRNA, US11 is the first example of a protein from a heterologous viral system performing such a role. It is not clear how US11 acts in this instance, but it is reasonable to propose that it either works by preventing the nuclear retention of these transcripts or by accessing the export pathway. Intron-containing retroviral transcripts may be retained in the nucleus either by being trapped in spliceosomes (Chang & Sharp, 1989) or by nuclear retention factors that interact with *cis*-acting regulatory sequences (CRS) (King *et al.,* 1998). The threshold level for Rev in infection and transfection assays is thought to arise because Rev is competing with spliceosomal components or factors which bind CRS to prevent nuclear retention and to direct them to export pathways, rather than a threshold for transport itself (Bakker *et al.*, 1996). US11 alone can support the cytoplasmic accumulation of a HTLV-1 spliceable *env* transcript, but not for the HIV-1 *env* RNA, which requires an unspliceable transcript (Diaz *et al.*, 1996). If US11 is acting to prevent nuclear retention this can, at least in part, be explained by the higher affinity US11 has for the XRE, allowing it to exclude or compete with retention factors. Removing the splice sites from the RRE or co-operation with Rev may bypasses the need for high affinity binding by US11. Alternatively, US11 may act to promote the nuclear export of these mRNAs by acting as an auxiliary protein, accessing and recruiting other factors. In the presence of US11 and sub-optimal

levels of Rev export is still dependent on the Crm-1 pathway and the Rev-NES, demonstrating that, in this instance, US11 is not the primary factor for export.

Why US11 binds the XRE and RRE is unclear, as they share no real sequence similarities with the HSV-1 RNAs bound by US11. The XRE and RRE are highly structured and may act as a substrate analogue for US11, mimicking the secondary structure adopted by its natural RNA partners.

In the monohybrid assay, immunoprecipitation and GST-pull down experiments US11 was shown to interact with the Rev protein, however no satisfactory explanation for this interaction can be given (Duc Dondon *et al.*, 2000). As these proteins share an RNA-binding partner, it is conceivable that in these experiments RNA was acting to bridge the proteins, rather than a direct protein-protein interaction.

One interesting aspect of the ability of US11 to co-operate with suboptimal amounts of Rev to promote viral replication, is that HSV-1 infection could increase the likelihood of HIV-1 replication and the progression of the disease to AIDS (Duc Dondon *et al.*, 2000). The potential interaction of HSV-1 and HSV-2 with HIV-1 has been studied in CD4+ T lymphocytes and in monocyte-macrophages and results in increased replication for both viruses. ICP0 and ICP27 have been implicated in the up-regulation of HIV-1 replication (Golden *et al.*, 1992). ICP0 increases transcription from the LTR of HIV-1 in association with NF-κB (Vlach & Pitha, 1993). ICP27 presumably acts either through its ability to bind RNA and promote 3' end processing, its involvement with RNA export, or its disruption of splicing components (Phelan *et al.*, 1996). US11, therefore, represents another factor that could contribute to the increased efficiency of HIV-1 replication with co-infection of HSV-1 or -2.

1.3.9.5 The XRP motif as an RNA-binding domain

As discussed in Section 1.3.3, the C-terminal domain of US11 consists almost entirely of a tandemly reiterated tripeptide, XRP (where X is any amino acid). This domain is predicted to form a poly-L-proline type II helix (PPII-helix) with 3 amino acids per turn. Both Roller *et al.* (1996) and Schaerer-Uthurralt *et al.* (1998) have found that deletion of this domain or within this domain disrupts RNA binding. This domain is an unusual RNA-binding domain, as most identified RNA binding motifs are either α-helical (such as the HIV-1 Rev ARM) or a β-hairpin (such as with HIV-1 Tat protein). RNA binding by β-hairpin motifs is predominantly directed by hydrogen bonds between the protein mainchain and the RNA, whereas, in interactions between α-helices and RNA the amino acid side chains provide the majority of interactions (Grate & Wilson, 1997). The PPIIH, because of its helical nature, would also presumably, rely more heavily on interactions directed by its amino acid side chains with RNA. The PPIIH is a flexible structure. RNA-protein binding reactions are frequently characterised by a structural transition either in the RNA, the protein or both. This involves regions of flexibility or disorder adopting more stable and energetically favourable conformations (Guzman, 1998; Williamson, 2000). The PPIIH represents a structure in which conformational changes are easily accommodated particularly in binding reactions.

The C-terminal domain is rich in arginine, a residue which is frequently implicated in protein-RNA interactions. Arginine is a very versatile amino acid in protein-RNA binding. It may be involved in charge neutralisation of the phosphate backbone and has the potential to form a network of hydrogen bonds. Additionally, van der Waals forces

and hydrophobic interactions from the long hydrocarbon side chain also play a major role in its involvement with the binding of RNA (Grate & Wilson, 1997; Weiss & Narayana, 1998). Proline, also over represented in the C-terminal domain of US11, can provide hydrophobic interactions and form hydrogen bonds with RNA. However, more frequently proline is involved in mediating protein-protein interactions, requiring a high degree of solvation for stability, and could be potentially involved in the formation of homomultimers by US11.

The only other XRP repeat protein that has been implicated in RNA binding is the EB2 protein of EBV. This protein, which shuttles between the nucleus and the cytoplasm, appears to be involved in RNA export and the processing of pre-mRNA (Buisson *et al.*, 1989; Forjot *et al.*, 2000). Both the EB2 protein and its 21 amino acid XRP repeat motif appear to bind RNA in a non-specific fashion. However, the XRP motif does not appear to be required for the effect of EB2 on RNA export or splicing (Buisson *et al.*, 1998).

1.4 Methods for identifying RNAs which interact with proteins

During the course of this work on US11, a method for the isolation and amplification of RNAs that interact with US11 was developed. Prior to this work there were no methods specifically available for the identification of native RNAs that interact with a particular protein. During the completion of this work however, several methods were published by which RNAs that interact with a particular protein may be isolated and identified. These methods are described below. In addition, SELEX, a method which allows the isolation of synthetic RNAs which bind a protein with high affinity, and the yeast three-hybrid system are discussed.

1.4.1 Systematic Evolution of Ligands by Exponential Enrichment

Systematic evolution of ligands by exponential enrichment (SELEX) was first developed by Tuerk & Gold (1990) to enable high affinity RNA ligands for proteins to be isolated from a pool of random RNA sequences. The protocol allows a randomised pool of RNAs to be selectively enriched for high affinity binders by successive rounds of protein binding, RT-PCR and *in vitro* transcription. A randomised sequence or a defined sequence mutated at specific sites is sandwiched between a 5′ promoter for the binding of a DNA-dependent RNA polymerase and a 3′ site for the hybridization of a primer for the initiation of reverse transcription. The DNA template is *in vitro* transcribed and the RNA is selected for the binding of a particular protein immobilized on a nitrocellulose membrane. The RNAs are eluted, reverse transcribed and PCR amplified (Fig. 1.14). The PCR products should therefore be over represented in DNA that encodes RNAs with an affinity for the protein in question. This pool of PCR products are again transcribed and the RNAs put through the same round of selection. In general, after approximately four rounds of selection the population of RNAs is sufficiently enriched for high affinity binders to allow the DNA templates to be cloned and sequenced.

This elegant technique has several advantages. It allows, without the need of any prior sequence information, the elucidation of high affinity RNA ligands for a protein of interest. From such information, a consensus binding site may be derived, particularly if there are existing native binding sites available for comparison. The establishment of a defined consensus sequence may enable the identification of the *in vivo* targets of a protein. Also, this information helps define the nucleic acid residues critical in the

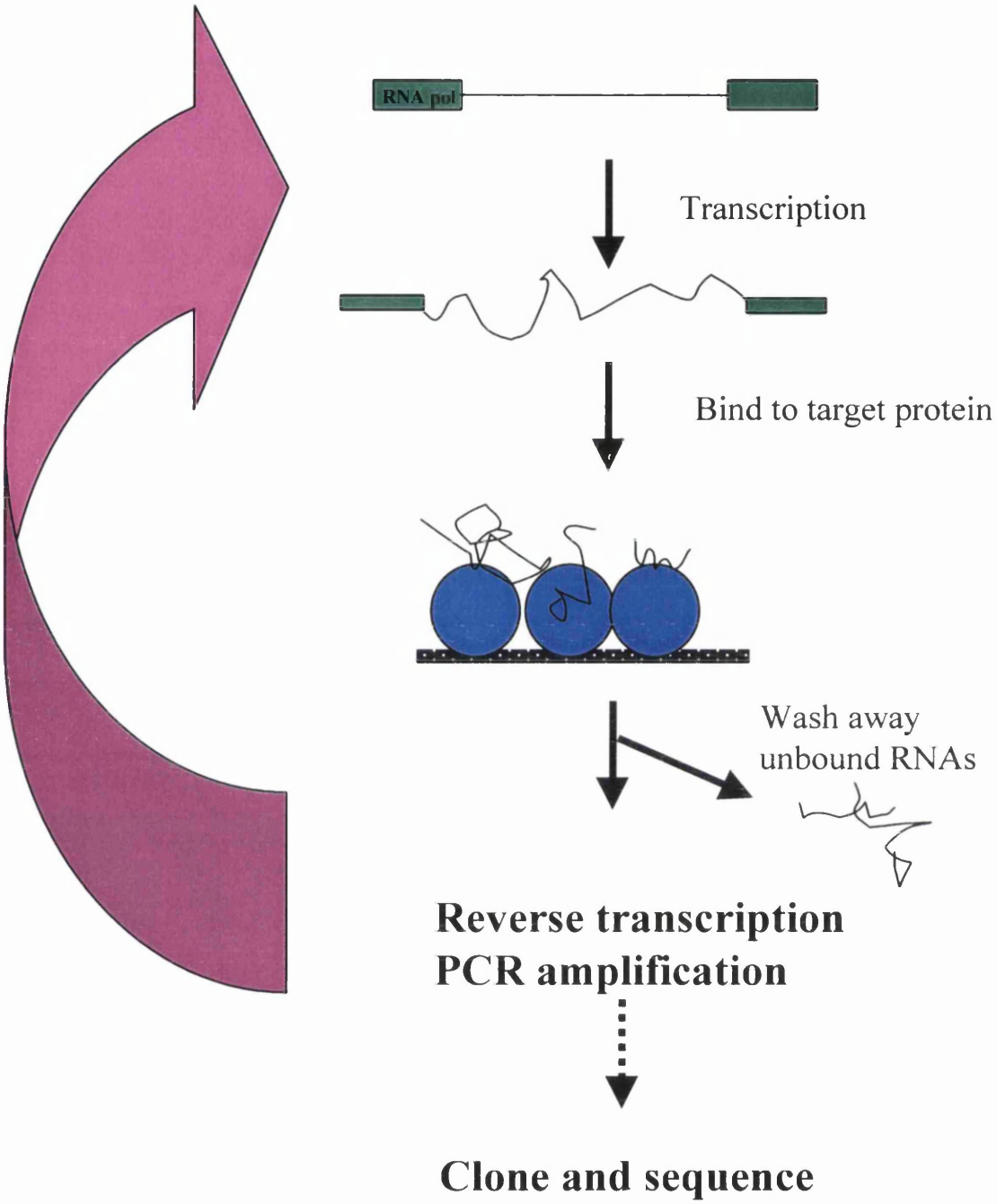


Fig. 1.14: A schematic diagram of SELEX.
Randomized DNA sequences (black line) flanked by priming sites for reverse transcription and PCR (green boxes) and a DNA-dependent RNA polymerase promoter (labelled RNA pol) site are transcribed. The RNAs are bound to the protein of interest (blue) immobilized on nitrocellulose (spotted box). The unbound RNAs are removed by washing and the bound RNAs eluted. The bound RNAs are reverse transcribed and amplified by PCR. The resulting pool of templates, enriched for sequences encoding RNA that interacts with the protein of interest, are subjected to further rounds of selection as before. After several rounds the templates are cloned and sequenced.

interaction. Therefore, SELEX can potentially save time over the mapping of binding sites and mutation of residues within the RNA element to define the consensus/critical residues. SELEX can also give information on whether the conservation of a particular RNA secondary structure is important in protein binding by showing if particular base-pairing is conserved during the evolution of RNA ligands.

SELEX is also being used to generate biologically active compounds for therapeutic applications (Gold *et al.*, 1995). High affinity RNA ligands can, in general, be selected for non-RNA binding proteins as well as RNA binding proteins. This is thought to be due to the tertiary structure adopted by RNAs which can mimic protein structure important in protein-protein interactions. Hence, for a given protein, either RNA or non-RNA binding, a high affinity RNA ligand can be selected which can act as a highly specific inhibitor of protein function. Thus, if introduced *in vivo,* this RNA may potentially inhibit a deleterious activity of a given protein. For example, SELEX has been used to generate an RNA molecule which can inhibit HIV-1 reverse transcriptase, which has an affinity for this enzyme over 100 times that of the natural substrate *in vitro* (Tuerk & MacDougal-Waugh, 1993; Kensch *et al.*, 2000) which may have potential therapeutic value in the suppression of AIDS in HIV positive individuals. Also, a DNA molecule (NX21909), generated by SELEX-like procedure to bind the active site of neutrophil released elastase modified by the addition of a valyl phosphonate moiety, can non-reversibly cross link with the active site serine of the enzymes catalytic site. This molecule has been shown to inhibit inflammation of the lung in mouse models, and represents a promising therapy for lung inflammatory diseases such as adult respiratory syndrome and ischemic reperfusion injury (Bless *et al.*, 1997).

In addition to the therapeutic applications of SELEX generated high affinity RNA ligands, these RNAs may also be used as diagnostic tools due to their high specificity acting similarly to antibody based diagnostic techniques (Jayasena, 1999).

The major disadvantage of SELEX is that the theoretical RNA substrates it generates may have no *in vivo* relevance. SELEX is performed with RNAs in vast molar excess of the protein in question to allow a high degree of competition between the RNAs for the binding site. Although this selects for very high affinity RNA ligands, an RNA more similar to the natural substrate may be lost in the battle for a binding site. Ultimately, the RNA ligands generated by SELEX are only as good as the initial randomised pool.

1.4.2 The Yeast three-hybrid system

The yeast three-hybrid system is an extension of the yeast two-hybrid system. Both systems rely on the fact that transcription factors can be divided into two domains the transcription activation domain and the DNA binding domain (Fields & Sternglanz, 1994). In the yeast two-hybrid system the fusion of two interacting proteins, one to the activation domain and one to the DNA binding domain, allows the two transcription factor domains to be brought into close proximity, stimulating transcription of a reporter gene down stream of a *cis*-acting DNA element which is the target of the DNA-binding domain. Using this system, a cDNA library can be screened for proteins which interact with a protein of interest (Fig. 1.15).

The three-hybrid system is an extension of this principle which allows the proteins fused to the activation domain and DNA binding domain to be bridged by a third interacting molecule, either RNA or protein (Zhang *et al.*, 1999). Where RNA is used as the bridging molecule, the DNA binding domain is fused to the bacteriophage MS2 coat

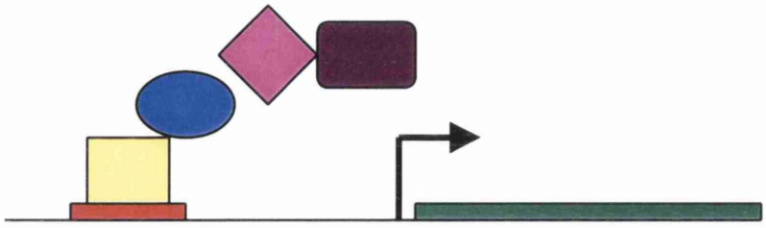


Fig. 1.15: A schematic representation of the yeast two-hybrid system.
The activation domain (purple) is brought into close proximity of a DNA binding domain (yellow) by the interaction of two proteins (blue and pink) so that transcription of a reporter gene (green) lying down stream of a *cis*-acting DNA element (red) bound by the DNA binding domain can be stimulated.

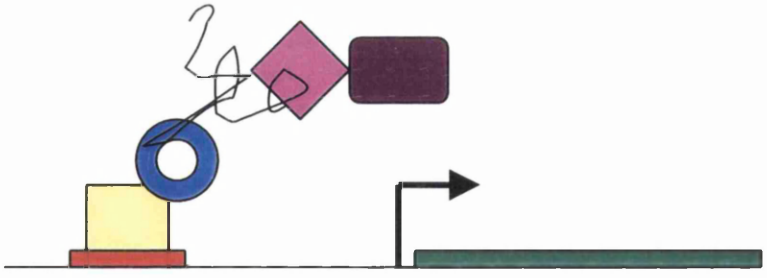


Fig. 1.16: A schematic representation of the yeast three-hybrid system.
The activation domain (purple) is brought into close proximity of a DNA binding domain (yellow) by the interaction of a protein (pink) with its target RNA which is fused to MS2 RNA (black line) which in turn interacts with the MS2 coat protein (blue) fused to the DNA binding domain (yellow). This allows the transcription of a reporter gene (green) lying down stream of a *cis*-acting DNA element (red) bound by the DNA binding domain.

protein and the protein of interest or a cDNA library to the activation domain. RNA is expressed from a third vector in which an RNA of interest is fused to two copies of MS2 RNA. In this way the activation domain is brought in close proximity to the DNA binding domain when the protein it is fused to interacts with the RNA of interest via the binding of the MS2 coat protein to its specific RNA (Fig.1.16).

1.4.3 RT-PCR based methods of isolating RNAs which interact with a protein of interest

1.4.3.1 Isolation of RNAs which interact with polycytidylate-binding protein α-complex protein 1

Polycytidylate-binding protein α-complex protein 1 (αCP1) has been implicated in mRNA stability and translational regulation. Trifflis *et al.* (1999) investigated the ability of a GST-αCP1 fusion protein to pull out RNAs from infected cell lysates which were subsequently amplified by RT-PCR.

GST or GST-αCP1 bound to glutathione-agarose were incubated with nuclear extracts, the complexes washed and the RNAs eluted. The RNAs were amplified by differential display RT-PCR (DD RT-PCR). Firstly, the RNA was reverse transcribed using T_{11} primers anchored at the 3′ end which anneal to the poly(A) tail, with one nucleotide (G, C or A). The cDNA was amplified by one round of PCR in the presence of [α-^{32}P] dCTP, one of the 3′ primers and random primers. The products were separated on a sequencing gel and those specific to GST-αCP1 were extracted and PCR amplified in the presence of the same primers. Ten different sequences were pulled out: four of which did not have any homologous sequences in data base searches, two mitochodrial

enzyme mRNAs, two membrane receptor RNAs, one ribosomal RNA, and managanese superoxidase dismutase mRNA.

1.4.3.2 Isolation of RNAs that interact with hnRNP A2

HnRNP A2 is a shuttling protein which associates with polysomes and has been implicated in the export of mRNAs, the regulation of translation and possibly the control of mRNA stability (Shyu & Wilkinson, 2000). Brooks & Rigby (2000) immunoprecipitated hnRNP A2-RNA complexes and amplified the co-immunoprecipitating RNAs using RT-PCR.

HnRNP A2 was immunoprecipitated from polysome preparations from a THP-1 myelomonocytic cell line. The isolated complexes were treated with proteinase K and the RNA extracted. The isolated RNAs were reverse transcribed in the presence of an oligo d(T) primer. The ss cDNA was homopolymer tailed with terminal deoxynucleotidyl transferase and dATP. The tailed cDNA was PCR amplified with an oligo d(T) primer, which could hybridize to both the 5′ and 3′ ends. The resulting products were examined by agarose gel electrophoresis. The products derived from immunoprecipitation performed with pre-immune serum yielded a smear ranging from 100-500bp, but those derived from hnRNP A2 immunoprecipitation produced a larger smear ranging from 100-2000bp. The products were labelled by random priming in the presence of [α-^{32}P] dCTP and used to probe a THP-1 Lambda Zap library. No overlap was observed of plaques which bound non-specific (pre-immune precipitated) species and hnRNP A2 immunoprecipitated species. 31 unique sequences were pulled out, of these 74% of the mRNAs contained U-rich elements and 13% were derived from RNAs encoding ribosomal proteins.

1.4.3.3 Isolation of RNAs that interact with fragile X mental retardation protein

Fragile X syndrome is one of the most common causes of mental retardation and is caused by the lack of expression of the Fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein which is thought to associate with a subset of foetal brain mRNAs, a few of which have been defined, FRMP mRNA, myelein basic protein (MBP) mRNA, and 60s rRNA (Ashley *et al.*, 1993; Siomi *et al.*, 1996).

Sung *et al.* (2000) used recombinant FMRP to pull out interacting RNAs and amplified the RNAs using DD RT-PCR. Biotinylated FMRP bound to avidin beads and incubated with total parietal cortex RNA, the complexes washed and the RNA extracted. First strand synthesis of cDNA was carried out using one of three poly(A) tail-dependent primers ($T_{12}GC$, $T_{12}GG$, or $T_{12}GA$). PCR amplification was carried out in the presence of [α-^{32}P]dCTP with one of the poly(A)-dependent primers and with one of six random 15-mer primers (this set of 18 primer combinations amplifies approximately 10-12% of the mRNA content of human brain mRNA). The products from this reaction were separated by electrophoresis, and bands specific to FMRP were excised from the gel and re-amplified with the corresponding primer sets. The amplified products were cloned into the pCR-3.1 vector and sequenced. In total nine DD RT-PCR bands were isolated and sequenced, one corresponding to the known binder, FRMP mRNA, three with homology to mRNAs and four representing unknown RNA, and one with homology to mitochondrial rRNA. Five of the sequences (excluding FMRP mRNA) were assayed for the binding of FRMP *in vitro* and all were found to bind.

1.5 Aims

To date, US11 has been shown to interact with five RNAs, which appear to share little in common in terms of sequence or biological role. Roller & Roizman (1990) first demonstrated that US11 possessed an RNA-binding ability, identifying an interaction with an *in vitro* derived transcript antisense to the 5′ UTR of US11. Subsequently, US11 was shown to bind and down-regulate Δ34, a truncated transcript of UL34 (Roller & Roizman, 1991). US11 has also been shown to be able to promote the cytoplasmic accumulation of HIV-1 and HTLV-1 RNAs containing export elements (Diaz *et al.*, 1996). *In vitro,* US11 also displays an affinity for rRNA derived form the 60s ribosome subunit (Roller & Roizman, 1992).

It would seem reasonable to suggest that US11 may bind to and influence the expression of other RNAs in infected cells. The aim of this project was therefore to test this hypothesis and characterise the interaction of US11 with any novel RNA partners discovered.

2.0 Materials & Methods

2.1 Materials

2.1.1 Plasmids

pQE-US11: Contains the US11 ORF cloned into the *Hind*III and *Sal*I sites in pQE-30; expresses 6xHis N-terminally tagged US11.

pQE-16: Enodes the dihydrofolate reductase gene C-terminally 6xHis tagged (Qiagen).

GST-US11 constructs: Schaerer-Uthurralt *et al.* (1998). A gift from J-J Diaz and J-J Madjar, Immuno-virologie Moleculaire et Cellulaire, Université Lyon, France.

2319: The entire US11 ORF cloned into the pGEX-2T vector at the *Sma*I and *Eco*RI sites; expresses full length GST-US11.

2450: The sequence encoding residues 85-149 of US11 cloned into the pGEX-2T vector at the *Sma*I and *Eco*RI sites; expresses GST-US11Δ1-84.

2625: The sequence encoding residues 1-87 of US11 cloned into the pGEX-2T at the *Sma*I and *Eco*RI sites; expresses GST-US11Δ88-149.

pGM1: Contains the γ-actin sequence (Amersham International).

pT-12/14: Sequence 585nt derived from "Sequence-Independent" RT-PCR of RNAs that interact with US11; homologous to 27332-26748nt of HSV-1 cloned into pGEM-T Easy.

pT-Δ34: A 451nt sequence (69462-69913nt) sequence derived from of the UL34 ORF of HSV-1 cloned into pGEM-T Easy.

2.1.2 Primers

The names and sequences of primers used in PCR for the cloning of inserts, the construction of *in vitro* transcription templates and for RT-PCR are given in Table 2.1.

2.1.3 Enzymes

Restriction enzymes were obtained from Boehringer Mannheim or New England Biolabs. T4 DNA ligase (10U/μl), RNase T1(100U/μl) and polynucleotide kinase (10U/μl) were obtained from Boehringer Mannheim. SuperScript™ reverse transcriptase (200U/μl) and terminal deoxynucleotydyl transferase were obtained from Gibco BRL. Taq DNA polymerase (5U/μl) was purchased from Applied Biosystems. Lysozyme was obtained from Sigma. RNase-free DNAse I (1U/μl) was obtained from Roche Diagnostics. T7 RNA polymerase (15U/μl) was obtained from Promega. SP6 RNA polymerase (50U/μl) was purchased from Stratagene.

2.1.3 Bacteria and culture media.

2.1.3.1 Bacteria

Plasmids were propagated and maintained in the *E.coli* strain DH5α: Φ80d *lac*ZΔM15, *rec*A, *end*A1, *gyr*A96, *thi*-1, *hsd*R17 (r_{k-}, m_{k+}), *sup*E44, *rel*A1, *deo*R, Δ(lacZY-argF) U169.

A: PCR primers for vector inserts

Insert	5′ primer	3′ primer
US11 ORF. Cloned into pQE-30. Expression of 6xHis US11.	GTAGGTCGACTCGAGATGAGCCAGACCCAAC	GATGAAGCTTGAGCGTATGCTCCATGTTGTG
Δ34. Cloned into pGEM-T Easy. *In vitro* transcription	GTGTGCACGGCGAGCTGCTC	AGCACCGACACACGCCGGTG

B: Primers for the construction of *in vitro* transcription templates

In vitro transcription template	5′ primer	3′ primer
T7-Δ34. *In vitro* transcription.	CAGAGATGTAATACTCACTATAGGGCCGTGT GCACGGCGAGCTGCTC	AGCACCGACACACGCCGGTG
T7-12/14. *In vitro* transcription.	CAGAGATGTAATACTCACTATAGGGCCTGGA GTTGGTGGTTAGCG	GTGGTTGTCAGCGGAAGACTGTTAG
1/6. *In vitro* transcription	CAGAGATGTAATACTCACTATAGGGCCAACC AGCTGTCGCCGGACTTC	GTGGTTGTCAGCGGAAGACTGTTAG
2/6. *In vitro* transcription	CAGAGATGTAATACTCACTATAGGGCGAGAC CAACACCCACGGCCTG	GTGGTTGTCAGCGGAAGACTGTTAG
3/6. *In vitro* transcription	CAGAGATGTAATACTCACTATAGGGCCGGAT CGGTGTATAAATTAC	GTGGTTGTCAGCGGAAGACTGTTAG
4/6. *In vitro* transcription	CAGAGATGTAATACTCACTATAGGGCCGCGC TGTCGTGAGAATCAG	GTGGTTGTCAGCGGAAGACTGTTAG
5/6. *In vitro* transcription	CAGAGATGTAATACTCACTATAGGGCCAGCA TGTCCGCCGGGACGC	GTGGTTGTCAGCGGAAGACTGTTAG

C: Primers used in reverse transcription

Δ34 RT-PCR	Δ34R: AGCACCGACACACGCCGGTG
PA RT-PCR	Oligo-d(T)$_{17}$: TTTTTTTTTTTTTTTT
SI RT-PCR	Random 9-mer primers

D: Primers used in the first round of PCR

RT-PCR	5′ primer	3′ primer
Δ34 RT-PCR	Δ34F: GTGTGCACGGCGAGCTGCTC	Δ34R: AGCACCGACACACGCCGGTG
PA RT-PCR	Abridged amplification primer (AAP): GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Oligo-d(T)$_{17}$-anchor primer: TCGAGCATCGGATCCTTACCTTTTTTTTTTTTTTTT
SI RT-PCR	Abridged amplification primer (AAP): GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Random 9-mer primers

E: Primers for second round of PCR

RT-PCR	5′ primer	3′ primer
Δ34 RT-PCR	Δ34NF: GCTGACGCGCGTGCTGTTGG	Δ34NR: GTAAGGGTTGCAGGGGACCTC
PA RT-PCR	Abridged universal amplification primer (AUAP): GGCCACGCGTCGACTAGTAC	3′ anchor site primer (3′ ASP): TCGAGCATCGGATCCTTACC
SI RT-PCR	Abridged universal amplification primer (AUAP): GGCCACGCGTCGACTAGTAC	Random 9-mer primers

Table 2.1: Primers. A: The primers used in the PCR of fragments for cloning. B: The primers used in the construction of *in vitro* transcription templates. C: Primers used in reverse transcription. D: Primers used in the first round PCR of ss cDNA. E: Primers used in the second round PCR of cDNA.

6xHis-tagged proteins were expressed in M15[pREP4]: *Nal*^{S}, *Str*^{S}, *rif*^{S}, *lac*$^{-}$, *ara*$^{-}$, *gal*$^{-}$, *mtl*, F^{-}, *rec*A^{+}, *uvr*$^{+}$, (Qiagen), was used to express 6xHis-US11.

GST-fusion proteins were expressed in the *E.coli* strain BL21: F^{-}, *omp*T, r^{-}_{B}, m^{-}_{B}.

2.1.3.4 Bacteria culture media

All bacterial strains were grown in L-Broth (10g NaCl, 16g Bactopeptone, 5g yeast extract in 1 litre H_2O, pH7.5) or in 2YT broth (5 g NaCl, 16g Bactopeptone, 10g yeast extract in 1 litre H_2O, pH7.5). LB agar plates were made from 1.5% (w/v) agar in L-broth. LB agar and media were supplemented with 25μg/ml kanamycin for the growth of M15[pREP4] and with 25μg/ml kanamycin and 100μg/ml ampicillin for M15[pREP4] transformed with pQE-30 or pQE-16. All other plasmids were propagated in the presence of 100μg/ml ampicillin.

2.1.4 Mammalian cells and cell culture

All cell culture media was obtained form Gibco BRL.

The baby hamster kidney clone 13 (BHK-21 C13) cell line was grown in Glasgow Modified Eagle's Medium (GMEM), 10% NBCS, 10% tryptose phosphate, 100U pencillin and 100μg/ml streptomycin.

HeLa cells, a human cervical carcinoma epithelial cell line, were grown Dulbecco's Modified Eagles Medium (DMEM), supplemented with 10% foetal calf serum (FCS), 5mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin. For [^{35}S] methionine metabolic labelling cells were incubated in the presence of Eagles medium containing 20% the normal methionine level, 2% NBCS and 30mCi/ml [^{35}S] l-

methionine.

2.1.5 Viruses

Two herpes variants were used in this study:

HSV-1 strain 17^+ the wild-type (*wt*) strain (Brown *et al.*, 1973).

HSV-1 $US11^-$ in which the expression of the US11 protein has been knocked out by the deletion of the Met-ATG by the deletion of four nucleotides at the *Sph*I site, at position 145166nt. A gift from Andrew Davison and Charles Cunningham, MRC Institute of Virology, Glasgow.

2.1.6 Antibodies

Anti-US11 antibodies

Anti-US11 mAb (28*); mouse mAb raised against a β-galactosidase-US11 fusion protein (Roller & Roizman, 1992). A gift from B. Roizman, University of Chicago.

Anti-US11 pAb: Rabbit pAb (14427); raised against the N-terminal 11 amino acids of US11 (MacLean *et al.*, 1987).

Anti-UL12 antibody

UL12 mAb (Q1); mouse monoclonal antibody raised against purified HSV-2 UL12. (Banks *et al.*, 1983).

Anti-UL13 antibodies

Rabbit anti-sera raised against amino acids 149-207 of UL13.

Rabbit anti-sera raised against 6xHis-UL13 of HSV-2. A gift from Y. Nishiyana, Nogoya University School of Medicine, Japan. (Daikoku et al., 1997).

Anti-UL14 antibody

UL14 pAb: Rabbit anti-sera raised against UL14 codons 58-218 fused to GST. A gift from J. Baines, Cornell University (Cunningham *et al.*, 2000).

Anti-His tag antibody

Anti-RSG-3xHis mAb: mouse mAb was obtained from Qiagen.

2.1.7 Radiochemicals

All radiochemicals were purchased from Dupont.

[γ-^{32}P] ATP	6000Ci/mmol, 10mCi/ml
[α-^{32}P] CTP	800Ci/mmol, 10mCi/ml
[^{35}S]-l-methionine	1175Ci/mmol, 10mCi/ml
[α-^{32}P] UTP	800Ci/mmol, 10mCi/ml

2.1.8 Chemicals and reagents

All chemicals were purchased from BDH Chemicals UK or Sigma, with the following exceptions:

Amersham International: ECL Western blotting reagents, Rainbow markers, Hybond-C nitrocellulose membrane, Hybond-N nitrocellulose membrane

Beecham Research: Ampicillin sodium BP (Penbritin®)

Bio-Rad: Ammonium persulphate (APS), Coomassie Brilliant Blue™, TEMED

Fluka: Formaldehyde, formamide

Gibco BRL: 1kb DNA ladder, Trizol reagent

Invitrogen: Recombinant RNasin (40U/μl)

Joseph Mills Ltd.: Ultrapure ethanol, methanol

Kodak: X-Omat film

Prolab: Butan-2-ol, glacial acetic acid, glycerol, isopropanol

Promega: Recombinant RNasin (40U/μl)

Sigma: Glutathione-agarose

2.1.10 Kits and miscellaneous

Gibco BRL: 5′ RACE System (Version 2.0)

Hybaid: Cosmid miniprep kit

Promega: *In vitro* trancription kit

Qiagen: Maxi-, midi-, and mini-prep plasmid isolation kits

Sartorius: 0.5ml Collodion bags

2.1.9 Solutions

30% acrylamide mix	29% (w/v) acrylamide, 1% (w/v) N, N'-methylene bis-acrylamide
40% acrylamide mix	38% acrylamide, 2% (w/v) N, N'-methylene bis-acrylamide

6x agarose gel loading buffer	0.25% (w/v) bromophenol blue
	0.25% (w/v) xylene cyanol FF
	30% (v/v) glycerol
Alkaline lysis:	
Solution I	50 mM Tris-HCl (pH 8.0)
	10 mM EDTA
Solution II	0.2 M NaOH
	1% (w/v) SDS
Solution III	3 M potassium acetate
	11.5% (v/v) glacial acetic acid
Bradford's reagent	0.01% (w/v) Commassie Brilliant Blue™
	0.0003% (w/v) SDS
	8.5% (v/v) phosphoric acid
	4.75% (v/v) ethanol
Coomassie stain solution	0.02% (w/v) Coomassie Brilliant Blue™
	50% (v/v) methanol
	7% (v/v) acetic acid

500 ml 100x Denhardt solution	10 g Ficoll 400
	10 g polyvinylpyrrolidone
	10 g BSA
Destain	5% (v/v) methanol
	10% (v/v) acetic acid
EMSA RNA binding buffer	10 mM Hepes, pH 7.6
(ERBB)	60 mM KCl
	3 mM $MgCl_2$
	1 mM DTT
	5% (v/v) glycerol
Fixing solution	40% (v/v) methanol
	10% (v/v) acetic acid
Giemsa stain	1.5% (w/v) suspension of Geimsa in glycerol
	heated to 56°C for 2h and diluted with an equal
	volume of methanol

Hybrid selection of cDNA

Pre-hybridization buffer	50% (v/v) formamide
	0.75 M NaCl
	0.1 M Pipes (pH 6.0)
	8 mM EDTA
	0.5% (w/v) SDS
	100 μg/ml salmon sperm DNA
Hybridization buffer	50% (v/v) formamide
	0.4 M NaCl
	0.1 M Pipes (pH 6.0)
	8 mM EDTA
	0.5% (w/v) SDS
	100 μg/ml salmon sperm DNA

Immunoprecipitation

Buffer E	100 mM Tris-HCl (pH 8.0)
	100 mM NaCl
	2 mM EDTA
	2 mM EGTA
	1% (v/v) NP40
	0.5% (w/v) sodium deoxycholate
	0.5 mM PMSF
Buffer EN	100 mM Tris-HCl (pH 8.0)
	500 mM NaCl
	2 mM EDTA
	2 mM EGTA
	1% (v/v) NP40
	0.5% (w/v) sodium deoxycholate
	0.5 mM PMSF
Buffer EB	Buffer E containing 2 mg/ml BSA

Immunoprecipitation of US11-RNA complexes:

RNA-binding buffer (RBB)	10 mM Tris (pH 7.9)
	150 mM NaCl
	50 mM KCl
	2 mM $MgCl_2$
	0.5 mM DTT
	0.2 mM EDTA
	0.2 mM PMSF
	20%(v/v) glycerol
10X MOPS	0.4 M MOPS
	0.1 M sodium acetate
	10 mM EDTA
	pH 7.2
Northern blot hybridization solution	50% (v/v) formamide
	5x SSC
	2x Denhardt
	5% (w/v) dextran sulphate
	0.5% (w/v) SDS
	250 μg/ml salmon sperm DNA

Northern blot pre-hybridization solution	50% (v/v) formamide 5x SSC 5x Denhardt 50 mM Na_2HPO_4 (pH 7.0) 0.1% (w/v) SDS 100 µg/ml salmon sperm DNA
PBS A	170 mM NaCl 3.4 mM KCl 10 mM Na_2HPO_4 (pH 7.5)
PBS complete	PBS A 1 g/l $CaCl_2.2H_2O$ 1 g/l $MgCl.6H_2O$
PBS-T	PBS A 0.05% (v/v) Tween 20

PCR reaction buffer	
(final concentration)	1 mM dATP, dCTP, dTTP and dGTP
	45 mM Tris-HCl, pH 8.8
	11 mM $(NH_4)_2SO_4$
	4.5 mM $MgCl_2$
	6.7 mM β-mercaptoethanol
	4.4 μM EDTA, pH 8.0
	113 μg/ml BSA
5x Polynucleotide kinase	350 μl ADP
(PNK) exchange reaction buffer	250 mM imidazole (pH 6.8)
	60 mM $MgCl_2$
	5 mM β-mercaptoethanol
3x Protein loading buffer	150 mM Tris-HCl (pH 6.8)
	30% (v/v) glycerol
	6% (w/v) SDS
	300 mM DTT
	0.3% (w/v) bromophenol blue
SDS-PAGE running buffer	0.05 M Tris base
	0.05 M glycine
	0.1% (w/v) SDS

2.5x Random priming buffer	0.5 M Hepes (pH 6.6)
	12.5 mM $MgCl_2$
	25 mM β-mercaptoethanol
	125 mM Tris (pH 8.0)
	50 mM dGTP
	50 mM dATP
	50 mM dTTP
5x RNA loading buffer	50% (v/v) glycerol
	1 mM EDTA
	0.4% (w/v) bromophenol blue

Small scale nuclear and cytoplasmic extracts

Buffer A	10 mM HEPES (pH 7.9)
	1.5 mM $MgCl_2$
	10 mM KCl
	0.5 mM DTT

Buffer C	20 mM HEPES (pH 7.9)
	25% (v/v) glycerol
	0.42 M NaCl
	1.5 mM $MgCl_2$
	0.2 mM EDTA
	0.5 mM PMSF
	0.5 mM DTT
Buffer D	20 mM HEPES (pH 7.9)
	20% (v/v) glycerol
	0.1 M KCl
	0.2 mM EDTA
	0.5 mM PMSF
	0.5 mM DTT
20x SSC	3 M NaCl
	0.3 M Tri-sodium citrate
	pH 7.0
50x TAE	0.2 M Tris base
	0.05 M EDTA (pH 8.0)

10x TBE	1.25 M Tris-HCl (pH 8.0) 0.14 M boric acid 10 mM EDTA (pH8.0)
TE	10 mM Tris-HCl 1 mM EDTA (pH 8.0)
Towbin (transfer buffer)	25 mM Tris base 192 mM glycine 20% (v/v) methanol
Tris-saline	140 mM NaCl 30 mM KCl 280 mM Na_2HPO_4 25 mM Tris-HCl (pH 8.0) 1 mg/ml glucose 0.1 mg/ml streptomycin 100 U/ml pencillin 0.0015% (w/v) phenol red
Trypsin	0.25% (w/v) trypsin in Tris-saline containing phenol red (pH 7.5)

Versene	0.6 mM EDTA 0.0002% (w/v) phenol red in PBS A

2.2 Methods

2.2.1 DNA manipulation, cloning and construction of templates for *in vitro* transcription.

2.2.1.1 Preparation of DNA.

For the small-scale isolation of plasmid DNA the Hybaid cosmid miniprep kit or the Qiagen plasmid miniprep kit were used according to the manufacturer's protocol. Alternatively, alkaline lysis, was performed as described here. 1.5-3ml of bacterial culture was pelleted by centrifugation at 12,000rpm in a benchtop centrifuge for 1min. The supernatant was removed and the pellet resuspended in 100µl of solution I. 200µl of solution II was added, mixed by inversion and incubated for 5min on ice. 150µl of solution III was added and mixed gently. Cell debris was pelleted by centrifugation at 12,000rpm for 10min. The supernatant was transferred to a fresh microfuge tube and extracted twice with phenol/chloroform and ethanol precipitated. The DNA pellet was washed with 70% ethanol and air-dried. The DNA was resuspended in 100µl of TE buffer and 10µg RNase A and incubated for 30min at 30°C.

For larger scale DNA preparations the Qiagen Midi- or Maxi-prep kit were used according to the manufacturer's protocol.

2.2.1.2 Phenol/chloroform extraction and ethanol precipitation of DNA

Typically, the volume of the DNA in solution was made up to 200µl with dH_2O and 200µl of 50% phenol/chloroform (v/v) was added. After vortexing for 30s the mixture

was centrifuged at 12,000rpm in a benchtop microfuge for 5min and the upper phase removed carefully and transferred to a clean microfuge tube. This was repeated until there was no precipitate visible at the interface (usually just once). The upper phase was transferred to a fresh microfuge tube and precipitated with the addition of 1/10 of a volume of 3M sodium acetate (pH 5.2), 2.5 volumes of ice-cold ethanol were added and incubated at -20°C for 1h. The DNA was recovered by centrifugation at 12,000rpm for 10min, washed once in 100µl of 70% ethanol and resuspended in the desired volume of TE or dH_2O.

2.2.1.3 Restriction endonuclease digestion of DNA and blunt-ending DNA

For restriction of vector DNA approximately 500ng of DNA was incubated in the presence of 2U of the restriction enzyme in 1x the appropriate buffer supplied by the manufacturers in a total volume of 20µl for 1h at 37°C. For the cloning of PCR products approximately 5µg of DNA was digested in the same manner for 3h at 37°C. For filling in over-hanging DNA ends 2U of Klenow large DNA polymerase subunit was incubated with DNA for 30min at 37°C in the presence of all four dNTPs at a final concentration of 1mM each.

2.2.1.4 Agarose gel electrophoresis of DNA

DNA was resolved in a horizontal slab gel of 1-1.5% (w/v) agarose in 1x TBE or 1x TAE containing 1µg/ml ethidium bromide and electrophoresised in a buffer of 1x TBE or 1x TAE. Electrophoresis was performed at 80-120V. DNA was visualized by UV-illumination.

2.2.1.5 Acrylamide gel electrophoresis of DNA

DNA fragments were separated in a 6% polyacrylamide/1x TBE gel by electrophoresis in a Bio-Rad mini-electrophoresis gel system. Electrophoresis was carried out at 100mV for 45-60min. The gel was stained with 5μg/ml ethidium bromide in 1x TBE, washed twice with 1x TBE and visualized under UV-illumination.

6% acrylamide gel:

Acrylamide (30%)	3ml
10x TBE	1.5ml
dH_2O	10.5
APS	100μl
TEMED	12μl

2.2.1.6 Isolation of DNA from agarose gels

DNA was extracted from agarose gel slices using Spin-X columns (Costar). The gel slice was frozen for 20min at -20°C in a Spin-X tube and then centrifuged in a benchtop centrifuge for 20min at 8,000rpm. The DNA was isolated from the eluate by phenol/chloroform extraction and ethanol precipitation.

2.2.1.7 Isolation of DNA from acrylamide gel slices

An acrylamide gel slice containing the DNA fragment of interest was excised. The DNA was eluted in 0.4M NaCl at 4°C o/n. The DNA was recovered by phenol/chloroform extraction followed by ethanol precipitation.

2.2.1.8 Ligation of insert into vector

Ligation of an insert into a vector with compatible ends was carried out by incubation of the insert with the vector at the molar ratios 3:1, 1:1, and 1:3. The ligation was performed in a volume of 10µl in the presence of 1µl of 10x ligation buffer and 1U of T4 DNA ligase at 16°C o/n.

For ligation into the pGEM-T Easy vector (Promega) 3µl of the insert was incubated with 0.5µl of the vector (25ng), 5µl of 2x rapid ligation buffer and 1µl of T4 ligase (3U/µl from Promega) and 0.5µl dH_2O. The reaction was incubated at RT for 1h.

2.2.1.9 Construction of the 6xHisUS11 expression plasmid

The ORF of US11 was PCR amplified from HSV-1 (17^+) *Eco*RI fragment H (*wt*) using primers GTAGGTCGACTCGAGATGAGCCAGACCCAAC and GATGAAGCTTG AGCGTATGCTCCATGTTGTG, which incorporated the *Sal*I and *Hind*III sites at the 5′ and 3′ ends of the US11 ORF respectively. The PCR product was digested with *Hind*III and *Sal*I and ligated into pQE-30 which had been linearised by the same

enzymes. The ligation mix was used to transform DH5α cells and the transformants plated out on agar plates 100μg/ml ampicillin. Ampicillin resistant colonies were screened for the presence of the US11 ORF in the pQE-30 backbone was checked by restriction enzyme analysis. The presence of correct insert was checked by sequencing with the QIAexpress sequencing primers 'Primer-Type III/IV' and 'Primer- Reverse Sequencing'.

2.2.1.10 Construction of the pT-12/14 and pTΔ34

The pT-12/14 insert was generated by SI RT-PCR and is described in Section 2.2.7.2. This insert was cloned into pGEM-T Easy (Fig. 2.1). pT-Δ34 was generated by the PCR amplification of the Δ34 sequence from the *Kpn*I C′ (*wt*) fragment of HSV-1 with the primers GTGTGCACGGCGAGCTGCTC and AGCACCGACACACGCCGGTG. The PCR product was purified and ligated directly into pGEM-T Easy (Fig. 2.1). The inserts were sequenced using T7 and SP6 promoter specific primers.

2.2.1.11 Construction of *in vitro* transcription templates of Δ34 and for 12/14 and 12/14 truncations

Templates for *in vitro* transcription of riboprobes the Δ34 RNA and the 12/14 RNA and progressive 5′ deletions of 12/14 RNA were generated by the inclusion of a 5′ T7 RNA Polymerase promoter sequence upstream of the sequence of interest by PCR. The sequences of the primers used are given in Table 2.1.

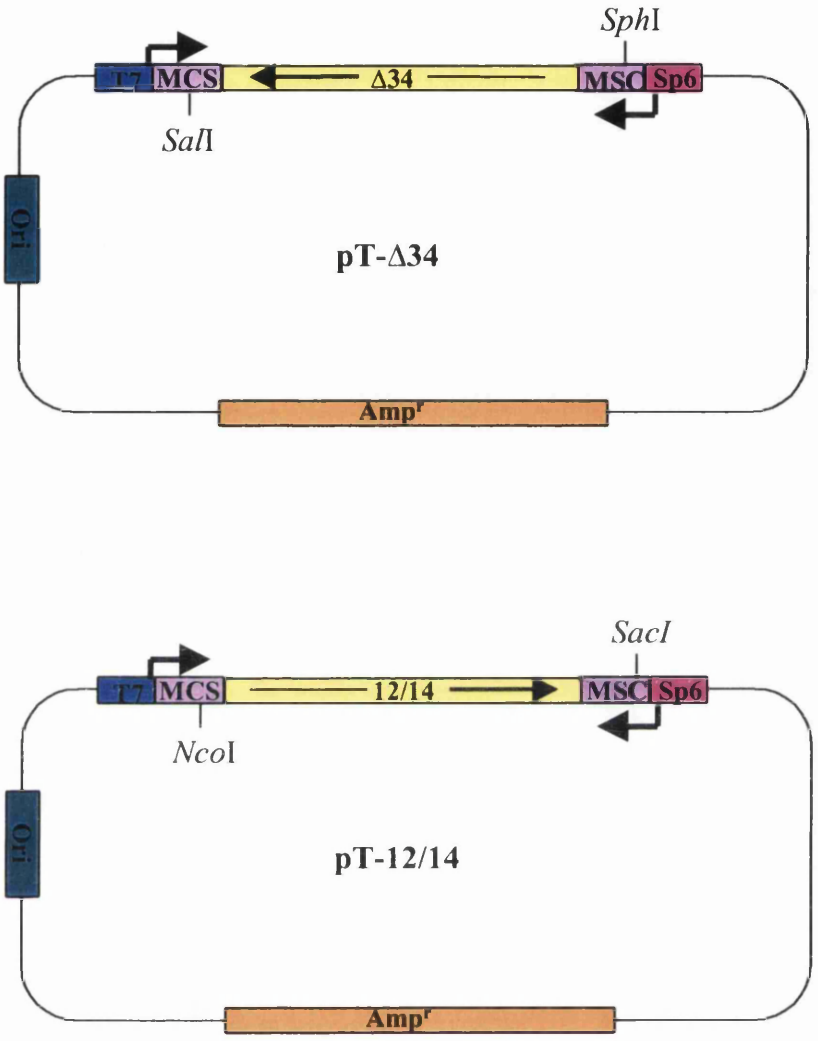


Fig. 2.1: Schematic diagram of pTΔ34 and pT-12/14.
pT-Δ34 was generated by ligation of a 451nt PCR fragment of the UL34 gene into pGEM-T Easy. pT-12/14 was generated by the ligation of the 585nt amplification product of SI RT-PCR into pGEM-T Easy. The insert is depicted by a yellow box, with the orientation of the RNA indicated by an arrow. The position of the restriction enzyme sites within the multiple cloning site (MCS, mauve box) used to linearize the vectors for the generation of run-off transcripts are indicated. RNA was generated by transcription with T7 or Sp6 RNA polymerase (the promoters are indicated by blue and pink boxes respectively, with a bent arrow). The Ori is depicted by a green box and the ampicillin resistance gene by an orange box.

2.2.2 Bacterial procedures

2.2.2.1 Making competent *E. coli*

10ml of L-broth was inoculated with bacteria and grown o/n at 37°C in a shaking incubator. 2.5ml of the o/n culture was used to inoculate 50ml of media and grown until an OD_{600} of 0.4 had been reached. The cells were pelleted by centrifugation at 5,000rpm for 5min at 4°C in a Sorvall RT 6000B centrifuge. The bacterial pellet was resuspended in 20ml of ice-cold 0.1M $CaCl_2$ and incubated on ice for 30min. The bacteria were pelleted as before, resuspended in 10ml of ice-cold 0.1M $CaCl_2$ and incubated on ice for 15min followed by centrifugation. The pellet was resuspended in 4ml of ice-cold 0.1M $CaCl_2$ with the addition of 15% glycerol, aliquoted, snap frozen and stored at -70°C.

2.2.2.2 Transformation of plasmids

10µl of competent *E. coli* and 10ng of circular plasmid or 5µl of ligation mix were incubated for 30min on ice in a pre-chilled 15ml falcon tube. Heat shock was carried out by incubation at 42°C for 90s. The bacteria were left to recover for 5-10min at RT. 1ml of L-broth was added to the transformation mix and incubated for 1h at 37°C in a shaking incubator. 200µl of the transformation mix was spread on LB agar plates containing the appropriate antibiotic and incubated o/n at 37°C.

2.2.2.3 Bacterial protein expression

i. 6xHisUS11 expression

Plasmid preparations were used to transform M15[pREP4] cells which were plated out on agar plates containing 25μg/ml kanamycin and 100μg/ml ampicillin. Resistant colonies were used to inoculate 10ml of L-broth, which was incubated o/n at 37°C in a shaking incubator. This 10ml culture was used to inoculate a 100ml L-broth which was grown at 25°C until an OD_{600} of 0.6 had been reached. The cultures were then induced with 1mM IPTG and grown for a further 5h at 25°C. 1ml of the culture was removed and pelleted by centrifugation at 12,000rpm for 1min in a benchtop centrifuge. The supernatant was removed and the bacterial pellet was resuspended in 100μl 1x protein gel loading buffer.

ii. Expression and purification of GST-US11

BL21 cells were transformed with pGEX-2T, or the pGEX-2T US11 constructs, 2319, 2540, or 2625, and plated out on agar plates containing 100μg/ml ampercillin. After incubation o/n at 37°C, resistant colonies were picked and used to innoculate 10ml of L-broth containing ampicillin. This starter culture was incubated o/n at 37°C in a shaking incubator. The starter culture was used to innoculate 100ml of L-broth. When an OD_{600} had been reached protein expression was induced with the addition of 0.1mM IPTG and incubated for a further 3h at 37°C.

The bacteria were pelleted by centrifugation at 9,000rpm in a GS3 rotor for 10min at 4°C and resuspended in 10ml of PBS. The bacteria were lysed by the addition of lmg/ml lysozyme and incubated on ice for 30min. 1% (v/v) Triton X-100 was added and the cell debris pelleted by centrifugation at 9,000rpm at 4°C. The supernatant was removed and added to 1ml of 50% suspension of glutathione-agarose in PBS and mixed on a rotating wheel for 2min. at RT. Three washes were performed with 50ml ice-cold PBS A and the pellet resuspended in 1ml of 5mM reduced glutathione/50mM Tris-HCl (pH8.0) and place on a rotating wheel for 2min at RT. The beads were pelleted by centrifugation 3,000rpm for 5s and the supernatant removed. The beads were subjected to a further two rounds of elution.

The purified protein was dialysed against 1x ERBB in 0.5ml Collodion bags at 4°C for 1h. The 1x ERBB was replaced with fresh ERBB and the protein sample dialyzed for a further 2h at 4°C. 20% glycerol (v/v) was added and the protein stored in 20µl aliquots at -70°C.

2.2.3 Cell culture and virus growth

2.2.3.1 Mammalian cell culture

Cells were grown in the appropriate media at 37°C supplied with 5% CO_2 in a humidified incubator.

Cells were harvested by washing with versene and removed by washing with trypsin/versene. Cells were diluted 1 in 10 in the appropriate medium for passaging.

2.2.3.2 Preparation of virus stocks

BHK21 C13 cells were grown in roller bottles to a confluency of 80% and infected at an moi of 1 in 300 in 40ml of media at 31°C for 3-5 days until a cytopathic effect (CPE) was observed. Cells were harvested by shaking the roller bottles to detach the cells and collected by centrifugation at 2,000rpm for 30min at 4°C in a Sorvall RT 6000B centrifuge. To harvest the cell released virus the supernatant was removed and centrifuged at 12,000rpm for 2h (Sorvall GSA rotor). The pellet was resuspended in 1ml/roller bottle, sonicated briefly, aliquoted, frozen on dry ice and stored at -70°C. The cell associated virus was harvested by resuspending the cell pellet in 5ml BHK medium and sonicated for 10min in a sonibath. The cell debris was pelleted by centifugation at 2,000rpm for 10min and the supernatant removed. The pellet was again resuspended in 5ml of BHK medium, sonicated and pelleted. The supernatant was added to that gained in the first round, aliquoted, frozen on dry ice and stored at -70°C.

Serial dilutions (10^{-1}-10^{-8}) of the virus stock were titrated in BHK21 C13 cells. 90% confluent BHK21 C13 cells in 60mm sterile dishes were infected for 1h at 37°C. The cells were overlaid with BHK medium/1% methylcellulose. After 2-3 days the media was removed and the cells stained with Giemsa stain for 2h, washed and the plaques counted under a dissecting microscope. The titre was calculated as plaque forming units/ml (pfu/ml).

Virus stocks were checked for contamination by streaking on to blood agar plates and incubated at 37°C for 5 days.

2.2.3.3 Infection of cells

80% confluent BHK or HeLa cell monolayers were infected with HSV-1 *wt* or HSV US11 null mutant at a multiplicity of 10 or no virus (mock-infected). Adsorption was for 1h at 37°C. The cells were washed with PBS, overlaid with the appropriate medium and infection allowed to proceed at 37°C in a humidified incubator supplied with 5% CO_2.

2.2.4 Preparation of cell extracts

2.2.4.1 Preparation of total cellular protein extracts

For preparation of cell extracts monolayers were washed with PBS, and cells lysed by suspension in 1ml cell extract buffer (50mM Hepes (pH 7.5), 50mM NaCl, 0.1% (v/v) NP-40, 10µl of protease inhibitor cocktail). Extracts were sonicated on ice, cell debris was pelleted, and the protein concentration determined by the Bradford assay.

2.2.4.2 Preparation of small-scale nuclear-cytoplasmic extracts

Cytoplasmic and nuclear fractionation was performed as described by Lee *et al.* (1990). Cells were harvested by scraping into PBS using a rubber policeman and washed twice in 30 packed cell volumes (PCV) of ice-cold PBS and pelleted by centrifugation at 3,000rpm in a benchtop centrifuge at 4°C. The cells were resuspended in 1 PCV of

buffer A and allowed to swell on ice for 15min. The cells were lysed using 6 strokes through a 26-gauge needle attached to a 1ml syringe. The nuclei were pelleted by centrifugation at 12,000rpm for 20s at 4°C. The supernatant was removed, aliquoted, snap frozen in liquid nitrogen and stored in liquid nitrogen. The nuclear pellet was resuspended in two-thirds PCV in buffer C and incubated on ice for 30min with occasional agitation. The nuclear debris was pelleted by centrifugation at 12,000rpm for 5min. at 4°C. The supernatant was removed and dialysed against buffer D in 0.5ml Collodion bags at 4°C for 2h. The dialysed nuclear fraction was aliquotted, snap frozen in liquid nitrogen and stored in liquid nitrogen.

2.2.5 Protein analysis

2.2.5.1 SDS-PAGE

SDS-PAGE was performed in a vertical gel system, using the Bio-Rad Mini Protean II™ electrophoresis apparatus. The resolving gel was poured between two glass plates which were separated by spacers. The resolving gel was overlaid with butan-2-ol and allowed to set at RT. The butanol was removed by washing with water. The stacking gel was poured on top of the resolving gel, into which a slot-forming comb was inserted. The stacking gel was left to set. The protein gels were assembled in a Bio-Rad electrophoresis cell with running buffer. Protein gel loading buffer was added to protein samples. The samples were boiled for 5min and loaded into the wells. The samples were electropherised at 150V until the dye front had reached the bottom of the gel.

	Resolving gel (10ml)			Stacking gel (5ml)
	10%	12%	15%	5%
Acrylamide (30%)	3.3 ml	4 ml	5 ml	0.83 ml
1.5M Tris-HCl (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	-
1M Tris-HCl (pH 6.8)	-	-	-	0.63 ml
10% (w/v) SDS	100 µl	100 µl	100 µl	50 µl
dH_2O	4 ml	3.3 ml	2.3 ml	3.4 ml
10% (w/v) APS	100 µl	100 µl	100 µl	50 µl
TEMED	4 µl	4 µl	4 µl	5 µl

2.2.5.2 Western blotting

Proteins were separated by SDS-PAGE and transferred to nitrocellulose using a Bio-Rad transblot cell. For transfer a sandwich was built in the following order: fibre pad, 3mm Whatman paper, SDS-PAGE gel, Hybond-C nitrocellulose, 3mm Whatman paper, fibre pad, pre-soaked in Towbin buffer. The sandwich was placed inside the Bio-Bad apparatus and transferred at 250mA for 1h or o/n at 30mA at 4°C.

The nitrocellulose membrane was blocked in PBS A/5% dried milk powder at RT for 1h or o/n at 4°C. The membrane was washed three times in PBS A. For the detection of a specific protein the antibody was diluted in PBS A/0.05% Tween-20 (PBS-T) and incubated with the membrane for 1h on a rotating shaker at RT. The membrane was washed three times in PBS-T followed by the addition of protein A-horse radish peroxidase (HRP) or anti-mouse IgG-HRP added at a dilution of 1/1000 in PBS-T and

incubated for 1h on a rotating shaker at RT. The membrane was washed with PBS-T five times. To visualise the protein of interest the membrane was incubated with ECL detection reagents for 1min (1:1, v/v), covered with clingfilm and exposed to X-Omat film.

2.2.5.3 Immunoprecipitation of UL13

HeLa were labelled with [^{35}S]-methionine (30 μCi/ml) for 6h in Eagle's medium containing 20% of the normal methionine level and 2% newborn calf serum. The monolayers were washed with PBS and total protein extracted as described in section 2.2.4.1. For immunoprecipitation 5μl of the anti-UL13 pAb and 50μl of immunoprecipitation buffer, buffer E, was added to 100μg of the cell extract and incubated at 4°C for 2h. 75μl of protein A-Sepharose (50% w/v) was added and mixed at 4°C for 1h. The beads were pelleted and were washed twice with buffer EB, once in buffer EN and four times with buffer E. 50μl of boiling buffer was added to the washed beads and the samples boiled for 3min. The samples were electrophoresed on a 10% SDS-PAGE gel and visualised by phosphoimager analysis.

2.2.6 Manipulation of RNA

2.2.6.1 Isolation of total RNA

Total cellular RNA was prepared by Trizol (Gibco BRL) extraction according to the manufacturer's protocol, phenol/chloroform extracted twice, chloroform extracted once and ethanol precipitated.

2.2.6.2 Isolation of cytoplasmic RNA

$1x10^7$ cells were harvested, pelleted at 2000rpm for 5min at 4°C and washed in 20ml of ice-cold PBS complete. The cells were resuspended in 2ml ice-cold lysis buffer (0.14M NaCl, 1.5mM $MgCl_2$, 10mM Tris-HCl (pH7.4), 5mM DTT, 0.5% (w/v) NP40) and incubated on ice for 15min. The cells were lysed by 5 strokes in a 1ml Dounce homogeniser and the nuclei pelleted by centrifugation at 3,300rpm for 15min at 4°C. The supernatant was removed, 100µg/ml proteinase K and 0.2% (w/v) SDS added and incubated at 37°C for 30min. The RNA was isolated by phenol/chloroform extraction and ethanol precipitation.

2.2.6.3 DNase treatment of RNA

RNA was incubated with 10µl New England Biolabs Restriction Enzyme Buffer 3, 80U of RNasin and made up to a volume of 100µl with DEPC-H_2O and incubated for 30min at 37°C. The RNA was extracted twice by phenol/chloroform extraction, once with chloroform and ethanol precipitated.

2.2.6.4 Phenol/chloroform extraction and ethanol precipitation of RNA

Typically, the volume of RNA in solution was made up to 200µl with dH_2O and 200µl phenol:chloroform:isoamyl alcohol (25:24:1) added. After vortexing for 30s the mixture was centrifuged at 12,000rpm in a benchtop microfuge for 5min and the upper phase

removed carefully and transferred to a clean microfuge tube. This was repeated until there was no precipitate visible at the interface (usually just once). The upper phase was transferred to a fresh microfuge tube and the RNA precipitated with the addition of 0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of ice-cold ethanol and incubated at -20°C for 1h. The RNA was recovered by centrifugation at 12,000rpm for 10min, washed once in 100µl of 70% ethanol and resuspended in the desired volume of TE or dH_2O.

2.2.6.5 Co-immunoprecipitation of US11 and RNA

The protocol used was taken from Roller & Roizman (1990). Pooled cytoplasmic and nuclear extracts (Section 2.2.4.2) were diluted with RBB to a concentration of 500µg/ml of protein. 1000U of RNasin was added and the US11 mAb (28*) was added at a dilution of 1 in 1000 and incubated at RT for 30min. 250µl of anti-mouse IgG-agarose (50% solution in RBB) was added This was mixed on a rotating wheel for 30min. RNase T1 was added to a final concentration of 3U/µl and the mixture incubated for a further 10min at RT (this step was omitted if the RNAs were to be reverse transcribed). This was followed by the addition of 1µg/µl of heparin and incubation for a further 10min. The beads were pelleted and washed three times in RBB containing 1mg/ml BSA, 500mM NaCl, and 5mg/ml heparin. The beads were washed once with binding buffer and the complexes solubilized by boiling for 2min in 500µl 0.5% (w/v) SDS/TE. The RNA was extracted twice by phenol/chloroform (1:1) extraction, once with chloroform and ethanol precipitated. The RNA was treated with DNase I followed by phenol/chloroform extraction and ethanol precipitation. The RNA pellet was resuspended in 20µl of dH_2O.

2.2.6.6 GST-US11 RNA pull downs

For the GST-fusion protein pull-downs purified 50μg recombinant GST-US11 or GST was pretreated with 50U of micrococcal nuclease at 30°C for 15min to remove contaminating bacterial RNA. The reaction was stopped by the addition of EGTA to a final concentration of 5mM and incubated for 10min. on ice. Glutathione-agarose beads were pre-swollen and washed in RBB and resuspended to form a 50% slurry. 100μl of the 50% slurry was incubated with 50μg of protein on a rotating wheel for 5min at RT. The beads were then pelleted, washed 5 times with RBB and resuspended in 100μl of RBB. The cell extracts (prepared as described above) were precleared with GST-glutathione-agarose by adding 100μl of GST-glutathione-agarose to 100μl total (pooled nuclear and cytoplasmic fractions) HeLa cell extract (diluted with RBB to 500μg/ml) and incubated for 30min at 4°C on a rotating wheel. The beads were then pelleted and the precleared extract used in the pull-down.

Heparin was added to the extract at 1μg/μl and 100μl of either GST-glutathione agarose or GST-US11-glutathione agarose and incubated at 4°C for 1h. RNase T1 was added to a final concentration of 3U/μl and the mixture incubated for 10min at RT (this step was omitted if the RNAs were to be reverse transcribed). The beads were pelleted and washed three times in RBB containing 5mg/ml BSA, once with RRB containing 500mM NaCl and three times with binding buffer. The beads were then resuspended in 500μl 0.5% (w/v) SDS/TE and boiled for 2min to elute the protein-RNA complexes. The RNA was extracted with phenol/chloroform twice and chloroform once and ethanol

precipitated. The RNA was treated with DNase I followed by phenol/chloroform extraction and ethanol precipitation. The RNA pellet was resuspended in 20µl of dH_2O.

2.2.6.7 T4 polynucleotide end-labelling

For 5′ end-labelling of nucleic acids with [^{32}P], 10µl of RNA or 5µl of 1kb DNA molecular weight marker was added to the end-labelling mix which consisted of 40U RNasin, 1x PNK exchange reaction buffer, 10µCi of [γ-^{32}P] ATP, followed by the addition of 10U of T4 PNK. The total reaction volume was 25µl. Following incubation at 37°C for 30min, 10µl of Sequenase kit stop solution (Amersham International) was added. The sample was heated to 100°C for 2min and 10µl of RNA or 2µl of 1kb DNA marker was loaded on to a 6% denaturing gel and electrophoresed for approximately 2h at 70 Watts.

6% denaturing gel:

Urea	40g
Acrylamide (40%)	10.1
10x TBE	10ml
dH_2O	40.5
10% APS	600µl
TEMED	50µl

A 6% denaturing gel mix was poured between two glass sequencing size plates separated by thin spacers. Wells were formed by the use of a sharks tooth comb.

Electrophoresis was performed using the Gibco BRL model S2 electrophoresis apparatus.

2.2.6.8 *In vitro* transcription

Riboprobes were transcribed *in vitro* from inserts cloned into pGEM-T Easy or templates with a T7 promoter at the 5′ end by the appropriate polymerase (either SP6, T7 RNA polymerase) in the presence of [α-^{32}P] rUTP using the Promega *in vitro* transcription system.

For transcription from the pGEM-T Easy plasmids the templates were prepared in the following fashion. The templates were linearized with the appropriate restriction enzyme, the positions of which are given in Fig. 2.1. For transcription with T7 RNA polymerase to generate the anti-sense transcript pT-Δ34 was digested with *Sal*I. For transcription with SP6 to generate the sense transcript, pT-Δ34 was digested with *Sph*I. For transcription of pT-12/14 to generate the sense transcript, pT-12/14 was digested with *Sac*I. For transcription of pT-12/14 to generate the anti-sense transcript, pT-12/14 was digested with *Nco*I. The templates were blunt-ended. The digested templates were separated by agarose gel electrophoresis and purified.

For transcription from the PCR-derived templates the PCR products were separated on an acrylamide gel and extracted. The templates were transcribed with T7 or SP6 RNA Polymerase as appropriate.

For *in vitro* transcription the following components were combined and the reaction incubated for 1h at 37°C.

5x transcription buffer	2.5µl
100mM DTT	1µl
RNasin (40U/µl)	0.5µl
2.5mM rATP, rGTP, rCTP (each)	2µl
100µM UTP	1.2µl
Template (1µg/µl)	0.5µl
[α-^{32}P] UTP	2.5µl
T7 or SP6 RNA polymerase	0.5µl

RNA loading buffer was added to the *in vitro* transcription reaction, and heated for 2min at 80°C. The riboprobes were loaded on to a 6% denaturing polyacrylamide gel and electrophoresed at 200V for 3-4h. The bands were excised after autoradiography. The probes were extracted by incubation with 0.5% (w/v) SDS/TE at 37°C o/n, followed by phenol/chloroform extraction and ethanol precipitation. The riboprobes were resuspended in 60µl 1x ERBB.

The components of a 6% denaturing gel are given in Section 2.2.6.7. The gel mixture was poured between two large plates separated by spacers and a comb inserted to form the wells according to the manufacture's instructions. Electrophoresis was performed using a Bio-Rad PROTEAN® xi cell kit.

2.2.6.9 Gel mobility shift assay

GST or GST-fusion protein was incubated on ice in 1x ERBB in the presence of 10ng of tRNA and 40U RNasin for 10min. 10,000cpm of probe was denatured for 1min at 80°C and followed by incubation on ice for 2min before being added to the binding reaction, which was then incubated at 4°C for 10min. Complexes were resolved on native 4% polyacrylamide gels electrophoresed at 200V for 4h at 4°C. The gel was dried under vacuum and exposed to X-Omat film with an intensifying screen at -70°C o/n.

4% non-denaturing polyacrylamide gel:

Acrylamide (30%)	2.5ml
10x TBE	6.7ml
50% glycerol	5ml
dH_2O	35ml
APS	500µl
TEMED	50µl

The gel was poured between two medium sized glass plates that were separated by spacers. Wells were formed by the insertion of a comb and electrophoresis carried out using Gibco BRL electrophoresis kit, model V15.17.

2.2.6.10 Dot blot RNA-binding assay

The RNA binding reaction was set up as in EMSA. Hybond-C nitrocellulose membrane was washed with dH_2O for 1h and then 1x EMSA RNA binding buffer (ERBB) for 30 min. The membrane was placed above 3mm Whatman paper (wetted with 1x ERBB) in dot blot apparatus and the apparatus assembled. The sides of the apparatus were taped to ensure a good seal and the holes not to be used were taped. 80µl of 1x ERBB was added to the RNA binding reaction on ice. The reaction mix was applied to the membrane and the vacuum was applied. When all the liquid had passed through the membrane the membrane was washed twice with 500µl of 1x ERBB. The vacuum was broken and the membrane retrieved. The regions of membrane to which the reactions had been applied were excised and counted by liquid scintillation counting.

2.2.6.11 Northern blotting

10µg of total RNA was dried under vacuum and resuspended in a buffer containing 50% (v/v) formamide, 16.6% (v/v) formaldehyde, and 1x MOPS. 3µl of 3x RNA loading buffer was added and the sample boiled for 2min and chilled on ice. The RNAs samples were loaded on to a 2.2% (v/v) formaldehyde/1.2% (w/v) agarose horizontal slab gel which had been pre-run for 30 min) and elctrophoresised in 1x MOPS buffer at 140mV until the dye front had migrated to two thirds of the gel. The marker lane was removed and stained in 5µg/ml acridine orange for 20min, washed twice in dH_2O and left to destain o/n in 500ml of dH_2O. The gel was stained in 5µg/ml of ethidium bromide in 1x MOPS and visualized under UV-illumination to ensure equal loading of

the samples. The gel was washed three times with 2x SSC and the transfer set up. For the transfer of RNA to Hybond-N nitrocellulose membrane a tower of 3mm Whatman paper, gel slab, membrane, and paper towels was constructed and 2x SSC allowed to move up thought the tower by capillary action. For this a 14 x 9 cm horizontal gel slab tray was stood upsides down in about 2cm of 2x SSC in a tray to be used as a support. To form the "wick" by which 2x SSC can travel into the tower two pieces of 14 x 18 cm 3mm Whatman filter paper were pre-wet in 2x SSC and laid over the support so the overhanging edges dipped into the 2x SSC. This was overlaid by four 14 x 9cm 3mm paper pre-wet in 2x SSC. The gel slab was placed on the filter paper and a nitrocellulose membrane cut to the same size as the gel was placed on top. The tower was completed by the addition of 2 pieces of 3mm paper cut to the same size as the gel and a stack of paper towels. A glass plate was placed on the top and the tower weighted down. To prevent the 2x SSC soaking though the top layers without passing through the gel-membrane sandwich areas exposed from the layers below were blocked by the used of strips of developed X-Omat film. At all times care was taken to ensure the removal of all air bubbles. Transfer was left to occur o/n at RT.

To prepare the blot for probing the tower was disassembled and the nitrocellulose membrane left to air dry. The RNA was cross-linked to the membrane for 2min by UV irradiation.

To probe the blot for specific RNAs a purified random primed probe was used. For this the membrane was pre-hybridized for 5h at 42°C in 5ml of pre-hybridization buffer. $2x10^6$ cpm of the purified, [^{32}P]-labelled DNA probe was denatured for 10min at 100°C, chilled on ice and added to 2ml hybridization buffer and incubated with the membrane o/n at 42°C. The blot was washed at 65°C, twice in 3xSSC/0.1% (w/v) SDS at 65°C and then twice with 1x SSC/0.1% (w/v) SDS at 65°C and monitored for radioactivity. If the

blot was still fairly radioactive (> 5cpm) it was washed a further 3x in 0.1x /0.1% (w/v) SDS at 65°C. The blot was sealed in a heat sealable bag and exposed to X-Omat film o/n (or longer if required) at –70°C.

2.2.7 RT-PCR procedures

2.2.7.1 Hybrid selection of cDNAs

In order to isolate cellular γ-actin RNA from a cDNA pool of total cytoplasmic RNA, hybrid selection was performed. The plasmid pGM1, containing a γ-actin sequence, was digested with *Xba*I and *Bgl*II and the products separated on a 1% agarose gel. A band of 840bp was excised and the DNA extracted. 10μg of the insert was spotted 1μl at a time on to (1cm^2 Hybond-N nitrocellulose which had been previously incubated in 0.4M NaOH for 30min) and air dried between applications. To remove unbound DNA the membrane was washed twice in ammonium acetate (pH 5.5) and 2 times in 2x SSC. After the membrane was air-dried the DNA was cross-linked by UV irradiation for 2min. The membrane was boiled in dH_2O for 1min, chilled on ice and washed five times with dH_2O. Pre-hybridization was performed in 2ml of pre-hybridization solution at 42°C for 4h. Cytoplasmic homopolymer tailed ss cDNAs were dried under vacuum and resuspended in 1ml of hybridization solution and incubated with the membrane o/n at 42°C. The membrane was washed 10 times at 50°C with 1x SSC/0.5% (w/v) SDS, five times with 5x SSC/0.5% (w/v) SDS, and once with 5mM Tris (pH 7.4), 1mM EDTA. To elute the captured cDNAs 1ml of elution buffer (5mM KCl, 2mM EDTA) was added to the membrane in a 1.5ml ependorf tube and boiled for 1min followed by snap freezing in a dry ice/ethanol bath. The eluate was removed and the procedure

repeated. The eluates were pooled and ethanol precipitated. The pellet was resuspended in 20µl of dH_2O.

2.2.7.2 RT-PCR

RT-PCR was carried out using the Gibco BRL 5′ RACE kit with some modifications to the protocol. The primers used for each stage are listed in Table 2.1. For reverse transcription 5µl of RNA, 2.5pmol of primer and dH_2O were mixed in a 0.5ml microfuge tube to give a total volume of 15.5µl and the mixture incubated at 70°C for 10min and on ice for 1min. The liquid was collected by a brief centifugation in a benchtop microfuge. To this mixture 2.5µl 10x Gibco BRL PCR buffer was added (200mM Tris-HCl (pH8.4), 500mM KCl), 2.5µl 25mM $MgCl_2$, 1µl dNTPs (10mM of each) and 2.5µl 0.1M DTT. This mixture was incubated at 42°C for 1min and 1µl of reverse transcriptase was added. The reaction mixture was incubated at 70°C for 50min. The liquid was collected by brief centrifugation and 1µl of RNase H was added followed by incubation at 37°C for 15min. The cDNA was isolated using GlassMAX spin columns (Gibco BRL) according to the manufacturer's protocol. For the amplification of unknown RNAs isolated by GST pull down the single stranded cDNA (ss cDNA) was homopolymer tailed with cytosine. For this 6.5µl dH_2O, 5µl 5x tailing buffer (50mM Tris-HCl (pH8.4), 125mM KCl, 7.5mM $MgCl_2$), 2.5µl 2mM dCTP and 10µl of purified ss cDNA were combined in a 0.5ml microfuge tube. The mixture was incubated for 2-3min at 94°C, then placed on ice for 1min. 1µl of terminal deoxynucleotidyl transferase was added and the reaction incubated at 37°C for 10min. The enzyme was inactivated by incubation at 65°C for 10min.

In the first round of PCR the reaction contained 5µl of dC-tailed ss cDNA, 20pmol of each primer and 1 mM dATP, dCTP, dTTP and dGTP, 45mM Tris-HCl (pH 8.8) 11mM $(NH_4)_2SO_4$, 4.5mM MgCl2, 6.7mM β-mercaptoethanol, 4.4µM EDTA, pH8.0, 113µg/ml BSA and 4U Taq DNA polymerase (Applied Biosystems) in a 0.2ml thin walled microfuge tube. The PCR cycle consisted of a hot start (3min at 95°C in which the DNA polymerase was added, followed by 2min at 72°C) followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and elongation at 72° for 4min. Followed by an elongation step at 72°C for 15 min. For the second round of PCR 5µl of a 1 in 100 dilution of the first PCR reaction was used in the same reaction conditions as detailed above. The products were visualised after separation on a 1.2% agarose gel in the presence of ethidium bromide under UV illumination.

2.2.8 Miscellaneous techniques

2.2.8.1 Random priming

Random priming was used to generate [^{32}P]-labelled DNA fragments for probing Northern blots. 100ng of DNA fragment in 6.75µl of dH_2O and 1.25µl of random hexamers (0.1 OD_{260} Units/ml) were combined in a 1.5ml microfuge tube and boiled for 2-5min. The mixture was collected by brief centrifugation in a benchtop centrifuge. To this mixture, 1µl of BSA (10mg/ml), 2.5x random priming buffer, 5µl [α-^{32}P] dCTP, and 1µl of Klenow was added. The reaction mix was mixed by vortexing, collected by brief centrifugation and incubated at 37°C for 1h. 1µl of Klenow was added and the reaction incubated for a further 1h at 37°C. The reaction volumn was made up to 60µl with dH_2O and the labelled DNA was purified using a mini-quick spin column.

2.2.8.2 Purification of DNA probes

The DNA was purified by mini-quick spin column. The cap was removed from the spin column, ensuring that no beads were lost and the base snapped off. The spin column was placed in a 1.5ml microfuge tube and centrifuged at 6,500rpm for 2min in a benchtop centrifuge. The spin column was placed in a clean microfuge tube. The volume of the probe to be purified was made up to 60µl with dH_2O and pipetted carefully on to the surface of the matrix. The column was centrifuged at 6,500rpm for 2min to elute the probe.

Chapter 3.0 Isolation of novel RNA binding partners for US11

To date, a limited number of RNA binding partners for US11 have been isolated; two HSV-1 derived RNAs, the anti-sense transcript of the US11 5′ UTR (Roller & Roizman, 1990), a truncated transcript of the UL34 gene, Δ34 RNA (Roller & Roizman, 1991); a cellular RNA, rRNA derived from the 60S subunit (Roller & Roizman, 1992); and two retroviral intronic export elements, the RRE of HIV-1 and the XRE of HTLV-1 (Diaz *et al.*, 1996).The role of these RNAs *in vivo* is unclear; the anti-sense transcript of the US11 5′ UTR and Δ34 RNA do not appear to encode a protein and as yet, have no clear role in infection and two are derived from heterologous viral systems. It would therefore be reasonable to hypothesise that they may serve as substrate analogues for US11, which is probable for at least the retroviral RNAs and that there are further HSV-1 or cellular transcripts which can bind US11 which have yet to be identified. In order to test this hypothesis it was necessary to attempt the isolation and identification of RNAs which bind US11 in infected cells.

Firstly, methods by which US11-RNA complexes could be isolated from infected cell lysates were examined. This required the production of recombinant US11, which is outlined in the first section of this Chapter. Secondly, RT-PCR was used as a means of identifying RNAs that interact with US11.

3.1 Production of recombinant US11

3.1.1 Production of recombinant 6xHis-US11

The system chosen for the bacterial expression of the US11 protein was the QIAexpress™ system (Qiagen). This system allows the gene of interest to be fused to an RSG-6xHis-tag either at the N- or C-terminus and the expressed 6xHis tagged proteins to be purified on a Ni-affinity column. In this purification system nitrilotriacetic acid (NTA) is bound to Sepharose® C6-LB beads. NTA is a tetradentate chelating agent. Ni^{2+} ions are chelated by NTA, which occupies four of the six available sites in the co-ordination sphere of Ni^{2+} ions. The remaining two sites are available to interact with the 6xHis tag. 6xHis tagged proteins can therefore be isolated from bacterial lysates by binding them to the Ni-NTA matrix and can be eluted from the column by washing with imidazole, which competes with histidine to chelate the Ni^{2+} ion.

The C-terminus of US11 has been shown to bind RNA (Roller *et al.*, 1996; Schaerer-Uthurralt *et al.*, 1998) and so the N-terminus of US11 was chosen as the location for the 6xHis tag to minimise the potential for interference with RNA binding.

The US11 ORF was PCR amplified and cloned into the pQE-30 vector as described in Materials and Methods and was termed pQE-US11 (Fig. 3.1). The pQE-US11 plasmid was used to transform *E.coli* M15(pREP4) cells. A 10ml starter culture was used to inoculate a 100ml culture which was grown at 37°C until an OD_{600} of 0.6 had been reached. The cultures were then induced with 1mM IPTG and grown for a further 5h at 37°C. Bacterial proteins were separated by SDS-PAGE gel electrophoresis and expression was examined by Coomassie staining or by Western blot analysis using an

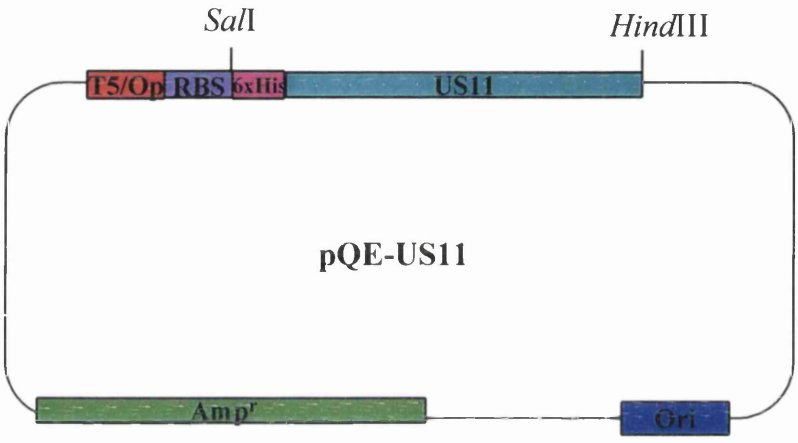


Fig. 3.1: A schematic diagram of pQE-US11.
The US11 ORF was PCR amplified incorporating a 5′ *Sal*I site and a 3′ *Hind*III site. The vector and insert were digested by *Sal*I and *Hind*III and ligated. The the turquoise box represents the US11 gene red box, the T5 promoter and *lac* operator sequence, the mauve box the ribosome binding site, the pink box 6xHis tag, the green box the ampicillin resistance gene (Amp^r) and the blue box the Ori.

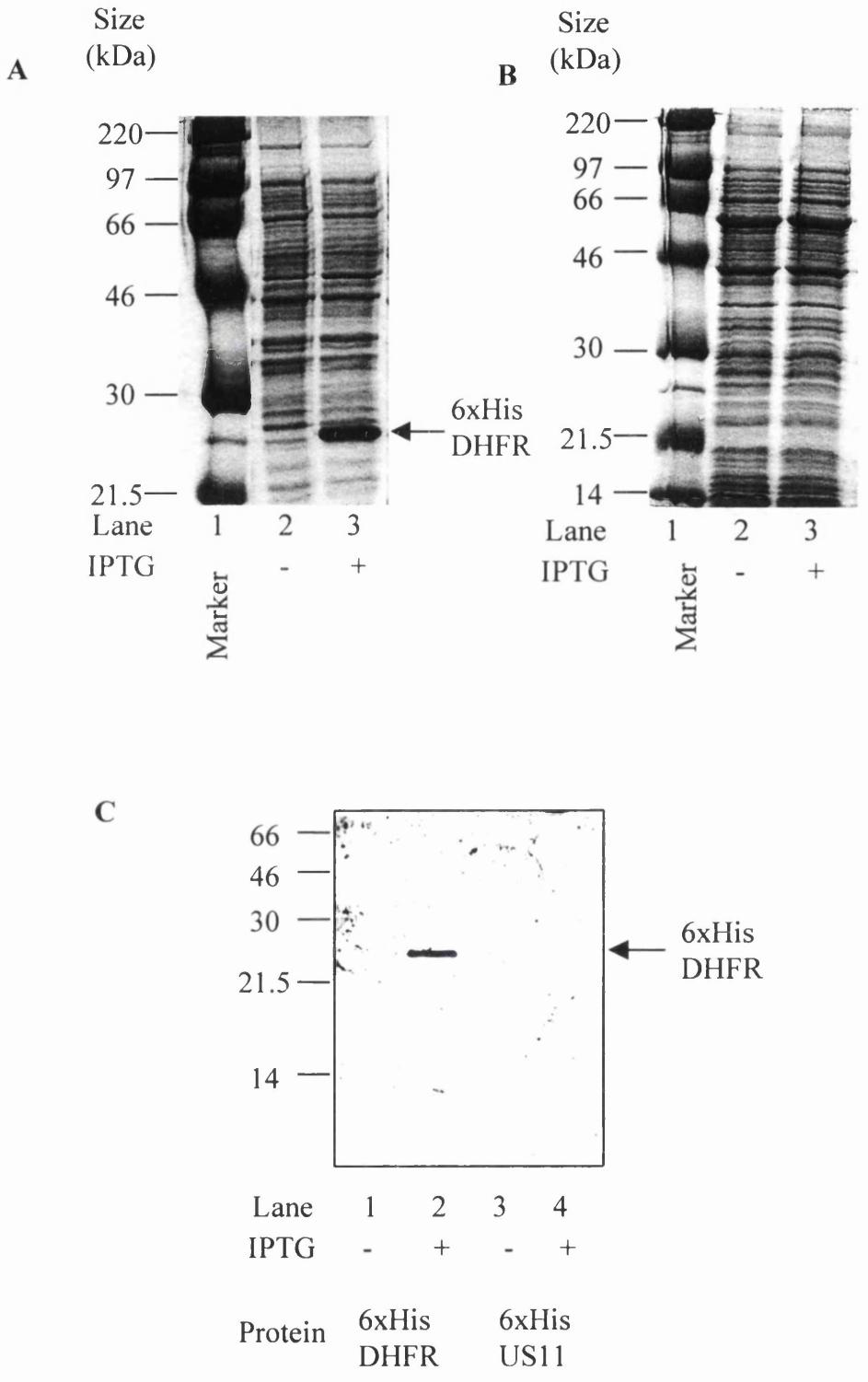
anti-RSG-His monoclonal antibody (mAb). The QIAexpress control plasmid, pQE-16, which encodes the dihydrofolate reductase (DHFR) gene C-terminally 6xHis tagged was used as a control for protein expression.

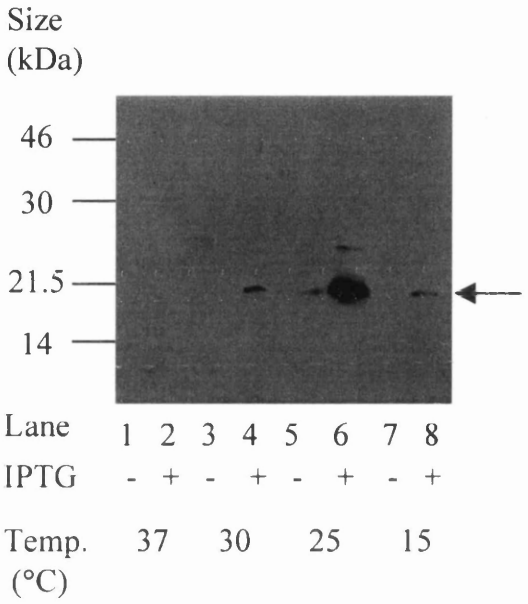
The 6xHis-DHFR protein can be readily visualised in induced cells by Coomassie staining of an SDS-PAGE gel migrating at 26kDa (Fig.3.2A). However, the recombinant 6xHis-US11 protein could not be detected in this fashion (Fig. 3.2B). In order to examine whether 6xHis-US11 is being expressed at levels too low to be detected by Coomassie staining, a Western blot of bacterial extracts was probed with the anti RSG-3xHis mAb (Fig.3.2C). Although expression of 6xHis-DHFR can be detected (lane 2), no expression of 6xHis US11 could be detected (lane 4).

A range of temperatures, 15, 25, 30 and 37°C, were tested to see if 6xHis-US11 could be expressed at any of these temperature (Fig. 3.3). A 10ml starter culture was used to inoculate 100ml of L-broth which was incubated at the respective temperatures. When the OD_{600} of the cultures reached 0.6 they were induced by the addition of 1mM IPTG and cultured at the repective temperature for a further 5h, except for the culture induced at 15°C which was induced for 12h due to the slow rate of growth. The expression of 6xHis-US11 was examined by probing a Western blot with the anti-RSG-3xHis mAb. At 37°C no expression of 6xHis-US11 could be observed (lanes 1 and 2). However, at 30, 25 and 15°C expression of 6xHis-US11 could be detected (lanes 3-8). The expression of the 6xHis-US11 was greatest at 25°C. Never the less expression of 6xHis-US11 could not be detected by Coomassie staining of SDS-PAGE gels of bacterially expressed proteins suggesting that it is expressed at very low levels (data not shown).

In order to confirm the identity of the recombinant protein, a Western blot of a bacterial extract induced for 5h at 25°C was probed with the anti-RSG-3xHis mAb and an anti-

Fig. 3.2: Expression 6xHis-US11 protein at 37°C.
1ml of the culture was pelleted at 12,000rpm and resuspended in 100µl of 1x protein gel loading buffer and boiled for 5min. 20µl were separated by electrophoresis on an SDS-PAGE gel and either stained with Coomassie or Western blotted. A: Coomassie stain of a 12% SDS-PAGE gel of 6xHis DHFR expression. Lane 2 shows the uninduced and lane 3 the induced protein profile. An arrow marks the position of the 26kDa DHFR band. B: Coomassie stain of a 15% SDS-PAGE gel of 6xHis US11 expression. Lane 2 shows the uninduced and lane 3 the induced protein profile. C: A Western blot of a 15% SDS-PAGE gel probed with an anti-RSG 3xHis antibody. Lanes 1 and 3 show uninduced 6xHis DHFR and 6xHis US11 respectively. Lanes 2 and 4 show induced 6xHis DHFR and 6xHis US11 respectively. An arrow indicates the position of 6xHis DHFR.


A
Size
(kDa)
220
97
66
46
30
21.5
6xHis
DHFR
Lane 1 2 3
IPTG - +
Marker
B
Size
(kDa)
220
97
66
46
30
21.5
14
Lane 1 2 3
IPTG - +
Marker
C
66
46
30
21.5
14
6xHis
DHFR
Lane 1 2 3 4
IPTG - + - +
Protein 6xHis DHFR 6xHis US11



3.3: Expression of 6xHis US11 at different temperatures.
Expression was carried out at different temperatures. 6xHis US11 was induced at 37°C (lane 2), at 30°C (lane 4), at 25°C (lane 6) and at 15°C (lane 8). Lanes 1,3,5 and 7 correspond to the uninduced cultures at the respective temperatures. All cultures were induced for 5h except for the culture at 15°C, which was induced for 12h. The proteins were separated on a 15% SDS-PAGE gel and Western blotted. The blot was probed with anti-RSG-3xHis antibody. An arrow indicates the position of 6xHis US11.

US11 polyclonal antibody (pAb). The anti-RSG-3xHis mAb and the anti-US11 pAb reacted with a doublet migrating below the 21.5kDa marker (Fig 3.4). This suggests that this doublet represents 6xHis-US11.

Despite attempts to optimize the conditions of expression, 6xHis-US11 could not be produced in sufficient amounts to be visualised by Coomassie staining. In addition, attempts were made to purify the 6xHis-US11 protein by Ni-NTA chromatography. However, even though the culture size was scaled up, sufficient amounts of 6xHis-US11 were not produced to allow visualisation by Coomassie staining of SDS-PAGE gels.

The low levels of 6xHis-US11 expression suggests that it is expressed very inefficiently. This inefficient expression may be due proteolytic degradation, a preponderance of codons not used in bacteria or that 6xHis-US11 is toxic. The lower temperature required to achieve expression suggests that toxicity or proteolysis plays a major role in the low levels of expression observed.

As it seemed unlikely that the problems with this system could be solved, another source of recombinant US11 was considered.

3.1.2. Production of recombinant GST-US11

Expression and purification of recombinant proteins tagged with glutathione *S*-transferase (GST) is commonly used. GST fusion proteins can be readily purified from bacterial lysates using glutathione, the substrate for GST, immobilized on agarose beads. The GST-fusion protein can be eluted from the beads using free glutathione.

A US11 (HSV-1 KOS strain) construct in which US11 is N-terminally tagged with GST (vector 2319) was obtained from J-J. Diaz and J-J. Madjar (Schaerer-Uthurralt *et al.*,1998). The KOS strain US11 is slightly smaller than 17^{+}, the only difference between

Lane	1	2	3	4	5
IPTG	-	+	Markers	+	-
Antibody	Anti-RSG 3xHis mAb			Anti-US11 pAb	

3.4: Western blot analysis of recombinant 6xHis-US11. Expression of 6xHis US11 was carried out at 25°C for 5h post-induction. The proteins were separated on a 15% SDS-PAGE gel and Western blotted. Lanes 1 and 5 represent uninduced and 2 and 4 induced 6xHis US11. The blot was probed with anti-RSG-3xHis antibody (lanes 1 and 2) and an anti-US11 pAb (lanes 4 and 5). Arrows indicate the positions of the cross reacting bands.

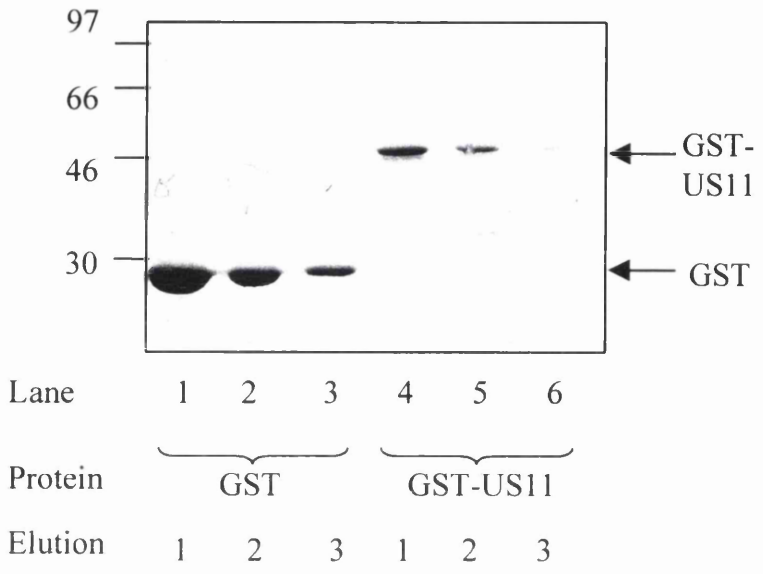
HSV-1 17^+ and KOS is that the former is 161 amino acids, and the latter, lacking two SPREPR repeats in the C-terminus is 149 amino acids in length.

Plasmids 2319 and pGEX-2T were transformed into BL21 cells. The GST-US11 constructs were expressed and purified as described by Schaerer-Uthurralt *et al.* (1998) and in Materials and Methods. In brief, a 10ml o/n culture was used to inoculate 100ml of LB media and when an OD_{600} of 0.4 was reached was induced by the addition of 0.1mM IPTG for 3h at 37°C. The bacteria were harvested by centrifugation and lysed and cell debris pelleted by centrifugation. The GST-fusion proteins were bound to glutathione-agarose beads which were washed extensively and eluted in three rounds with free reduced glutathione. GST-US11 could be purified in one step as a single band free of contaminants as visualised by Coomassie staining of SDS-PAGE gels (Fig 3.5A), as previously demonstrated by Schaerer-Uthurralt *et al.* (1998). However, the stability of the full-length GST-US11 diminished rapidly either with storage at 4°C or -70°C, even with the addition of 20% glycerol and breakdown products could be seen sometimes within a month with storage at -70°C, and in less time at 4°C.

To assess the functionality of the purified protein the RNA binding activity of the GST-US11 protein was tested by electrophoretic gel shift mobility assay (EMSA) with the known RNA substrate, Δ34. The basis of EMSA is the ability of a protein that binds an RNA to retard its progress in a non-denaturing polyacrylamide gel. An *in vitro* transcribed [α-^{32}P] UTP labelled Δ34 riboprobe was incubated in the absence of protein, or in the presence of 12.5ng/μl of GST or GST-US11 at 4°C for 10min and the products resolved on a 4% non-denaturing polyacrylamide gel (Fig. 3.5B). Without the addition of protein the Δ34 riboprobe migrates as a free unbound species (lane 1) and with addition of GST the probe also migrates as an unbound species (lane 2). However, with the addition of GST-US11 the probe is retarded, demonstrating that the probe is in

Fig. 3.5: Purification of GST-US11.
A: GST and GST-US11 were expressed in *E. coli* BL21 cells. The recombinant proteins were bound to glutathione-agarose beads and eluted with three washes in 1ml of 5mM reduced glutathione. Protein loading buffer was added to 20µl of each elution and the samples boiled for 5min. The samples were electrophoresed on a 12% SDS-PAGE gel and Coomassie stained . Lanes 1-3 GST, elutions 1-3 respectively. Lanes 4-6 GST-US11, elutions 1-3 respectively. B: 10,000cpm of a [^{32}P]-labelled Δ34 riboprobe was incubated without protein (lane 1) or 12.5ng/µl of GST (lane 2) or GST-US11. The Complexes were resolved on a native 4% polyacrylamide gel and visualised by autoradiography.

A


97
66
46
30
GST-
US11
GST
Lane 1 2 3 4 5 6
Protein GST GST-US11
Elution 1 2 3 1 2 3

B


Lane 1 2 3
Protein - GST GST-US11

complex with the recombinant protein (lane 3). The recombinant protein therefore behaved as previously shown by Schaerer-Uthurralt *et al.* (1998).

It was observed that the full length GST-US11 fusion protein rapidly lost RNA binding activity when stored at -70°C. In addition, when glycerol stocks of BL21 cells transformed with 2319 DNA (encoding GST-US11) were used to inoculate the 10ml o/n starter culture the yield of and RNA binding activity of the purified recombinant protein was diminished. The production of GST-US11 therefore required that BL21 cells were transformed immediately prior to expression, as opposed to using glycerol stocks. Also, different batches of protein could differ significantly in their ability to bind RNA. Therefore new batches were titrated against Δ34 RNA in EMSA to ensure that fully active protein was used.

3.2 Isolation of US11-RNA complexes

The first step in developing the assay was to isolate US11-RNA complexes from infected cells. Roller & Roizman (1991) achieved this by immunoprecipitating US11 from infected cell lysates, treating the complexes with RNase T1, extracting the protected RNA species and 5′ end-labelling the RNA using T4 polynucleotidyl kinase (PNK) in the presence of [α-^{32}P] ATP. The RNAs were electrophoresed on a denaturing gel and visualised by autoradiography (Fig. 1.13). Roller & Roizman (1991) did not detect any RNAs using extracts from mock infected, US11^{-} HSV-1 infected cells, or without the addition of the secondary antibody. However, numerous RNA species of varying size were co-immunoprecipitated from HSV-1 infected cell lysates. The end-labelled RNAs were used to probe a Southern blot of a *Bam*HI digest of HSV-1 DNA. The only fragment that hybridized contained the UL34 sequence and subsequent

analysis identified the Δ34 RNA as a binding partner for US11. As only one RNA hybridized, this suggests that other RNAs may have been present but at levels below the limit of detection by Southern blot hybridization and autoradiography. Alternatively, some of the labelled RNAs may have been derived from cellular RNAs.

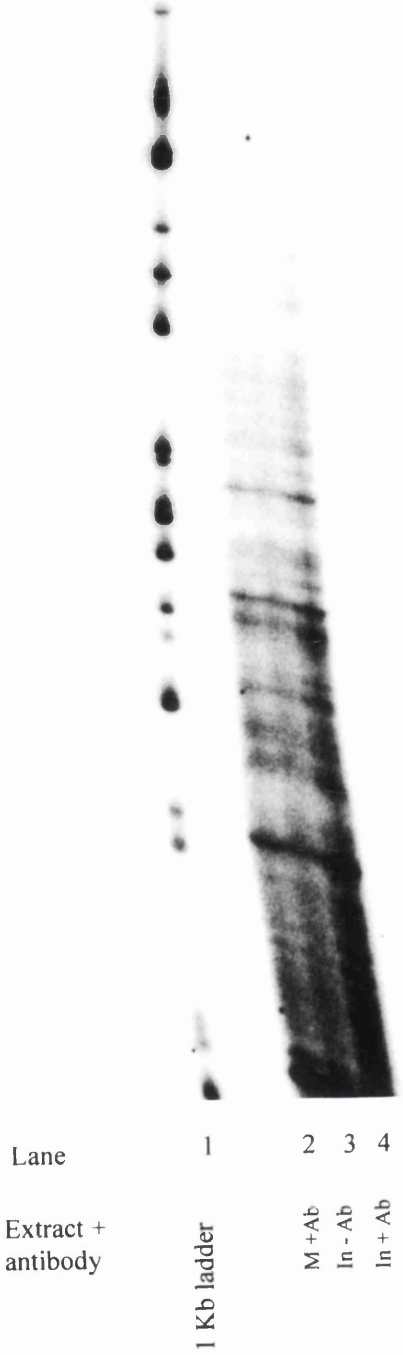
An alternative approach to immunoprecipitating native US11-RNA complexes from infected cell lysates is to use the GST-US11 fusion protein to isolate RNAs which bind US11. GST-US11 protein bound to a glutathione-agarose could be incubated with infected cell extracts, the complexes pelleted and washed and the RNAs extracted. This method would allow high concentrations of US11 to be used, far in excess of virus infected cellular levels of US11, thus the potential for isolating interacting RNAs is increased. However, US11 is phosphorylated *in vivo* and one potential problem is that recombinant US11 may not possess the necessary post-translational modifications required for RNA binding.

3.2.1 Co-immunoprecipitation of US11-RNA complexes

In order determine whether the co-immunoprecipitation of US11 and RNA employed by Roller & Roizman (1991) could be used in conjunction with an RT-PCR based amplification the protocol was repeated.

An anti-US11 monoclonal antibody (28*) was used to immunoprecipitate US11 from mock infected and infected HeLa cell lysates (16h pi). The immunoprecipitation and end-labelling of RNAs was preformed as described by Roller & Roizman (1991). However, in the example shown in Fig. 3.6, the following modifications were made to the protocol. The cell lysates were pre-cleared with the secondary antibody, and the isolated RNA was pre-treated with DNase I in an attempt to reduce the non-specificity

A



B

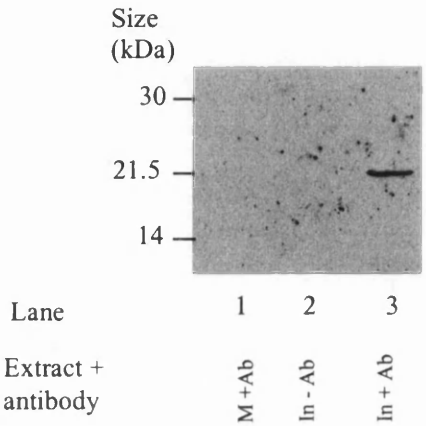


Fig. 3.6: Co-immunoprecipitation of US11 and RNA.
A: An autoradiograph of a 6% denaturing gel of [^{32}P]-end labelled RNAs. US11 was immunoprecipitated from infected cell extracts (lane 4), a control immunprecipitation was performed using mock infected cell extracts (lanes 2), or without the anti-US11 antibody (lane 3). A 1kb ladder was end labelled as a control for the reaction (lane 1). B: The corresponding Western blot of immunoprecipitated proteins electrophoresed on a 12% SDS-PAGE probed with a US11 antibody. Abbreviations: Anti-US11 monoclonal antibody (Ab), infected cell extract (In), mock infected cell extract (M).

of the immunoprecipitation as described below. RNAs which co-immunoprecipitated with US11 were isolated by boiling the beads for 2min in 0.5% (w/v) SDS/TE. The RNA was recovered by phenol/chloroform extraction and ethanol precipitation. The isolated RNAs were end-labelled with [γ-^{32}P] ATP in the presence of PNK. The end-labelled RNAs were resolved on a denaturing gel. Fig. 3.6A shows the labelled RNAs generated by such a pull down. Many RNA species were isolated from both mock infected cells plus the anti-US11 mAb and infected cells minus the anti-US11 mAb (lanes 2 and 3). The labelling seems to be slightly heavier with immunoprecipitation from infected cells plus the anti-US11 mAb (lane 4), but in general, the pattern of products is similar. Many species were isolated and the pattern obtained varied between experiments, but overall appeared to be similar for mock infected, infected minus secondary antibody and infected cell lysates. Western blot analysis demonstrated that US11 was co-immunoprecipiated (Fig. 3.6B). The reason for the non-specific reactivity of the secondary antibody is not clear. The apparent lack of US11-specific RNA species may be due to the conditions not being optimal for US11-RNA interactions or that insufficient amounts of US11 were precipitated and hence the RNA co-immunoprecipitated could not be visualized by end-labelling especially in light of the high background labelling. Therefore, alternative solutions to isolating RNAs that bind to US11 were explored.

3.2.2 Isolation of GST-US11/RNA complexes from infected cell lysates

Trifillis *et al.* (1999) used a recombinant GST-αCP1fusion protein to successfully pull out interacting RNAs. The ability of GST-US11 to pull down RNAs from infected cells was examined (a schematic outline of the protocol is given in Fig. 3.7). Firstly, 50μg of

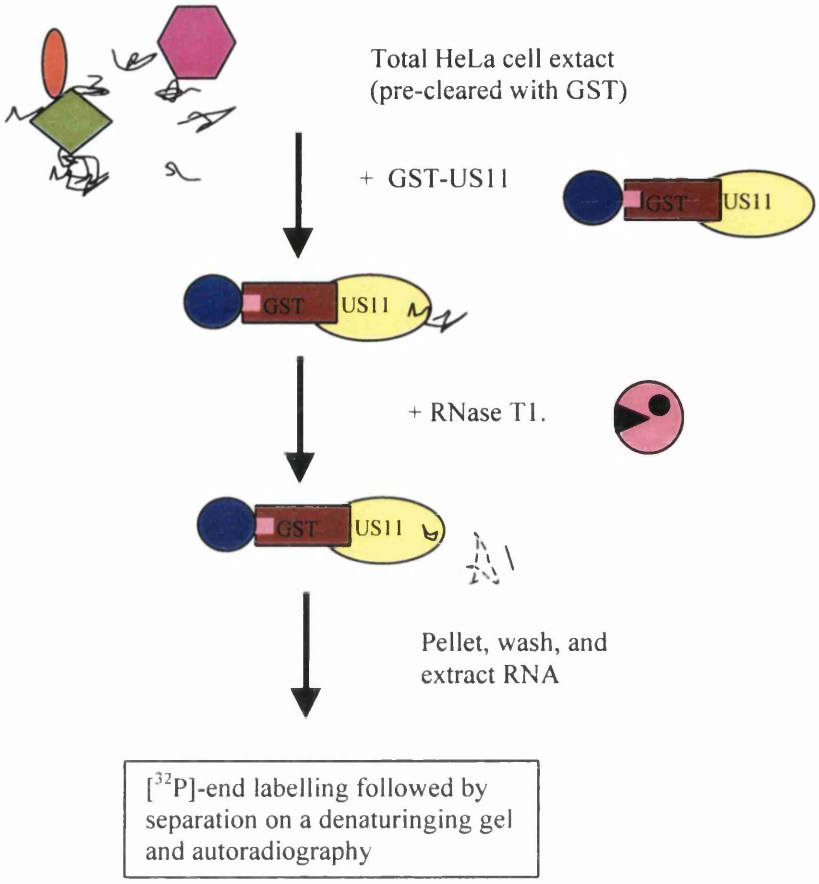


Fig. 3.7: Schematic diagram of the pull down of GST-US11/RNA complexes.

Schematic diagram of the method used to isolate US11-RNA complexes. GST-US11 bound to glutathione-agarose is incubated with infected HeLa cell extracts. The reaction is incubated with RNase T1. The complexes are washed and the RNA extracted.

purified GST or GST-US11 were pretreated with miccrococal nuclease to remove any contaminating bacterial RNA and then bound to glutathione-agarose beads in RNA binding buffer (RBB). Infected HeLa cell extracts were pre-cleared by incubation with GST-glutathione-agarose to remove RNAs that inadvertently bind GST or the glutathione-agarose beads. The pre-cleared extract was incubated with GST- or GST-US11-glutathione-agarose beads. RNase T1 was added and the complexes washed extensively to remove non-specific RNAs. The beads were resuspended in 0.5% (w/v) SDS/TE and boiled for 2min. The RNA was extracted by phenol/chloroform and ethanol precipitated. The RNA was 5′ end-labelled with PNK in the presence of [γ-^{32}P] ATP, and the RNAs resolved on a denaturing gel (Fig. 3.8A).

Both from the pull down with GST and GST-US11 no labelled species could be detected (lanes 3 and 4). A 47nt RNA derived from RSV (a gift from Lindsay Rowlands) was used as a positve control for RNA end-labelling reaction (lane 1).

Reverse transcription coupled with PCR amplification (RT-PCR) is a very sensitive method to detect low abundance RNAs. Therefore, in order to assess whether RNAs were present at an amount to low to be detected by end-labelling, the presence of Δ34 RNA, known to bind US11, was examined by RT-PCR (Fig. 3.8B). The pull-down was performed as before except that the RNase T1 step was omitted. The RNA was reverse transcribed in the presence of a Δ34 sequence-specific primer (Δ34R). The ss cDNA was subjected to two rounds of PCR in the presence of Δ34 specific primers. In the second round of PCR the primers were nested inside the sequence of the first. With GST alone no amplification product was observed, however, with GST-US11 a band migrating at approximately 250bp was seen corresponding to the expected amplification product of Δ34. This species was isolated, cloned into pGEM-T Easy and its identity as Δ34 confirmed by sequencing.

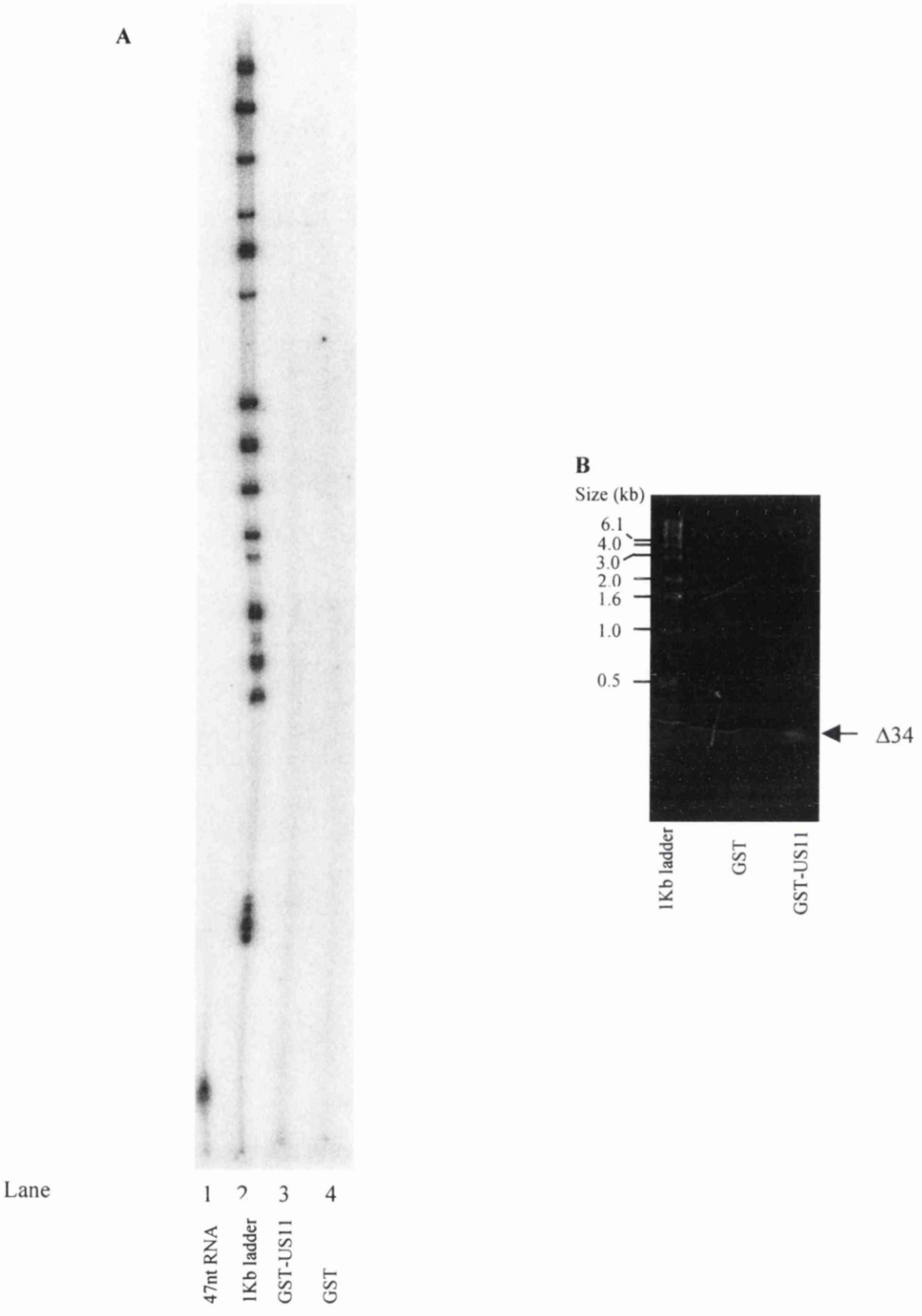


Fig. 3.8 Pull down of GST-US11/RNA complexes.
A: Autoradiograph of 6% denaturing gel. GST (lane 4) or GST-US11 (lane 3) were used to in a GST pull down, the RNA extracted, [^{32}P]-end labelled and separated on a sequencing gel. A 49nt RNA derived from RSV was used as positive controls for [^{32}P] RNA labelling in the end labelling reaction (lane 1). Lane 2 contains an end labelled 1Kb marker. B: GST or GST-US11 were used to pull out RNAs from infected cell lysates. The RT-PCR of the RNA was performed with primers specific for Δ34 RNA. 5μl of the products were separated on a 1.5% agarose gel. The position of the amplified product is indicated with an arrow.

3.3 RT-PCR based assay for detecting RNAs bound by US11

Using GST-US11 bound to glutathione-agarose beads it was possible to isolate a known binder RNA, Δ34 RNA, which could be detected using RT-PCR with specific primers. Other RNAs which interact with US11 could also be represented. Therefore, a RT-PCR method which allowed the RNAs to be reverse-transcribed and amplified without the need for sequence information had to be devised. Two methods were tested, one that depended on the RNA species being polyadenylated, and the other which was independent of the presence of a poly(A)$^+$ tail.

3.3.1 Poly(A) tail dependent RT-PCR assay.

The first method examined was based on a combination of 3′ and 5′ Rapid Amplification of cDNA Ends (RACE). 3′ RACE relies on the presence of a poly(A)$^+$ tail, which is added to the 3′ end of mRNA. Reverse transcription is carried out using a oligo-d(T) primer, and subsequent rounds of amplification utilize a gene specific primer and a primer specific for the poly(A):(T) sequence or an anchor site that has been incorporated during RT or the first round of PCR. In 5′ RACE a gene specific primer is used in RT and the resultant cDNA is homopolymer tailed with one of the dNTPs using terminal deoxynucleotidyl transferase (TdT). This serves as primer site in subsequent PCR cycles in conjunction with a gene specific primer.

Using the poly(A)$^+$ tail and homopolymer tailing as a primer hybridization site in RT-PCR, it was reasoned that this would bypass the need for any gene specific sequence information for RT-PCR and hence allow the amplification of unknown RNA species.

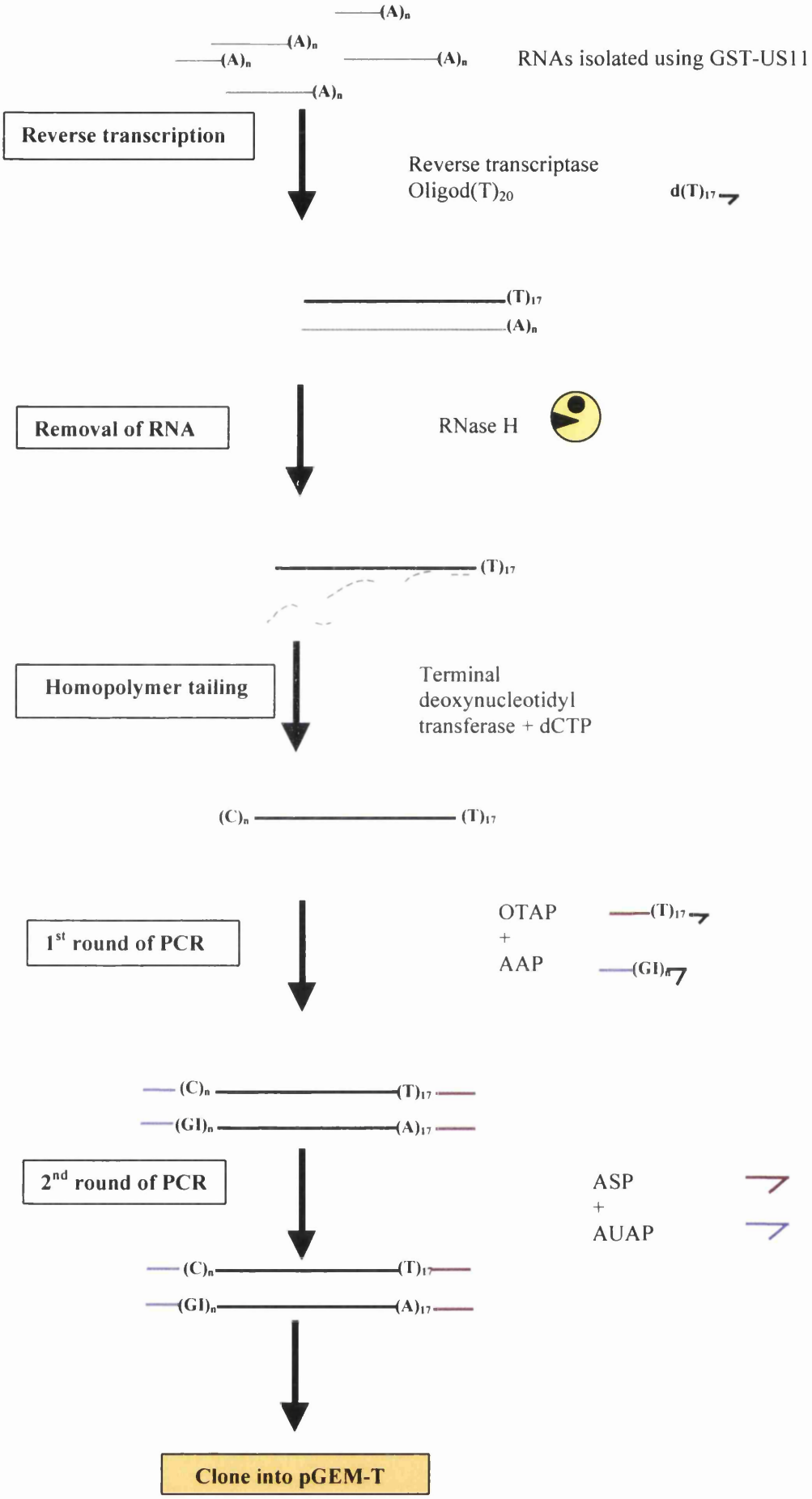
This method was termed poly(A) dependent RT-PCR (PA RT-PCR). Thermostable Taq DNA polymerase adds a single deoxyadenosine to the 3′ ends of amplified product. By using a vector such as pGEM-T Easy, which possesses a single deoxythymidine overhang, PCR products can be directly ligated into the vector and subsequently sequenced. This method is described in detail below and schematically illustrated in Fig. 3.9.

The RNA is reverse transcribed in the presence of an oligo $d(T)_{17}$ primer and the RNA is removed by digestion with RNase H. The single stranded (ss) cDNA is homopolymertailed with dCTP by TdT. The C-tailed ss cDNA is amplified by two rounds of PCR with primers specific for the 5′ and 3′ ends of the template. In the first round of amplification the Gibco BRL 5′ RACE primer, Abridged Anchor Primer (AAP) and Primer Oligo-$d(T)_{17}$-Anchor Primer (OTAP) were used. The AAP contains a 3′ GI sequence of 16nt (GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG) that can hybridize to the homopolymer tail of the cDNA and a 5′ anchor sequence, which serves as site for the primer to anneal to in the second round of PCR. The OTAP primer contains an $oligo(T)_{17}$ sequence and an anchor site for hybridization in the second round of PCR (TCGAGCATCGGATCCTTACCTTTTTTTTTTTTTTTT). The products of the first round of PCR are diluted 1 in 100, and 5µl used in the second round of PCR. In the second round of PCR the Gibco BRL 5′ RACE primer, Abridged Universal Amplification Primer (AUAP) and 3′ Anchor Site Primer (3′ ASP) were used. The AUAP (GGCCACGCGTCGACTAGTAC), anneals to the anchor site incorporated by the AAP in the first round of PCR and 3′ ASP (TCGAGCATCGGATCCTTACC) anneals to the anchor sequence incorporated by the OTAP primer.

This method was first tested using a known substrate, γ-actin. Cytoplasmic Hela cell RNA was reverse transcribed using an oligo-$d(T)_{17}$, treated with RNase H and purified .

Fig. 3.9: Schematic diagram of poly(A)-dependent RT-PCR.

A schematic diagram for reverse transcribing and PCR amplifying RNA that interacts with US11. Reverse transcription is performed using an oligo-$d(T)_{17}$ primer and SuperScript™ reverse transcriptase. RNA in RNA/DNA hybrids is digested with RNase H. The cDNAs are homopolymer tailed with d(C) using terminal deoxynucleotidyl transferase. Two rounds of PCR are performed. The first using the primers Abridged Amplification Primer (AAP) and Oligo-$d(T)_{17}$-Anchor Primer (OTAP) both containing anchor sites for the hybridization of the primers in the next round. In the second round of PCR the primers Abridged Universal Amplification Primer (AUAP) and the 3′ Anchor Site Primer (3′ASP) are used. The products of the reaction can be resolved on an agarose gel, excised and cloned into pGEM-T Easy for subsequent sequencing.


(A)n
(A)n
(A)n
(A)n
(A)n
RNAs isolated using GST-US11
Reverse transcription
Reverse transcriptase
Oligod(T)20
d(T)17
(T)17
(A)n
Removal of RNA
RNase H
(T)17
Homopolymer tailing
Terminal
deoxynucleotidyl
transferase + dCTP
(C)n
(T)17
1st round of PCR
OTAP
+
AAP
(T)17
(GI)n
(C)n
(T)17
(GI)n
(A)17
2nd round of PCR
ASP
+
AUAP
(C)n
(T)17
(GI)n
(A)17
Clone into pGEM-T

The cDNA was homopolymer tailed with dCTP in the presence of TdT. A technique known as hybrid selection of cDNAs was used to isolate the γ-actin homopolymer tailed cDNA. In brief, a DNA fragment containing 840bp of the γ-actin sequence was UV cross-linked to Hybond-N nitrocellulose membrane. The poly(A) reverse transcribed, C-tailed ss cDNA was hybridized to the membrane, washed and the cDNA eluted by boiling and snap freezing the membrane. The eluate was subjected to ethanol precipitation and the pellet resuspended in dH_2O. The eluted RNA was amplified by two rounds of PCR as described above. As the OTAP, AAP, ASP, and AUAP primer have such different annealing temperatures, 34, 52, 62 and 60°C, respectively, the PCR was therefore performed at a range of different temperatures to determine whether an optimum temperature to yield a product could be found (Fig. 3.10).

At an annealing temperature of 60°C no PCR products are observed. However, when the annealing temperature is lowered to 57°C a smear running from 1kb to the bottom of the gel is observed. At an annealing temperature of 54°C no products are observed but, at 51°C specific amplification of γ-actin RNA is seen, at 49°C a smear running at about 0.2kb is seen. From this it appears that the specific amplification of cDNA has a limited temperature range, 51°C producing a specific product, which the annealing temperatures 49°C and 54°C are unable to do. The non-specific smeared products may result from the formation of primer dimers or annealing at multiple sites within the γ-actin sequence. 51°C was therefore chosen as the temperature to perform PA RT-PCR with.

In order to isolate RNAs which interact with US11 the pull-down of GST-US11/RNA complexes was achieved as detailed above, but as this required full length mRNA the RNase T1 step was omitted. RT-PCR was carried out as described above and the products separated by agarose gel electrophoresis. A typical profile gained from such an amplification protocol is shown in Fig. 3.11. With GST alone a low molecular weight

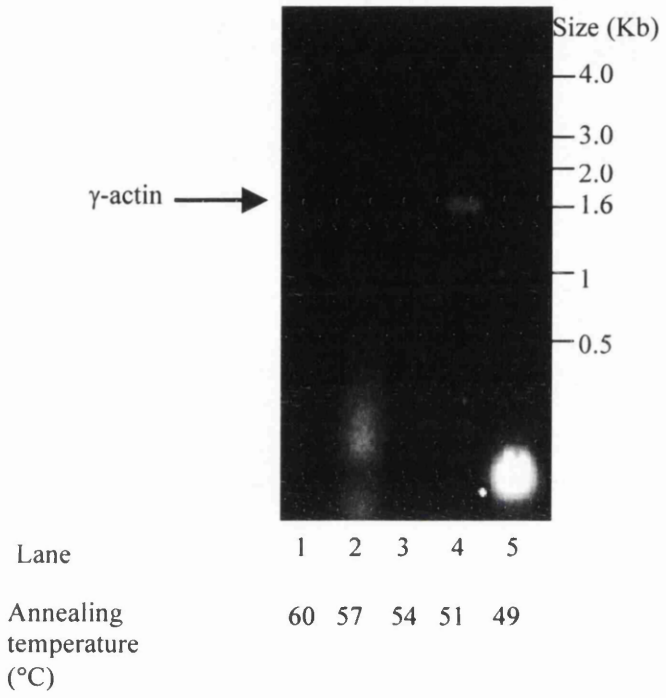


Fig. 3.10: Poly(A)-dependent RT-PCR of γ-actin cDNA at different PCR annealing temperatures.

Total cytoplasmic RNA was reverse transcribed using an oligo $d(T)_{17}$ primer. γ-actin RNA was pulled out of the cDNA pool by hybrid selection and subjected to two rounds of PCR amplification by the PA RT-PCR scheme outlined in panel A. The two rounds of PCR were performed at different annealing temperatures, 60, 57, 54, 51, and 49°C. The 5µl of the products were separated on a 1.5% agarose gel. The band was excised and cloned into pGEM-T and its identity as γ-actin confirmed by sequencing.

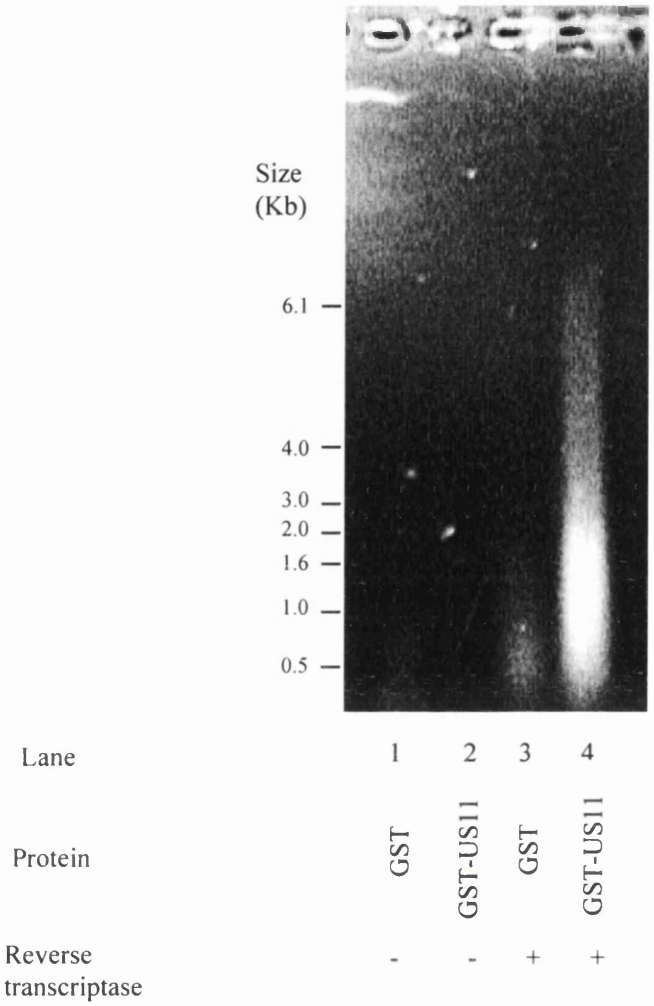


Fig. 3.11: Poly(A)-dependent RT-PCR of RNAs pulled down with GST-US11.

GST (lanes 1 and 3) or GST-US11 (lanes 2 and 4) were used to pull out RNAs from infected cell lysates (16h p.i.). The RNAs were reverse transcribed (lanes 3 and 4) and amplified by two rounds of PA RT-PCR. The amplified products were resolved on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV-illumination.

smear of between 0.4 and 1.6kb was observed, whereas with GST-US11 a product smear is located between 0.4 and 6 kb. The smeared nature of the products probably arises from the primers mis-hybridizing on the cDNA template, hybridization of the oligo-$d(T)_{17}$ primer in various locations within the RNA poly(A) tail or the fragmentation of RNA. In order to see if a RT-PCR method could be developed which resulted in a more distinct banding pattern of amplified products another RT-PCR method was examined.

3.3.2 Sequence-independent RT-PCR assay.

The second RT-PCR method examined is based on the 5′ RACE method in combination with random primers. In brief, GST and GST-US11 were used to pull down RNA from infected cell extracts as before. The RNA was reverse transcribed using random 9-mer primers and the RNA removed using RNase H. The resultant ss cDNA was homopolymer tailed using terminal TdT and dCTP. The C-tailed ss cDNA was amplified by two rounds of PCR. In the first round the random 9-mer primers and the AAP were used. The products were diluted 1 in 100 and amplified in the second round of PCR using random 9-mer primers and the AUAP.

Fig. 3.12 shows a schematic illustration of this protocol. This method theoretically allows RT-PCR without sequence bias i.e. independent of the presence of a poly(A) tail. This method was therefore termed sequence-independent RT-PCR (SI RT-PCR). US11 binds at least one non-polyadenylated transcript, Δ34 RNA (Roller & Roizman, 1991) and therefore, a method which does not rely on the presence of such a sequence would be advantageous in the isolation of RNA which binds to this protein. Fig. 3.13A shows the results of such an assay. Δ34-specific primers were used in reverse transcription and PCR amplification of the RNA as a positive control, with GST alone no amplification

Fig 3.12: A schematic diagram of sequence-independent RT-PCR.
Scheme of the sequence-independent amplification of RNAs that interact with US11. RNAs are reverse transcribed using 9-mer random primers and SuperScript^{TM} reverse transcriptase. The RNA in DNA:RNA hybrids is digested with RNase H. The cDNA is homopolymer tailed with deoxycytosine using terminal deoxynucleotidyl transferase. The C-tailed cDNAs are subjected to two rounds of PCR. The first round with random 9-mer primers and AAP. The second round is performed with random 9-mer primers and AUAP. The products of this reaction are separated on an agarose gel, the bands excised and cloned into pGEM-T Easy.

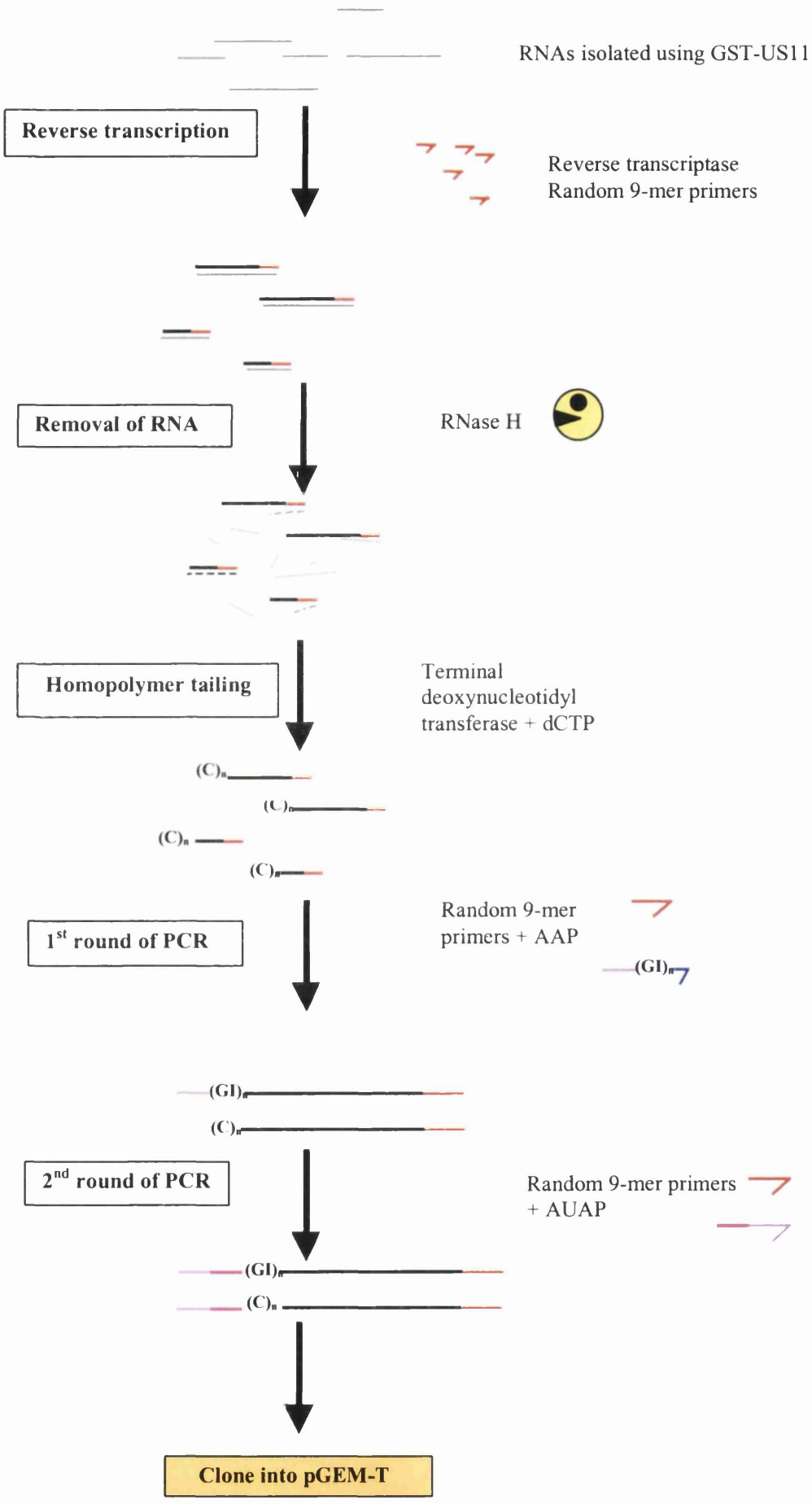
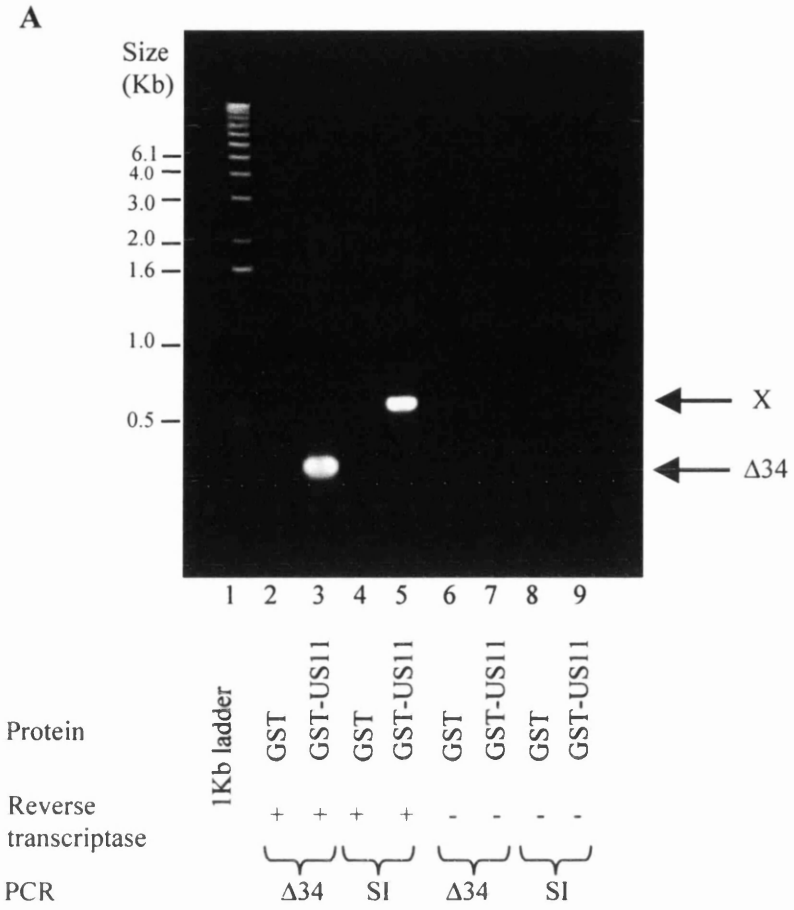
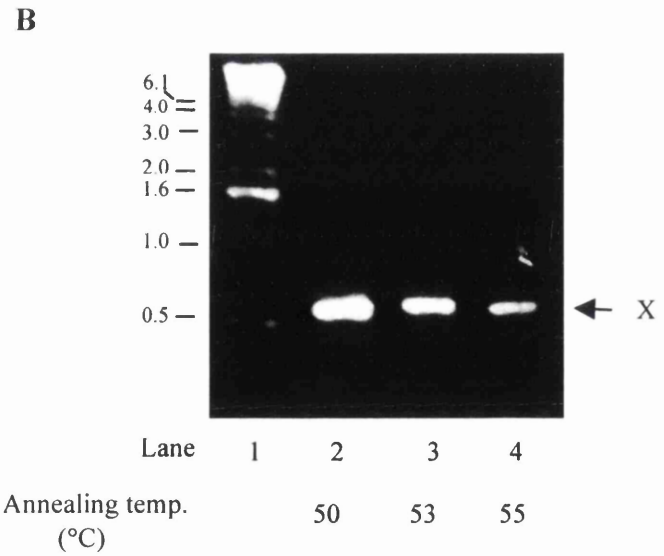

RNAs isolated using GST-US11
Reverse transcription
Reverse transcriptase
Random 9-mer primers
Removal of RNA
RNase H
Homopolymer tailing
Terminal
deoxynucleotidyl
transferase + dCTP
(C)n
(C)n
(C)n
(C)n
1st round of PCR
Random 9-mer
primers + AAP
(GI)n
(GI)n
(C)n
2nd round of PCR
Random 9-mer primers
+ AUAP
(GI)n
(C)n
Clone into pGEM-T

Figure 3.13: Sequence-independent RT-PCR of RNAs that interact with GST-US11.

A: Separation on a 1.5% agarose gel of 20μl of the reaction products derived from the SI RT-PCR of RNAs pulled down with either GST (Lanes 2, 4, 6 and 8) or GST-US11 (Lanes 3, 5, 7, and 9), with (lanes 2-5), or without (lanes 6-9) reverse transcription. Gene specific primers to the Δ34 RNA were used as a positive control for US11-RNA interactions (lanes 2, 3, 6 and 7). Sequence-independent (SI) RT-PCR was performed on the RNA derived from the pull down (lanes 4, 5, 8 and 9). Arrows show the position of the Δ34 product amplified by gene specific primers and the unknown amplification product "X" amplified by SI RT-PCR. B: The annealing temperature of both PCR cycles for SI-PCR of RNAs that interact with GST-US11 were performed at 50 (lane 2), 53 (lane 3) and 55°C (lane 4). The products were resolved on a 1.5% agarose gel.


A
Size
(Kb)
6.1
4.0
3.0
2.0
1.6
1.0
0.5
X
Δ34
1 2 3 4 5 6 7 8 9
1Kb ladder
GST
GST-US11
GST
GST-US11
GST
GST-US11
GST
GST-US11
Protein
Reverse
transcriptase
+ + + + - - - -
PCR
Δ34
SI
Δ34
SI


B
6.1
4.0
3.0
2.0
1.6
1.0
0.5
X
Lane
1 2 3 4
Annealing temp.
(°C)
50 53 55

products are observed (lane 2), but with GST-US11, an amplifcation product migrating at approximately 250bp is seen (lane 3). The identity of this species as Δ34 was confirmed by cloning and sequencing. SI RT-PCR was performed on the pulled down RNAs. With GST alone no amplification products are observed (lane 4), but with GST-US11 a single product migrating at approximately 600bp is seen (lane 5). Without reverse transcription no amplified products are seen (lane 8 and 9). To determine whether any further products could be amplified the annealing temperature of PCR cycles was varied. Lowering of the annealing temperature of the PCR should allow less stringent hybridization between the random primers and the cDNA template, therefore, potentially more PCR products will be generated. However, when the annealing temperature was lowered to 53°C or 50°C, although the amount of the 600bp product was increased, no novel bands were observed (Fig. 3.13B). Touch-down PCR, in which the first 5 rounds of amplification were performed at 60°C, and the annealing temperature was lowered by 0.5°C for each subsequent round for 30 cycles, did not yield any further products (data not shown).

3.4 A sequence derived by SI RT-PCR is a potential RNA ligand for US11

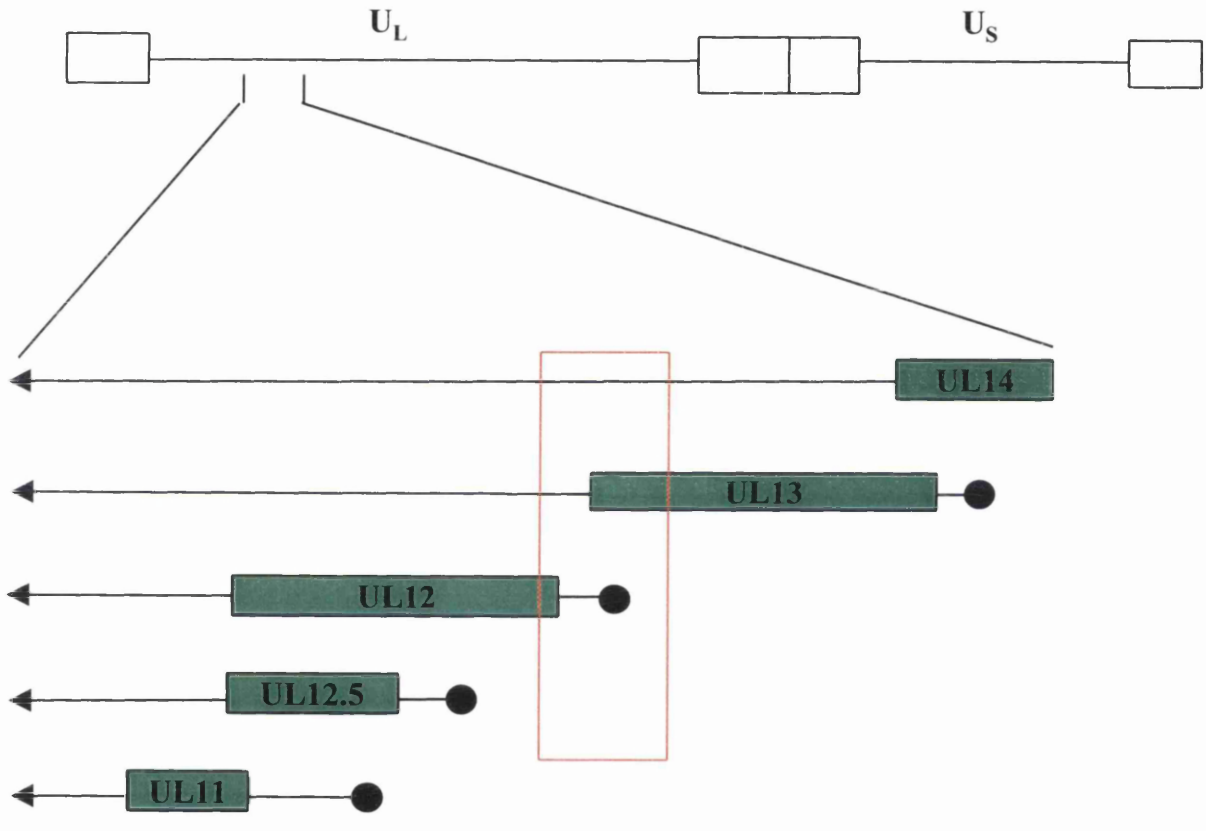
The 600bp fragment was excised from the gel and cloned into pGEM-T Easy and sequenced. The sequence was analysed by BLAST-N (see Appendix) and was found to have homology to a 585nt sequence in the U_L region containing a 3′ co-terminal RNA family encoding UL12, UL13 and UL14. The region lies within the 3′UTR of UL14, the 3′ ORF and 3′ UTR of UL13 and the 5′ UTR and 5′ ORF of UL12 mRNA (Fig. 3.14). The plasmid was therefore named pT-12/14. Δ34 is pulled out by GST-US11, as confirmed by RT-PCR with gene-specifc primers, but it is not amplified in the SI RT-

B

```
1   CUGGAGUUGGUGGUUAGCGUGUACGUCGCCCCGAGCCUGGGCGUACCCGU
51  GACCCGGUUUCCCGGUUACCAGUAUUUUAACAACCAGCUGUCGCCGGACU
101 UCGCCCUGGCCCUGCUCGCCUAUCGCUGCGUGCUGCACCCAGCCCUGUUU
151 GUCAACUCGGCCGAGACCAACACCCACGGCCUGGCGUAUGACGUCCCAGA
201 GGGCAUCCGGCGCCACCUCCGCAAUCCCAAGAUUCGGCGCGCGUUUACGG
251 AUCGGUGUAUAAAUUACCAGCACACACACAAGGCGAUACUGUCGUCGGUG
301 GCGCUGCCUCCCGAGCUUAAGCCUCUCCUGGUGCUGGUGUCCCGCCUGUG
351 UCACACCAACCCGUGCGCGCGGCACGCGCUGUCGUGAGAAUCAGCGUUCA
401 CCCGGCGGCGCGCUCAACCACCGCUCCCCCCACGUCGUCUCGGAAAUGGA
451 GUCCACGGUAGGCCCAGCAUGUCCGCCGGGACGCACCGUGACUAAGCGUC
501 CCUGGGCCCUGGCCGAGGACACCCCUCGUGGCCCCGACAGCCCCCCCAAG
551 CGCCCCCGCCCUAACAGUCUUCCGCUGACAACCAC-585
```

Fig. 3.14: Location and sequence of UL12-14.
A: The genomic location of the UL12-14 genes.The upper line shows a schematic diagram of the HSV-1 genome, the unique elements are represented by lines and major repeat elements by boxes. The expanded region of the genome, UL11-UL14 is depicted below. The arrow heads represent the 3′ terminus and the circles the 5′ terminus of the RNAs. The 585nt region of homology with the sequence derived from SI RT-PCR is highlighted by a red box. B: The RNA sequence of the region highlighted in B. The the transcription iniation site of UL12 is indicated by an arrow, stop codon of UL13 is highlighted in blue and the Met-AUG of UL12 in red.

A



A

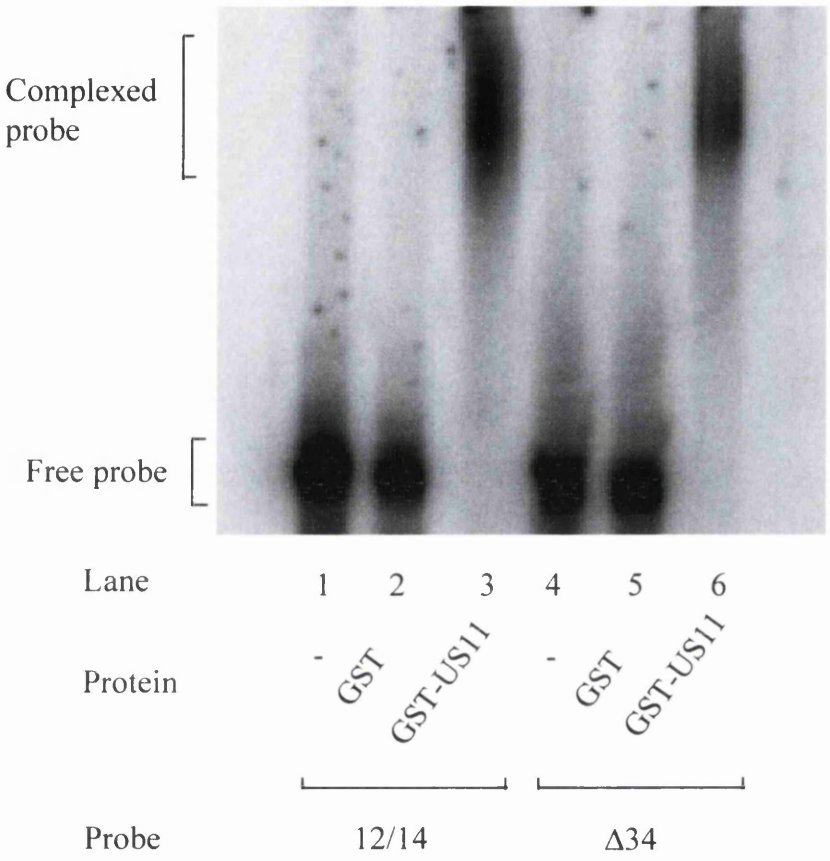


Fig. 3.15: EMSA of US11 interaction with 12/14 RNA.
10,000cpm of *in vitro* transcribed 12/14 RNA (lanes 1-3) or Δ34 RNA (lanes 4-6) was incubated without the addition of protein (lanes 1 and 4), in the presence of 12.5ng/μl GST (lanes 2 and 5) or 12.5ng/μl GST-US11 (lanes 3 and 6). Complexes were resolved on a 4% non-denaturing gel and visualised by autoradiography.

SI RT-PCR.This does indicate that either some bias has been introduced during the RT-PCR procedure, either as sequence-bias or due to the relative abundance of these transcripts.

The sequence pulled out by GST-US11, termed the 12/14 RNA, was tested for authenticity with regards to an interaction with US11. 12/14 RNA was transcribed from linearized pT-12/14 with T7 RNA polymerase and was radiolabelled with [α-^{32}P] UTP. *In vitro* binding of US11 to the 12/14 sequence was assayed using EMSA (Fig. 3.15). The Δ34 and 12/14 riboprobe was incubated either without the addition of protein or in the presence of GST or GST-US11. Without the addition of protein both the Δ34 and 12/14 riboprobe migrate as free, unbound RNA (lanes 1 and 4). No change in the mobility of the riboprobes is detected when incubated in the presence of GST (lanes 2 and 5). When incubated with GST-US11 a shift in the mobility of both riboprobes is generated (lanes 3 and 6), demonstrating that the US11 component can bind both the positive control RNA, Δ34, and the RNA amplified by SI RT-PCR, 12/14 RNA. Therefore, it can be concluded that the 12/14 fragment has the potential to interact with the US11 protein. The authenticity of this interaction is examined in subsequent Chapters.

3.5 Summary and discussion of results.

This Chapter describes the development of a protocol to enable the isolation and identification of unknown RNAs which bind a specific protein of interest. US11 is known to bind a limited number of RNAs: the RRE and the XRE, the anti-sense transcript of its own gene's 5′ UTR and a truncated RNA derived from the UL34 gene, Δ34. As the function of these interactions in HSV-1 infection is unclear it was hypothesised that US11 could potentially bind other RNAs of significance.

Other methods are now published for the identification of RNAs which interact with a particular protein and are discussed in Chapter 1. These methods are compared to the protocol described here in Chapter 7. This section shall deal with the comparison of methods used in the identification of RNA partners for US11.

Three methods were used previously to identify US11-RNA interactions: (1) the *in vitro* transcription of HSV-1 DNA fragments coupled with EMSA (Roller & Roizman, 1990), (2) the immunoprecipitation of US11-RNA complexes followed by radiolabelling of RNAs and hybridization with HSV-1 genomic fragments (Roller & Roizman, 1991); (3) testing candidate RNAs for US11 binding, the assumptions being based on the biological and physical properties of the protein (Diaz *et al.*, 1996; Roller *et al.*, 1992). All have their limitations based on the assumptions they make. The first two are limited to the identification of HSV-1 RNAs. The former could potentially involve a large amount of screening and the latter is limited by the sensitivity of detection. The third method is limited to what RNAs are reasonable to test, and is therefore self limiting.

RT-PCR can overcome the limitations outlined above. It is a highly sensitive method allowing the identification of low abundance RNAs. It can potentially lead to the

identification of both cellular and viral RNAs and it is a relatively quick method, taking approximately 5 days from the pull down to the picking of positive colonies.

The first step in developing the protocol was to isolate US11-RNA complexes from infected cell lysates. Immunoprecipitation of US11 yielded a non-specific profile of RNAs. A GST-US11 pull down did not yield any RNAs detectable by end-labelling, but RNAs could be detected using RT-PCR, suggesting that an RT-PCR based protocol may allow the identification of interacting RNAs as it is a more sensitive method.

Two RT-PCR methods were tested, a poly(A)-dependent RT-PCR (PA RT-PCR) protocol and a sequence-independent RT-PCR (SI RT-PCR) protocol. The products of PA RT-PCR migrated as a smear on agarose gel electrophoresis. From this it was difficult to isolate specific products. The smeary nature of the products probably results from aberrant primer hybridization, multiple priming sites within the poly(A) tails or RNA fragmentation. SI RT-PCR was advantageous in one major way. US11 can bind non-polyadenylated RNAs and these would be selected against using PA RT-PCR. SI RT-PCR therefore allows the non-bias amplification of RNAs. Using random 9-mer primers in SI RT-PCR one product was detected. Unexpectedly, when this product was sequenced it was found not to be the known binder, Δ34 RNA, but a sequence derived from the 3′ co-terminal RNAs of UL12, UL13 and UL14. This demonstrates that there is some degree of bias, in that the Δ34 RNA is present in the RNAs pulled down, as detected by gene specific primers, but is not amplified in this assay. However, despite its limitations, SI RT-PCR has allowed another potential RNA binding partner for US11 to be found.

4.0 Investigation into the *in vitro* binding of the 12/14 RNA by US11

In an attempt to isolate novel RNAs which associate with US11 in infected cells a protocol was devised to allow US11-RNA complexes to be captured and the RNA amplified and cloned by RT-PCR. One species of RNA was identified from this procedure with homology to a sequence found in three 3' co-terminal HSV-1 RNAs, UL12, UL13 and UL14 mRNAs, and has been designated 12/14 RNA. In order to examine whether this interaction is authentic, the interaction between US11 and the 12/14 RNA was examined *in vitro*.

4.1 Authentic 12/14 derived RNA can bind to US11

To confirm that the sequence identified from the SI RT-PCR assay was derived from the HSV-1 U_L region encoding UL12-14, and did not arise due to contamination of the sample, the corresponding region from the genome was used to construct a template for *in vitro* transcription. The 585nt region containing the 12/14 sequence and a 451nt sequence encoding Δ34 RNA were PCR amplified from the *Eco*RI D and the *Kpn*I C' fragments respectively, of HSV-1 using primers to incorporate a T7 promoter sequence upstream. These templates were transcribed *in vitro* in the presence of [α–^{32}P] UTP and the interaction with US11and the riboprobes examined by EMSA.

10,000cpm of the 12/14 or the Δ34 riboprobe were incubated without protein, with GST or with GST-US11 and the complexes resolved by native polyacrylamide gel electrophoresis (Fig. 4.1). Without the addition of protein, or with the addition of GST both the 12/14 probe and the Δ34 probe migrated as free species (lanes 1, 2, 4 and 5).

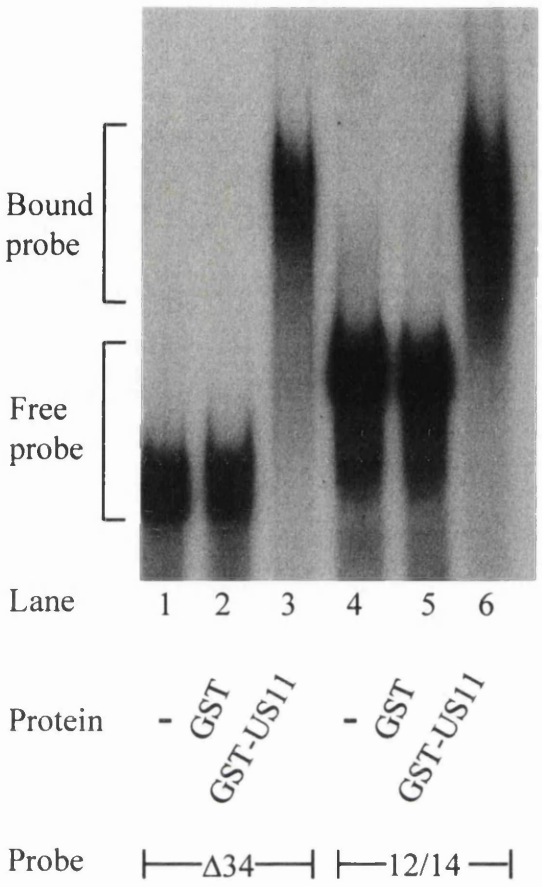


Fig. 4.1: EMSA of the interaction between authentic 12/14 RNA US11.

10,000cpm of *in vitro* transcribed [^{32}P]-labelled Δ34 (lanes 1-3) or 12/14 RNA (lanes 4-6) were incubated without protein (lanes 1 and 4), with 12.5ng/ ng/μl GST (lanes 2 and 5) or with 12.5ng/μl GST-US11 (lanes 3 and 6). The complexes were resolved on a 4% non-denaturing acrylamide gel and the complexes visualised by autoradiography.

However, when GST-US11 was included in the reaction the mobility of both probes was retarded, indicating that they are able to form a complex with US11 (lanes 3 and 6). This confirms that US11 can bind RNA transcribed from a fragment derived from the U_L region of HSV-1 containing the transcripts of UL12-UL14 *in vitro*.

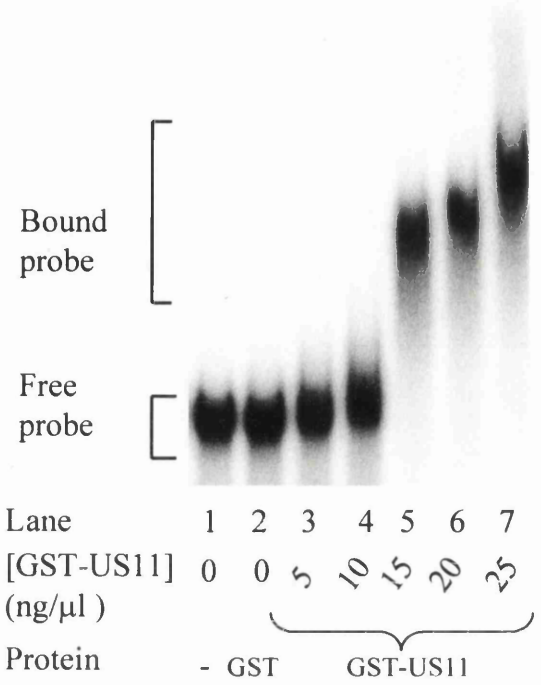
4.2 The effect of protein concentration on the interaction of US11 and 12/14 RNA

It has been proposed that GST-US11 can multimerise when in complex with RNA. In EMSAs with US11 and the Δ34 RNA, the XRE or the RRE RNAs, increasing concentrations of GST-US11 leads to the progressive retardation of the riboprobe indicating that the size of the complex is increasing (Schaerer-Uthurralt *et al.*, 1998). This has been suggested to be a result of the homomultimerization of US11. Additionally, by sucrose gradient centrifugation of infected cell extracts, US11 has been shown to associate in large complexes, which are thought to comprise largely of US11 homomultimers (Diaz *et al.*, 1993).

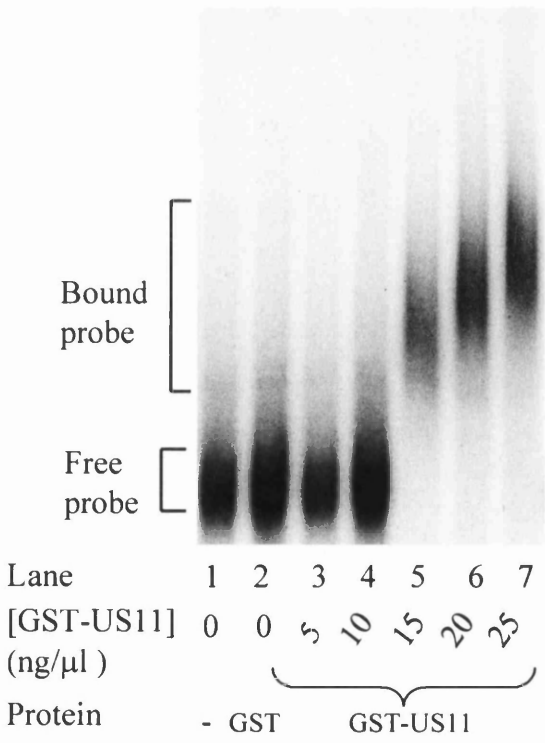
To examine whether 12/14 RNA is bound in a similar fashion and to compare the strength of interaction of US11 with the known binder, Δ34, the effect of protein concentration on binding was examined on the binding of 12/14 and Δ34 RNA (Fig. 4.2). 10,000cpm of the Δ34 or the 12/14 riboprobe was incubated in the absence of protein, or in the presence of 25ng/μl GST or increasing concentrations of GST-US11 from 5ng/μl to 25ng/μl and the complexes resolved on a non-denaturing polyacrylamide gel (Fig 4.2A). In the absence of protein or in the presence of GST or 5ng/μl or 10ng/μl GST-US11 the Δ34 riboprobe migrated as free, unbound species (lanes 1-4). When 15ng/μl GST-US11 was incubated with the probe a mobility shift was observed (lane 5). In the presence of 20ng/μl GST-US11 the mobility shift generated is greater that that

Fig. 4.2. EMSA of 12/14 and Δ34 RNA incubated with increasing concentrations of GST-US11. 10,000cpm of the Δ34 (panel A) or the 12/14 (panel B) riboprobe were incubated either without protein (lane 1), with 25ng/μl GST (lane 2) or with increasing concentrations of GST US11 (lanes 3-7). Complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

A


Bound probe
Free probe
Lane 1 2 3 4 5 6 7
[GST-US11] (ng/µl) 0 0 5 10 15 20 25
Protein - GST GST-US11

B


Bound probe
Free probe
Lane 1 2 3 4 5 6 7
[GST-US11] (ng/µl) 0 0 5 10 15 20 25
Protein - GST GST-US11

generated with 15ng/μl GST-US11 (lane 6). Incubation of the riboprobe with a higher concentration of GST-US11 (25ng/μl) again results in a further increase in the retardation of the riboprobe (lane 7).

As seen with the Δ34 riboprobe, the 12/14 riboprobe migrated as a free, unbound probe in the absence of protein and in the presence of GST or 5ng/μl and 10ng/μl concentrations of GST-US11 (Fig. 4.2B, lanes 1-4). In the presence of 15ng/μl GST-US11 the probe is retarded (lane 5). The retardation is increased in the presence of 20ng/μl GST-US11 and even more so in the presence of 25ng/μl GST-US11 (lanes 6 and 7).

This indicates that GST-US11 has a similar affinity for Δ34 and 12/14 RNA, both being bound at 15ng/μl GST-US11. As previously observed in the interaction between US11 and RNA, the size of the RNA/ GST-US11 complexes increase in size as increasing amounts of GST-US11 are added, indicating that larger multimers are being formed.

4.3 The specificity of the 12/14 interaction with US11

To test the sequence specificity of the interaction between US11 and the 12/14 probe, binding assays were performed with antisense transcripts of Δ34 and 12/14 and competition assays were performed in the presence of [^{32}P]-labelled 12/14 RNA and unlabelled RNA.

To examine the sequence-specificity of the US11-RNA interaction its binding of an antisense transcript of Δ34 (Δ34AS) was tested (Fig. 4.3A). 10,000cpm of Δ34 or Δ34AS riboprobe were incubated in the presence of 25ng/μl GST or 25ng/μl GST-US11 and the complexes resolved on a non-denaturing polyacrylamide gel. With GST both the Δ34 and the Δ34AS riboprobes migrate as free species (lanes 1 and 3). With the

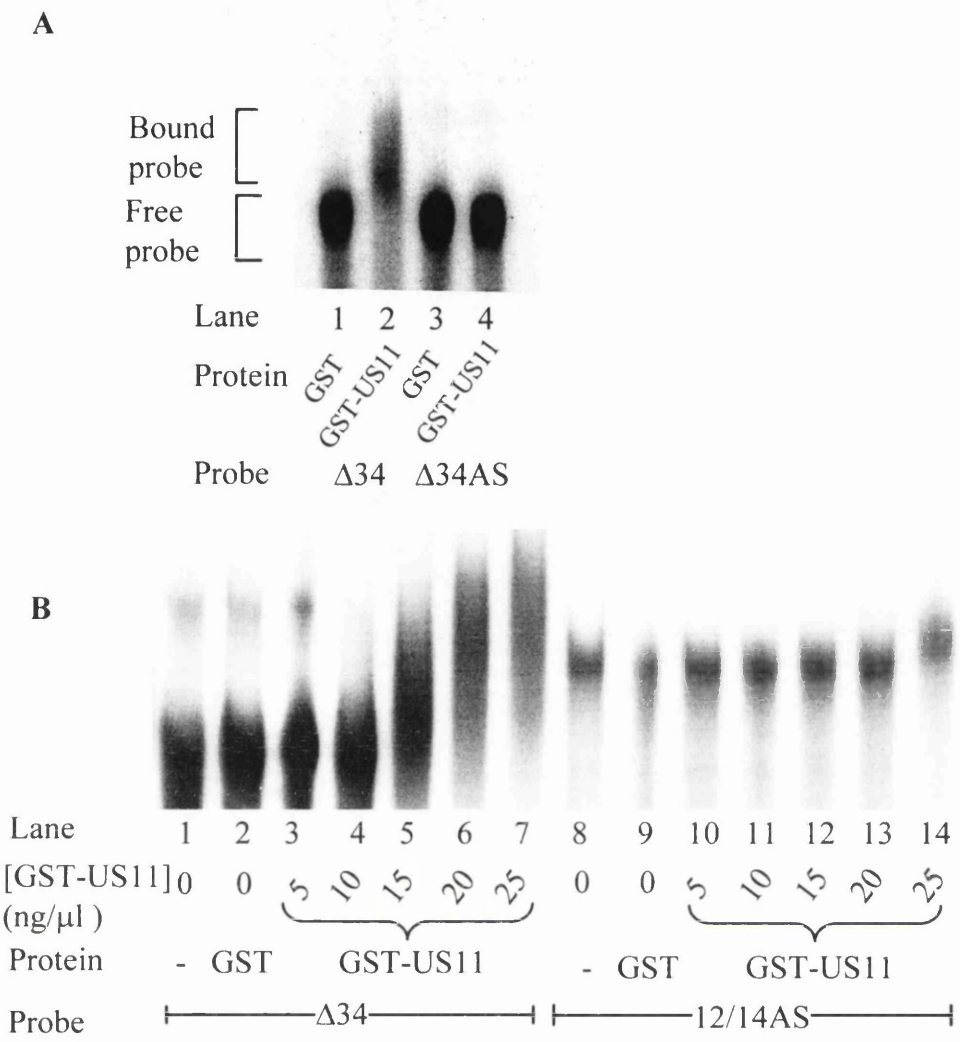


Fig. 4.3: EMSA of US11 interaction with antisense RNA.
A: 10,000cpm of [^{32}P]-labelled Δ34 (lanes 1 and 2) or Δ34AS riboprobe (lanes 3 and 4) were incubated with 25ng/μl GST (lane 1 and 3) or 25ng/μl GST-US11(lane 2 and 4). B: 10,000cpm of Δ34 (lanes 1-7) or 12/14AS (lanes 8-14) riboprobe were incubated in the absence of protein (lanes 1 and 8), or in the presence of 25ng/μl GST (lane 2 and 9) or in the presence of increasing concentrations of GST-US11 (lanes 3-7 and 10-14). The complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

addition of GST-US11 the Δ34 riboprobe is retarded in the gel indicating that a complex between GST-US11 and Δ34 has been formed (lane 2). However, when Δ34AS is incubated in the presence of GST-US11 no retardation of the riboprobe is observed. Therefore, under the conditions that permit GST-US11 to bind Δ34, GST-US11 cannot interact with the antisense transcript of Δ34.

To further examine the sequence-specificity of the US11 interaction with RNA, its binding of a 12/14 antisense riboprobe (12/14AS) riboprobe was examined by EMSA (Fig. 4.3B). Δ34 RNA was used as a control for binding. The riboprobes were incubated in the absence of protein, or in the presence of 25ng/μl GST or increasing concentrations of GST-US11 (ranging from 5-25ng/μl). In the absence of protein, or in the presence of 5-10ng/μl of GST-US11, the Δ34 riboprobe migrates as a free species (lanes 1-4). In the presence of 15ng/μl GST-US11 a shift in the mobility of the probe is observed (lane 5). As demonstrated in Section 4.2, upon the addition of 20ng/μl GST-US11 the riboprobe is retarded further and even more so with the addition of 25ng/μl GST-US11 (lanes 6 and 7). In comparison, the incubation of 12/14AS in the absence of protein or with the addition of GST or 5ng/μl-20ng/μl GST-US11 produces no retardation of the riboprobe (lanes 8-13). At 25ng/μl GST-US11 a small shift is detectable (lane 14), but this is not of the order of magnitude seen with Δ34 RNA. Therefore, it would appear that at only at high concentrations of GST-US11 (25ng/μl), a small amount of binding of the 12/14AS riboprobe could be observed.

The sequence-specificity of 12/14 RNA binding was further assayed by competing non-specific and specific RNA with US11. The principle of a competition assay is that a protein is incubated in the presence of a radiolabelled RNA probe and increasing concentrations of an unlabelled RNA. If a particular unlabelled RNA can bind to the

same site on the protein it should displace the labelled probe which should run as free, unbound probe.

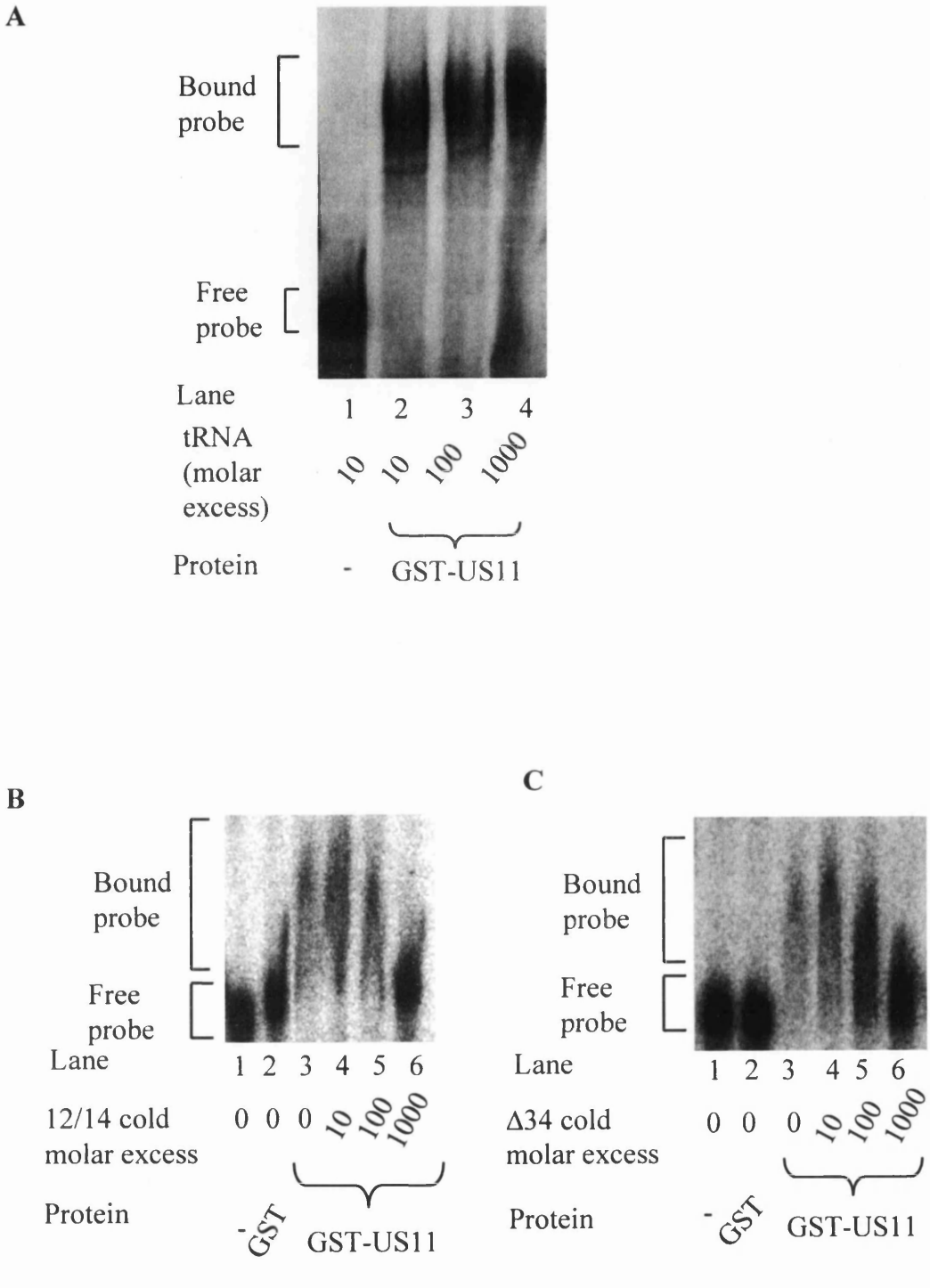
In the first instance yeast tRNA, a highly structured RNA, was competed against 12/14 RNA for the binding of US11. US11 was incubated in the presence of 3.3μM [^{32}P]-labelled 12/14 riboprobe and increasing concentrations of unlabelled yeast tRNA (10-, 100-, and 1000-fold molar excess) (Fig. 4.4A). In the absence of protein the 12/14 riboprobe migrates as a free species (lane 1). In the presence of 10-fold molar excess tRNA and 333nM GST-US11 a shift in mobility is observed (lane 2). In the presence of 100-fold molar excess tRNA no change in the GST-US11/12/14 riboprobe complex is observed (lane 3). Similarly, in the presence of 1000-fold molar excess tRNA no competition for the binding of US11 is observed (lane 4). Therefore, a non-specific, highly structured RNA cannot compete with 12/14 RNA for the binding of US11.

To further characterise the binding of 12/14 RNA by US11 the specific RNAs Δ34 and 12/14 were assayed for competition.

3.3μM of [^{32}P]-labelled 12/14 riboprobe was incubated in the absence of competitor probe or the presence of increasing amounts of unlabelled 12/14 riboprobe at a 10-, 100- and 1000-fold molar excess (Fig. 4.4B). When incubated without the addition of protein the riboprobe migrates as a free unbound species (lane 1). With the addition of GST, in this instance a slight decrease in the mobility of the riboprobe is observed (lane 2). The addition of 333nM GST-US11 causes the riboprobe to be significantly retarded (lane 3). When 10-fold molar excess unlabelled 12/14 RNA is included in the reaction no competition is observed (lane 4). At 100-fold molar excess of specific competitor RNA a decrease in complex mobility is observed (lane 5) and at 1000-fold molar excess complete dissociation is observed (lane 6). A decrease in complex size is apparent when 100-fold molar excess unlabelled 12/14 RNA is used to compete off labelled 12/14

Fig. 4.4: Competition assays.

A: 3.3μM of [^{32}P] labelled 12/14 riboprobe was incubated in the absence of protein (lane 1), or with 333nM of GST-US11 (lanes 2-4), and with the addition of unlabelled yeast tRNA in 10- (lane 1 & 2), 100- (lane 3), and 1000- (lane 4) fold molar excess of the labelled probe. B: 3.3μM of [^{32}P]-labelled 12/14 riboprobe was incubated in the absence of protein (lane 1), with 500nM GST (lane 2) or with 333nM of GST-US11 (lanes 3-6), without the additional RNA (lane 3), or with the addition of unlabelled 12/14 RNA at 10- (lane 4), 100- (lane 5), and 1000- (lane 6) fold molar excess of the labelled probe. C: 3.3μM of [^{32}P]-labelled 12/14 riboprobe was incubated in the absence of protein (lane 1), with 500nM GST (lane 2) or with 333nM of GST-US11 (lanes 3-6), without addition of unlabelled RNA (lane 3), or with the addition of unlabelled Δ34 RNA at 10- (lane 4), 100- (lane 5), and 1000- (lane 6) fold molar excess of the labelled probe.The complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by phosphoimaging.


A
Bound probe
Free probe
Lane
1 2 3 4
tRNA (molar excess)
10 10 100 1000
Protein
- GST-US11
B
Bound probe
Free probe
Lane
1 2 3 4 5 6
12/14 cold molar excess
0 0 0 10 100 1000
Protein
- GST GST-US11
C
Bound probe
Free probe
Lane
1 2 3 4 5 6
Δ34 cold molar excess
0 0 0 10 100 1000
Protein
- GST GST-US11

RNA, and at 1000-fold molar excess complete dissociation of the labelled complex is observed. This indicates that there is a specific, saturable, RNA binding site within US11 for the 12/14 RNA.

The addition of unlabelled Δ34 RNA as competitor for US11 binding with 12/14 labelled RNA gave a similar pattern (Fig. 4.4C). Without the addition of protein or the addition of GST the 12/14 riboprobe migrates as an unbound species (lanes 1 and 2). With the addition of 333nM GST-US11 the riboprobe is retarded (lane 3). The addition of 10-fold molar excess does not result in a change in the complex (lane 4). As with the competition with 12/14 RNA, a slight decrease in mobility seen at 100-fold molar excess (lane 5). Almost complete dissociation of the 12/14 riboprobe observed at 1000-fold molar excess cold Δ34 RNA (lane 6). As Δ34 RNA can compete with US11 for the binding by US11, this would suggest that the 12/14 and the Δ34 RNA are bound by the same site in US11. The competition of the unlabelled Δ34 riboprobe with the [^{32}P] - labelled 12/14 riboprobe is slightly less than that for the unlabelled 12/14 riboprobe. This may indicate that 12/14 RNA is a better substrate for binding than Δ34. However, 12/14 did not appear to be a better substrate for binding when the effect of increasing GST-US11 concentrations were compared for each substrate (Section 4.2). A more likely explanation is that they do bind the same site in US11, but, given their lack of sequence similarity, they do not interact with exactly the same specific residues in US11.

The only published competition assay for a US11-RNA interaction available for comparison is for the binding of the antisense transcript of the 5′ UTR of US11 (Roller & Roizman, 1990). When the binding of the antisense transcript of the US11 5′ UTR was competed against the same unlabelled transcript no competition was observed at 100-fold molar excess competitor. Competition was seen at 300-fold molar excess. At

this ratio, a decrease in the size of complex is observed. At 1000-fold molar excess the size of the complex decreases further, but the competition is by no means complete.

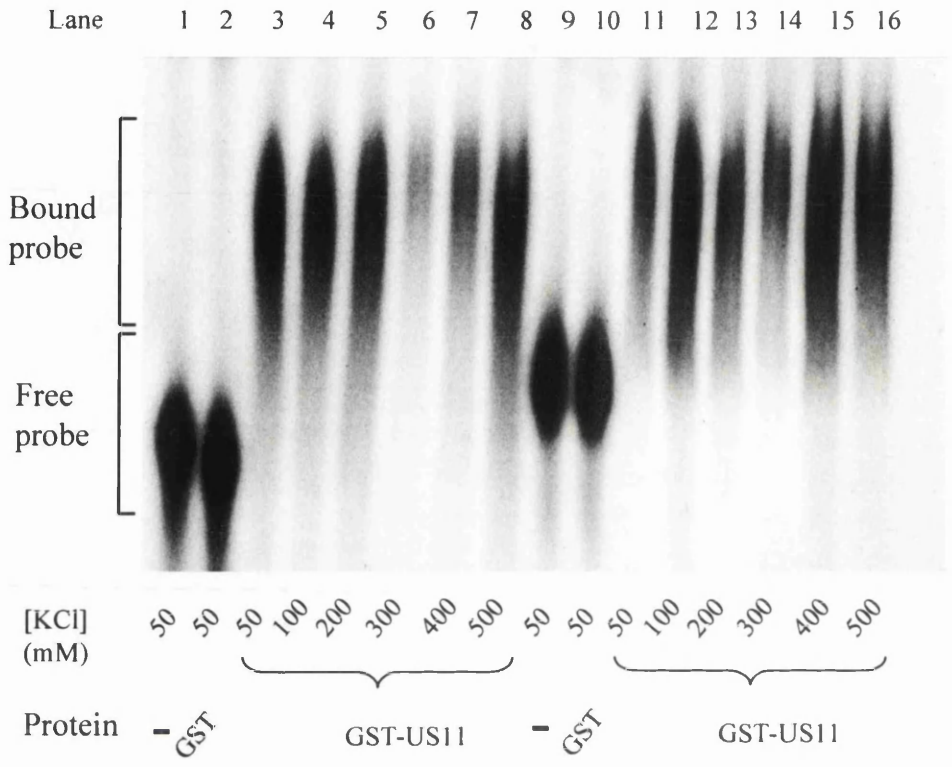
It is interesting to note that here and with the anti-sense 5′ UTR of US11, rather than a gradual dissociation of the bound probe from the complex in the presence of unlabelled competitor RNA, there is a decrease in the mobility of the labelled RNA-GST-US11 complex. This may indicate that US11 existing in large multimers in complex with radiolabelled RNA can dissociate to complex with specific competitor RNA, forming smaller multimers.

4.4 The effect of salt concentration on the binding of the 12/14 RNA by US11

The effect of salt concentration on the binding of the 12/14 probe by US11 was tested by increasing the NaCl or KCl concentration in the binding reaction. The final concentration of KCl in the binding reaction was tested at 50, 100, 200, 300, 400 and 500mM (Fig. 4.5A). In the absence of protein or in the presence of GST, both the Δ34 and the 12/14 riboprobe migrated as free, unbound species (lanes 1,2, 9 and 10). In the presence of GST-US11 a shift in mobility of Δ34 and the 12/14 riboprobe is observed (lanes 3 and 11). The shift in mobility of the Δ34 and the 12/14 riboprobes induced by GST-US11 are not decreased at KCl concentrations of 100, 200, 300, 400 or 500mM (lanes 4-8 and 12-16). The binding of both the 12/14 probe and the Δ34 probe by US11 did not appear to be effected at any of the KCl concentrations tested.

The variation of the NaCl concentration, 50, 100, 200, 300, 400, and 500mM, was examined (Fig. 4.5B). In the absence of protein and in the presence of GST the Δ34 and the 12/14 riboprobes migrate as free species (lanes 1, 2, 9 and 10). The addition of GST-US11 causes a decrease in mobility of both riboprobes (lane 3 and 12). The addition of

A


Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Bound probe
Free probe
[KCl] (mM)
50 50 50 100 200 300 400 500 50 50 50 100 200 300 400 500
Protein
- GST
GST-US11
- GST
GST-US11

B

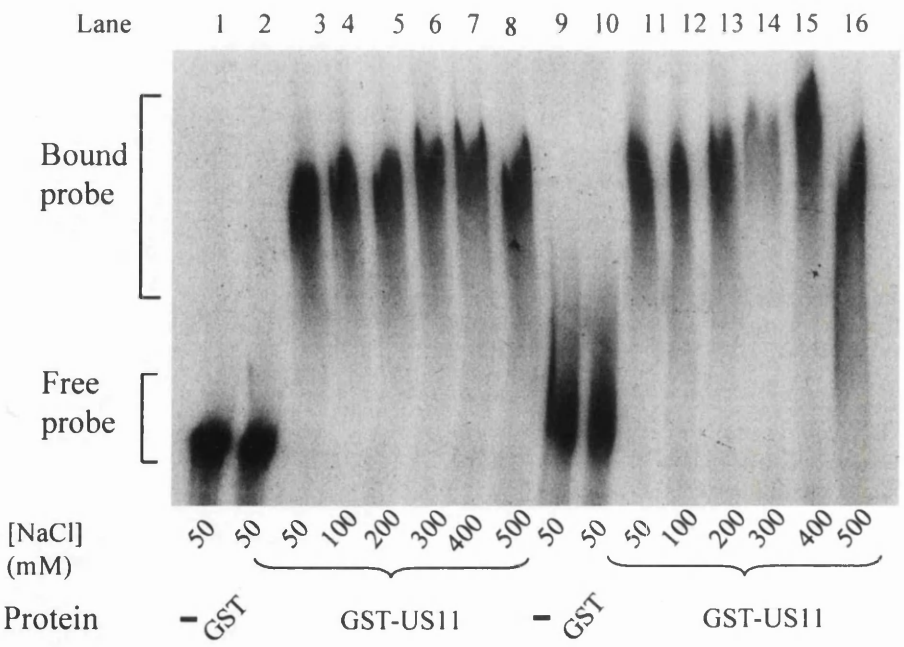

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Bound probe
Free probe
[NaCl] (mM)
50 50 50 100 200 300 400 500 50 50 50 100 200 300 400 500
Protein
- GST
GST-US11
- GST
GST-US11

Fig. 4.5: EMSA of US11 binding of 12/14 and Δ34 RNA under different salt concentrations.

In vitro transcribed, [^{32}P]-labelled Δ34 (lanes 1-8) or 12/14 RNA (lanes 9-16) were incubated without protein (lanes 1 and 9), with 15ng/μl of GST (lanes 2 and 10) or with 15ng/μl of GST-US11 (lanes 3-8 and 11-16). KCl (A) and NaCl (B) concentrations were varied in each reaction, 50mM (lanes 1-3 and 9-11, 100mM (lane 4 and 12), 200mM (lanes 5 and 13), 300mM (lanes 6 and 14), 400mM (lanes 7 and 15), and 500mM (lanes 8 and 16). The complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

100mM or 200mM NaCl does not appear to affect the mobility shift generated by GST-US11 (lanes 4, 5, 12 and 13). There appears be a slight increase the mobility of both riboprobes at 300mM and 400mM NaCl (lanes 6, 7, 14 and 15; the difference the intensity of the riboprobe in lane 14 is due to the under loading of the sample). But whether this is due to the aberrant running of samples due to the high salt concentrations or a genuine effect is unclear. Upon the addition of 500mM NaCl a small decrease in the retardation of the Δ34 riboprobe is observed (lane 8). A greater reduction in the retardation of 12/14 riboprobe is seen in the presence of 500mM NaCl (lane 16). Again, whether this is due to the aberrant running of samples at high salt concentrations or a genuine effect is unclear. In general the binding of both the Δ34 RNA and the 12/14 RNA are not perturbed to any great extent in the presence of up to 500mM NaCl.

The binding of 12/14 and Δ34 RNA does not appear to be greatly perturbed by KCl and NaCl concentrations. However, it appears that 500mM NaCl may affect RNA binding to some extent and influence 12/14 binding a little greater than that of Δ34 RNA. This may be a result of the disruption of ionic interactions between the protein and the RNA and the difference in the decrease of retardation of Δ34 RNA compared with 12/14 RNA is slightly greater for the latter, perhaps reflecting the different specific interactions between RNA and the binding domain of US11. Alternatively, the decrease in complex size may be due to dissociation of US11 homomultimers.

4.5 Mapping the US11 RNA binding site for 12/14 RNA

The RNA-binding domain of US11 has been mapped to the C-terminal domain by Roller *et al.* (1996) and Schaerer-Uthurralt *et al.* (1998). The structure and properties of the C-terminal domain are discussed in detail in Chapter 1. In brief, the C-terminal

domain of US11 consists almost entirely of a tandemly repeated tripeptide, XRP, where X is any amino acid (Lonsdale *et al.*, 1979; Rixon & McGeoch, 1984). The C-terminal domain of US11 is predicted to form a poly-L-proline type II helix (PPII-helix), a flexible structure with three amino acids per turn (Roller *et al.*, 1996; Schaerer-Uthurralt *et al.*, 1998)

In addition to full length GST-US11, Schaerer-Uthurralt *et al.* (1998) also constructed several GST-US11 vectors in which US11 was truncated. Two vectors encoding N- and C-terminal domain deletions of the US11 protein fused to a GST moiety, GST-US11Δ1-84 (named 2540), and GST-US11Δ88-149 (named 2625), were provided by J-J Diaz and J-J Madjar. The GST-US11 truncations were expressed and purified as described in Chapter 3 and by Schaerer-Uthurralt *et al.* (1998). Fig. 4.6 shows a Coomassie stained SDS-PAGE gel of the purified GST-US11 truncated proteins.

To examine whether US11 binds to the 12/14 RNA in a manner similar to that of the antisense transcript of the US11 5′ UTR, RRE, XRE and Δ34 RNAs, the binding of GST-US11 fusion proteins deleted in the N- or C-terminal domains were examined by EMSA (Fig. 4.7). Without the addition of protein or GST, the 12/14 RNA migrates as a free, unbound species (lanes 1 and 2). With the inclusion of full length GST-US11 the riboprobe displays a decrease in mobility (lane 3). In the presence of the C-terminal domain deletion mutant (GST-US11Δ88-149) the riboprobe migrates as a free species, indicating that this truncated protein cannot bind the RNA (lane 4). In contrast the N-terminal deletion mutant, GST-US11Δ1-84, is able to retard the probe (lane 5). This indicates that like the other RNA substrates of US11, 12/14 RNA is bound by the C-terminal domain of US11.

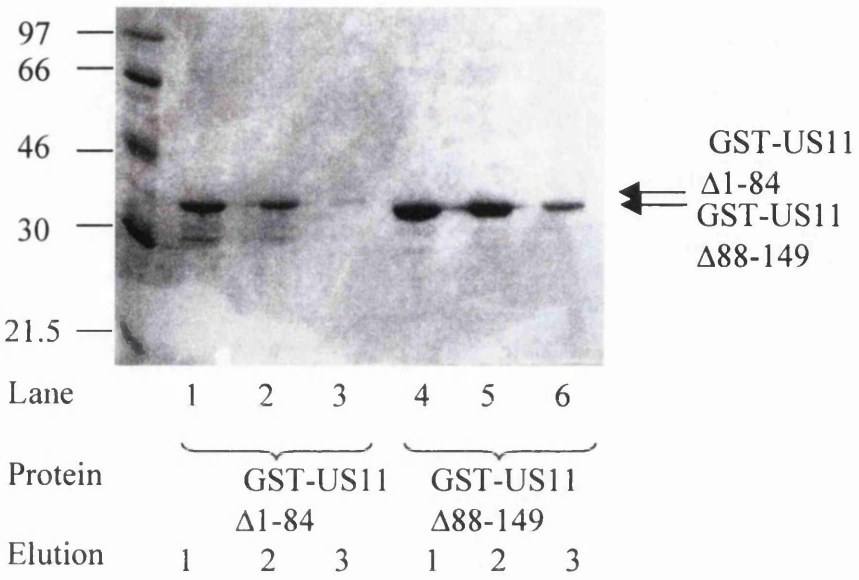


Fig. 4.6: Purification of GST-US11Δ1-84 and GST-US11Δ88-149. The GST-US11 deletion mutants, GST-US11Δ1-84 and GST-US11Δ88-149 were expressed in *E. coli* BL21 cells. The GST-fusion proteins were isolated using glutathione-agarose. The GST-fusion proteins were eluted three times with reduced glutathione. Protein gel loading buffer was added to 20µl of each fraction and boiled for 5min. The samples were separated by electrophoresis on a 12% SDS-PAGE gel and stained with Coomassie. Lanes 1-3 GST-US11Δ1-84, elutions 1-3 respectively. Lanes 4-6 GST-US11Δ88-149, elutions 1-3 respectively.

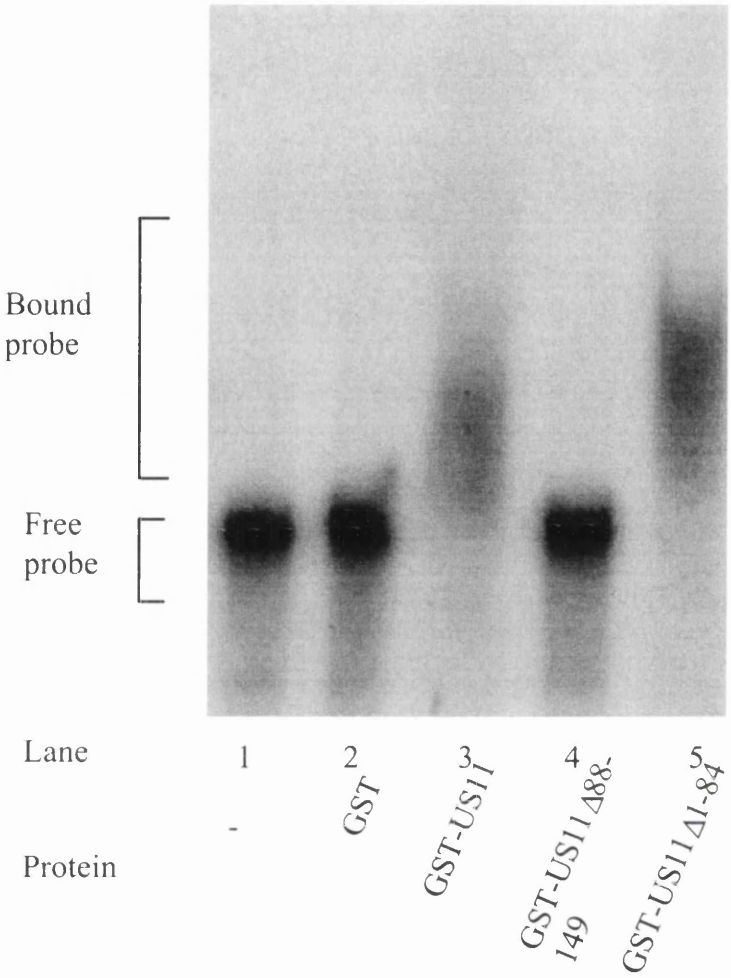


Fig. 4.7: Mapping of the US11 12/14 RNA binding domain by EMSA. 10,000cpm of the 12/14 riboprobe were incubated either without protein (lane 1),with 25ng/μl of GST (lane 2) with 25ng/μl of GST-US11 (lane 3), with 25ng/μl of GST-US11Δ88-149 (lane 4), or with 25ng/μl of GST-US11 Δ1-84 (lane 5). Complexes were resolved on a 4% non-denaturing acrylamide gel and visualised by autoradiography.

4.6 The effect of increasing concentration of GST-US11Δ1-84 on the binding of 12-14 RNA

Both the RNA-binding activity and the ability to form homomultimers resides in the C-terminal domain (Schaerer-Uthurralt, *et al.*, 1998). The ability of the N-terminal domain deletion mutant was therefore tested for the ability to multimerise in complex with the 12/14 RNA (Fig. 4.8). The Δ34 and 12/14 riboprobes were incubated without protein or with the addition of 50ng/μl GST or GST-US11Δ1-84 at concentrations ranging from 2.5ng/μl to 50ng/μl. Both riboprobes migrated as free, unbound species without the addition of protein and with GST alone (lanes 1, 2, 9 and 10). With as little as 2.5ng/μl GST-US11Δ1-84 a very slight shift in the mobility of the Δ34 riboprobe was generated (lane 3). Δ34 was increasingly retarded with increasing concentrations of GST-US11Δ1-84 (lane 4-8). The ability to multimerize was not affected by deletion of the N-terminal domain as demonstrated by the increase in complex size with increasing protein concentrations.

However, the generation of a mobility shift with GST-US11Δ1-84 and the 12/14 riboprobe required higher concentrations of protein. The 12/14 riboprobe migrated as a free, unbound species in the absence of protein or with the inclusion of GST (lane 9 and 10). No mobility shift was observed at GST-US11Δ1-84 concentrations between 2.5ng/μl and 12.5ng/μl (lane 11-13). In the presence of 25ng/μl GST-US11Δ1-84 the 12/14 riboprobe is retarded, and the retardation increased upon the addition of higher concentrations of protein indicating the formation of multimers (lane 14-16).

The affinity of the N-terminal domain for 12/14 RNA therefore appears to be less than that for Δ34 RNA.

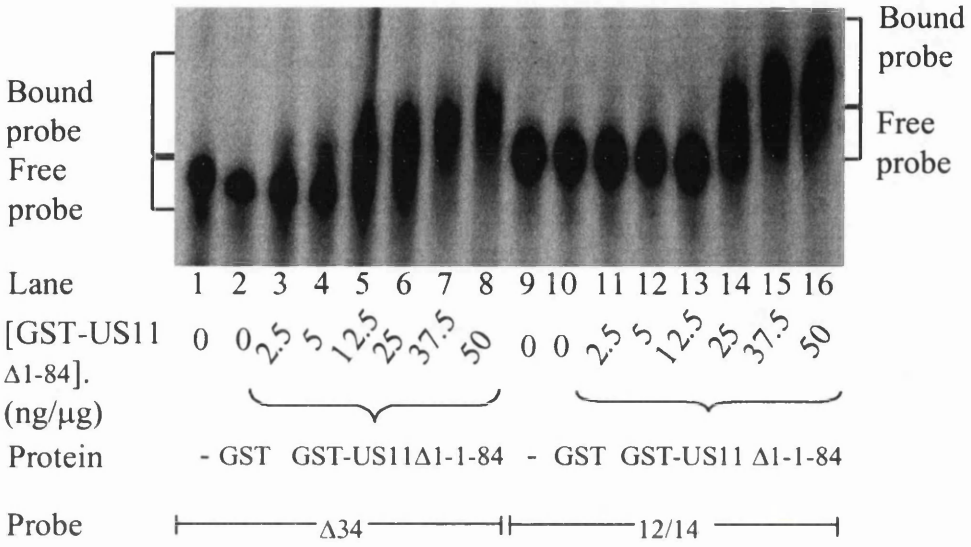


Fig. 4.8: EMSA of 12/14 and Δ34 RNA incubated with increasing concentrations of GST-US11 Δ1-84.

10,000cpm of the Δ34 (lanes 1-8) or the 12/14 (9-16) riboprobe were incubated either without protein (lanes 1 and 9), with 50ng/μg GST (lanes 2 and 10) or with increasing concentrations of GST-US11Δ1-84 (lanes 3-8 and 11-16). Complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

12/14 riboprobe is retarded, and the retardation increased upon the addition of higher concentrations of protein indicating the formation of multimers (lane 14-16).

The affinity of the C-terminal domain for 12/14 RNA therefore appears to be less than that for Δ34 RNA.

4.7 Discussion and summary of results

This chapter deals with the *in vitro* analysis of the US11-12/14 RNA interaction. It confirms that US11 can interact with a fragment derived from HSV-1 which encodes the entire 5' UTR of UL12 and the 5' end of the UL12 ORF, the 3' UTR and coding region of UL13 and the 3' UTR of UL14. This binding is sequence-specific in that it can be competed off with the specific competitors 12/14 and Δ34 RNA, but not with tRNA and US11 does not bind an antisense transcript of Δ34 and displays a low affinity for antisense 12/14 RNA.

Roller *et al.* (1996) and Schaerer-Uthurralt *et al.* (1998) have mapped the RNA binding domain of US11 to the C-terminal XRP repeat domain for the XRE, RRE, Δ34 and anti-sense US11 5′ UTR RNAs. 12/14 RNA is also bound by the C-terminal domain of US11.

It is interesting to note deletion of the N-terminal domain of US11 causes 12/14 RNA to be bound with less affinity than Δ34 RNA, indicating that they are not bound in exactly the same way by US11. This difference may be explained if that deletion of the N-terminal domain causes the C-terminal domain to adopt a less favourable structure for the binding of 12/14 RNA. Alternatively, the binding of 12/14 RNA may be stabilized by interacting with residues in the N-terminal domain, whereas Δ34 binding is independent of this domain.

5.0 The mapping of the RNA-binding site for US11 in 12/14 RNA

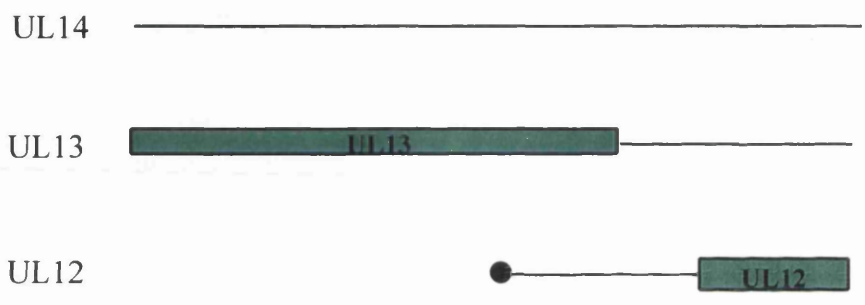
This Chapter deals with the *in vitro* deletion mapping of the binding site in the 12/14 RNA. This work illustrates the potential problems of mapping RNA binding sites by deletion, namely, that deletion of sequences outlying the binding site may lead to structural re-arrangement of the RNA, making the interpretation of such data difficult.

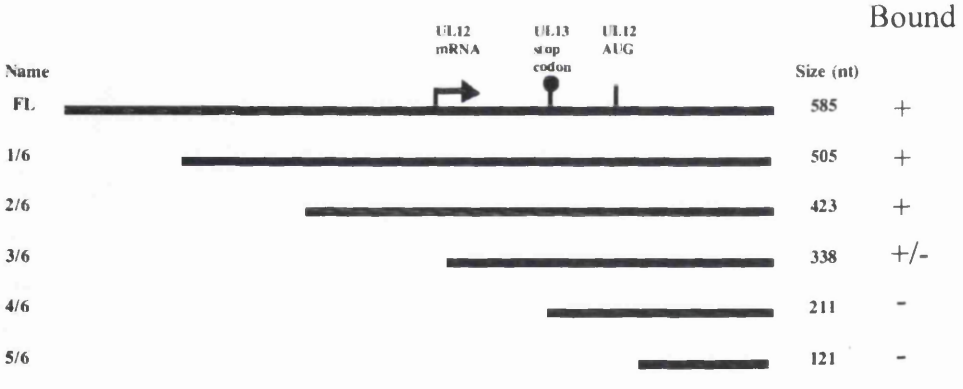
5.1 Mapping of the US11 binding site in the 12/14 RNA

In order to map the site of US11 interaction with the 12/14 RNA, templates were constructed with sequential 5' deletions. The templates were constructed by PCR amplification and incorporation of a T7 promoter 5' to the sequence to be transcribed *in vitro* (Fig.5.1A).

GST or GST-US11 was incubated with one of seven [^{32}P]-labelled *in vitro* transcribed RNA probes (Fig.5.1B). When incubated with the Δ34 riboprobe or the 12/14 riboprobe, a mobility shift was generated by the inclusion of GST-US11 (lanes 2 and 4) but not with GST alone (lanes 1 and 3). Deletion of 80 nucleotides from the 5' end of the 12/14 RNA (riboprobe 1/6) did not negate binding of US11, the probe being retarded by the addition of GST-US11 (lanes 5 and 6). Deletion of 162 nucleotides from the 5' end of the 12/14 RNA (riboprobe 2/6) lead to a slight decrease in incorporation into a complex with GST-US11 to retard the probe in EMSA, but it did retain significant binding activity (lanes 7 and 8). However, the loss of 247 nucleotides from the 5' end of 12/14 RNA (riboprobe 3/6) resulted in a severe decrease in binding (lanes

A


UL14
UL13
UL13
UL12
UL12


Bound
UL12 mRNA
UL13 stop codon
UL12 AUG
Name
Size (nt)
FL
585
+
1/6
505
+
2/6
423
+
3/6
338
+/-
4/6
211
-
5/6
121
-

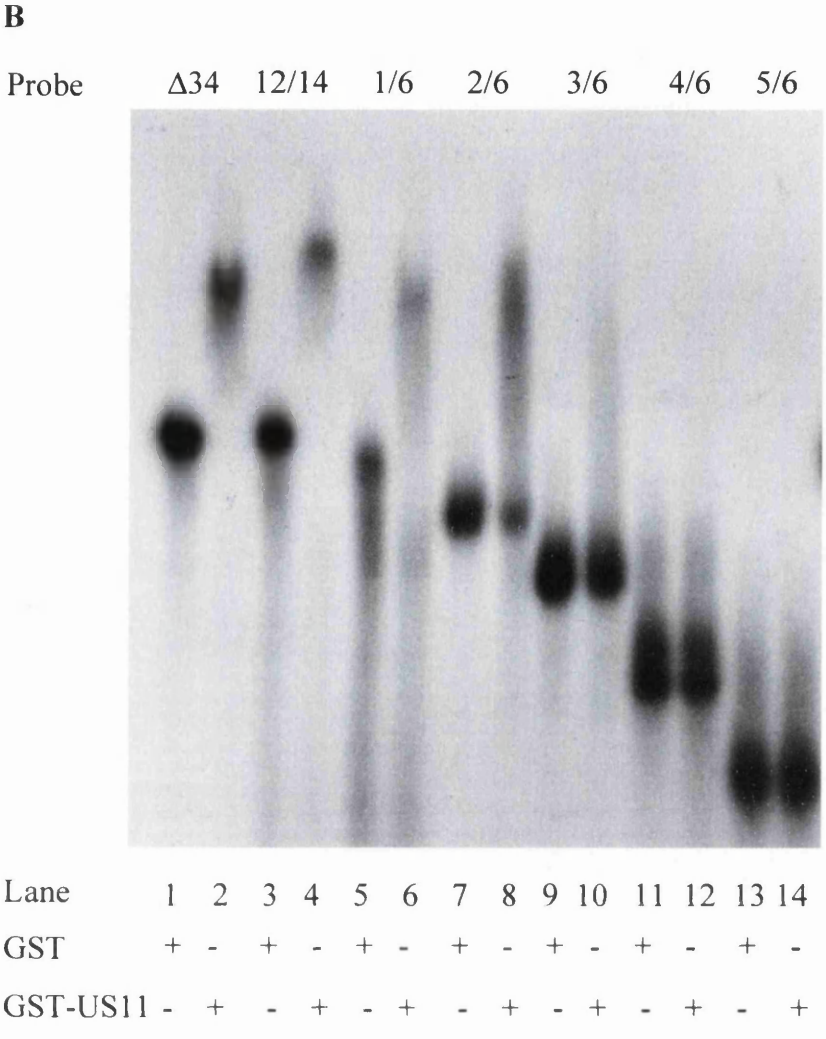


Fig. 5.1: Mapping of the US11 RNA binding site of US11 in 12/14 RNA.
A: At the top of the diagram the UL12, UL13 and UL14 RNA regions represented in the 12/14 riboprobe are indicated, a black dot indicates the 5′ end of UL12 mRNA, a line represents an UTR and a green box an ORF. Below is a schematic diagram of the riboprobes used to map the RNA binding site of US11. The position of the UL12 mRNA initiation start is indicated by an arrow, the UL13 translational stop codon and the UL12 translation initiation start codon, AUG are indicated on the 12/14 RNA. The incorporation of a particular riboprobe into a complex with GST-US11 as indicated by an increase in mobility of the probe is indicated by + in panel A and non-binding riboprobes are indicated by a -. B: 25ng/μl of either GST (lanes 1, 3, 5, 7, 9, 11 and 13) or 25ng/μl GST-US11(lanes 2, 4, 6, 8, 10, 12, and 14) were incubated in the presence of Δ34 (lanes 1and 2) or 12/14 (lanes 3 and 4) riboprobes or with riboprobes with successive deletions from the 5′ end of the 12/14 RNA; riboprobes 1/6 (lanes 5 and 6), 2/6 (lanes 7 and 8), 3/6 (lanes 9 and 10), 4/6 (lanes 11 and 12) and 5/6 (lanes 13 and 14). Complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

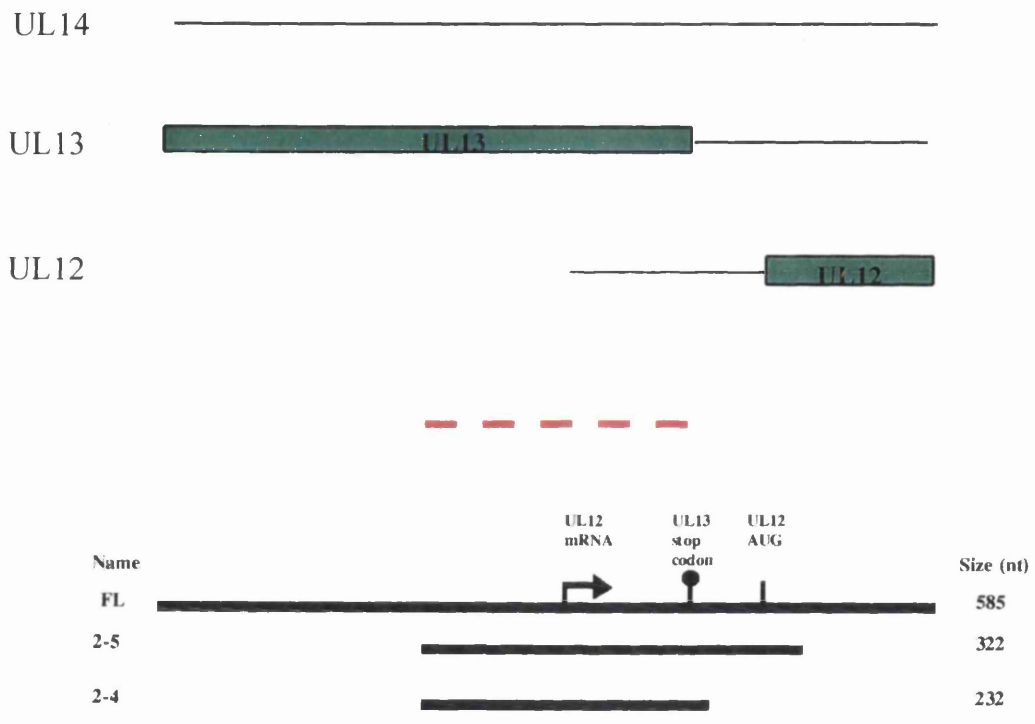
9 and 10). Consistently, with this probe, a faint smear was seen above the free probe in the presence of GST-US11, indicting that there may be some binding occuring. GST-US11 could not retard a riboprobe with 375 (riboprobe 4/6) or 464 nucleotides deleted from the 12/14 RNA in EMSA (lanes 11-14).

From this it can be concluded that deletion of up to 162 nucleotides from the 5' end of the 12/14 RNA does not have a major impact on US11 binding. Deletion of a further 85 nucleotides results in a severe decrease in binding, indicating that this region may be important in the interaction between US11 and the 12/14 RNA. Deletion of a further 127 nucleotides completely abolished binding.

The site of US11 binding was mapped further by the construction of templates for the *in vitro* transcription of two regions of the 12/14 RNA (Fig. 5.2A). Both probes lacked the 5' 162 nucleotides, the deletion of which does not fully negate the interaction between US11 and the 12/14 RNA. The riboprobes were additionally deleted from the 3' end of the 12/14 RNA. Riboprobe 2-5 had 100 nucleotides deleted from the 3' end and the 2-4 riboprobe 191 nucleotides deleted from the 3' end. GST or GST-US11 were incubated with either 10,000 cpm of the 12/14, 2-5 or 2-4 riboprobe and binding assayed by EMSA (Fig. 5.2B). As expected, the 12/14 riboprobe was retarded by GST-US11, but not by GST (lanes 1 and 2). Both the 2-5 and 2-4 riboprobes were retarded by the addition of GST-US11 and not GST, indicating that they contain a binding site for US11 (lanes 3-6). Therefore, deletion of 162 nucleotides from the 5' end and 191 nucleotides from the 3' end of the 12/14 RNA does not appear to negate US11 binding. This indicates that these regions are not absolutely required for US11 binding and that the binding site for US11 is contained within a 232 nucleotide portion of the 12/14 RNA.

When compared to the RNAs encoded by this region, the US11 binding site maps

A


UL14
UL13
UL13
UL12
UL12
UL12
mRNA
UL13
stop
codon
UL12
AUG
Name
Size (nt)
FL
585
2-5
322
2-4
232

B

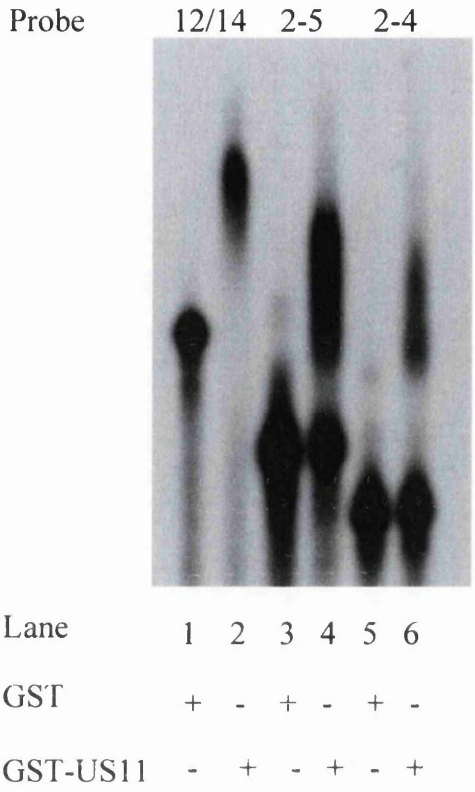


Fig. 5.2: Further mapping of the RNA binding site of US11 within the12/14 RNA.

A: At the top of the diagram the UL12, UL13 and UL14 RNA regions represented in the 12/14 riboprobe are indicated, a black dot indicates the position of the UL12 mRNA 5′ end, a line represents an UTR and a green box an ORF. A red dashed line hightlights the region of these mRNAs corresponding to the 2-4 riboprobe. Below a schematic diagram of the deletions. The position of the UL12 mRNA initiation start is indicated by an arrow, the UL13 translational stop codon and the UL12 translation initiation start codon, AUG are indicated on the 12/14 RNA. B: 25ng/μl GST (lanes 1, 3 and 5) or 25ng/μl GST-US11 (lanes 2, 4 and 6) was incubated in the presence of either the 12/14 riboprobe (lanes 1 and 2) or with a riboprobe with 162nt deleted from the 5′ end and 100nt from the 3′ end of the 12/14 probe (named 2-5) (lanes 3 and 4), or 162nt deleted from the 5′ end and 191nt from the 3′ end of the 12/14 probe (named 2-4) (lanes 5 and 6). Complexes were resolved by electrophoresis on a 4% native polyacrylamide gel and visualised by autoradiography.

within the 3' UTR of the UL14 mRNA, 225nt of the 3' ORF and 7 nt of the 3' UTR of the UL13 gene and 108nt of the UL12 5' UTR (Fig 5.2A).

5.2 Secondary RNA structure of the 2-4 RNA

The complex formation between the 2-4 riboprobe and US11 was investigated further for the following reasons; the purified 2-4 riboprobe migrated as two species in non-denaturing gel electrophoresis and 2-4/GST-US11 complexes migrated as two species in EMSA. This is illustrated in Fig. 5.3. With GST alone, the 2-4 riboprobe migrates as two species, labeled A and B (in order of fastest migrating) (lane 1). When incubated with GST-US11, the intensity of species A is decreased and species B is absence and two bound complexes are observed, labelled C and D (in order of the fastest migrating) (lane 2). This raises the question of whether the two unbound species represent alternative structures formed by the 2-4 riboprobe and whether the two bound species represent these alternative structures in complex with US11.

To examine whether these extra bands result from the formation of alternative secondary structures, the 2-4 riboprobe was purified on a denaturing polyacrylamide gel one band was excised, eluted and electrophoresed on a non-denaturing gel (Fig. 5.4). In non-denaturing gel electrophoresis the ribo probe migrated as two species. Extraction of the two species from the non-denaturing gel and subsequent electrophoresis of these species on a denaturing gel revealed that they migrated to the same position on the gel. It was therefore concluded that these species represent different structures adopted by the 2-4 riboprobe. An alternative explaination may be that the faster migrating species (A) represents monomeric RNA, whereas the slower migrating species (B) represents RNA dimers.

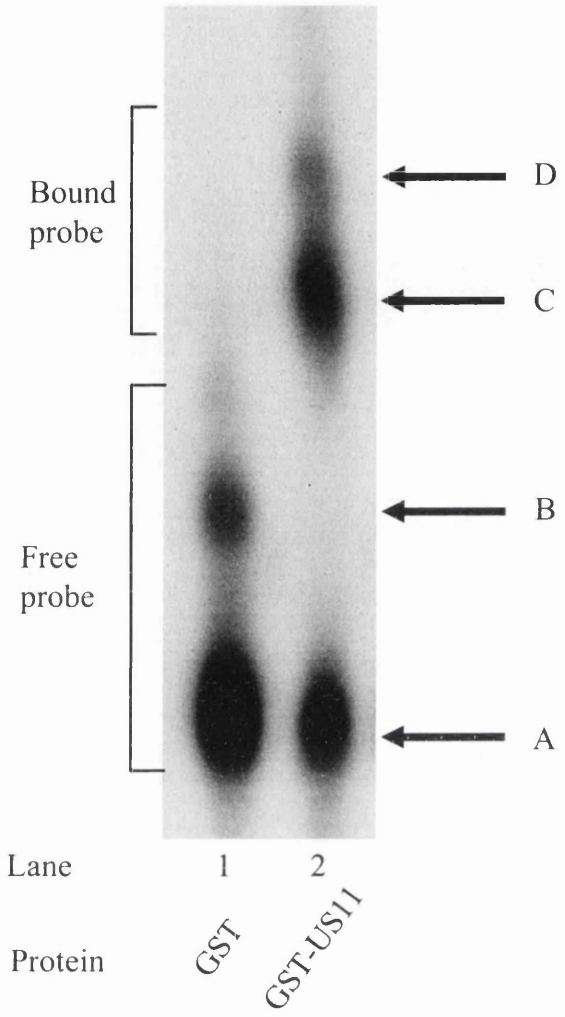


Fig. 5.3: EMSA of the interaction between the 2-4 species and US11. The 2-4 riboprobe was incubated in the presence of 25ng/μl of GST (lane 1) or 25ng/μl of GST-US11 (lane 2). The positions of multiple unbound secondary structures formed by the 2-4 riboprobe are indicated by arrows labelled A and B. The positions of multiple bound secondary structures formed by the 2-4 riboprobe in complex with US11 are indicated by arrows labelled C and D. The complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

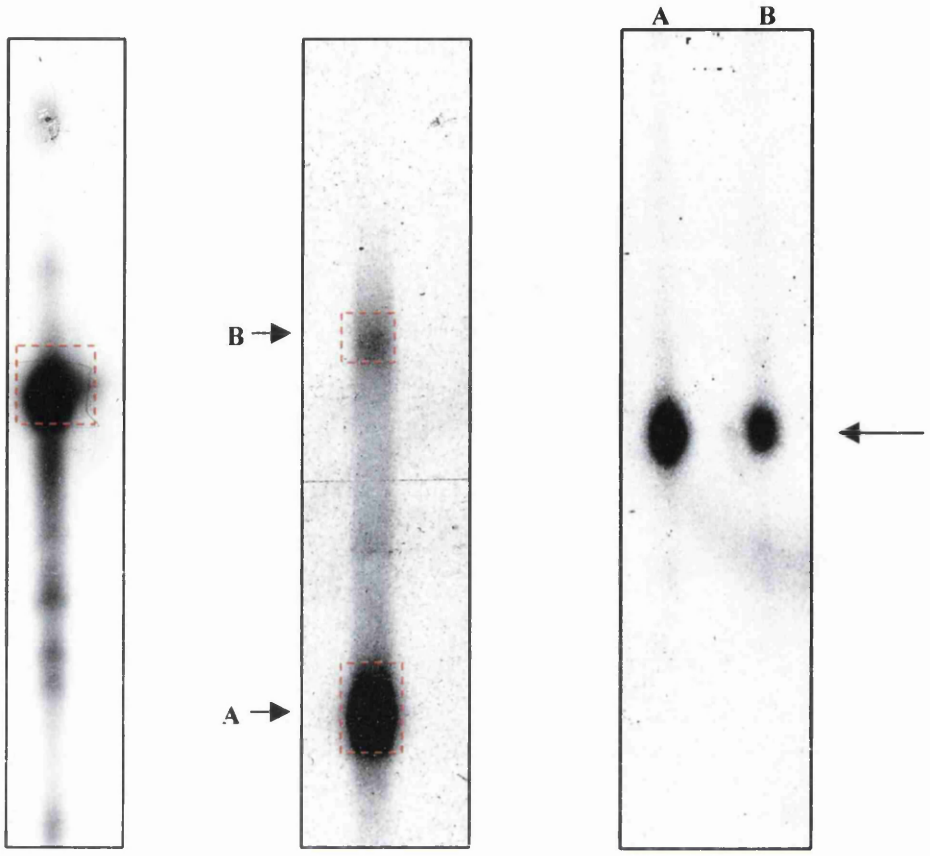


Fig. 5.4: Investigation of the 2-4 riboprobe to form alternative secondary structures *in vitro*.

The 2-4 template was transcribed *in vitro* in the presence of [α-^{32}P] UTP and electrophoresised on a 6% denaturing gel and visualised by autoradiography (left-hand panel). The band (boxed) was excised, and the RNA eluted. 5 x 10^6cpm of the probe was separated on a 4% non-denaturing gel, exposed to X-Omat film, the two species, A and B were excised (boxed bands) and eluted (central panel). 20,000cpm of species A and B were separated on a 6% denaturing gel and visualised by autoradiography (right-hand panel).

5.3 Differential binding of the 2-4 RNA secondary structures by GST-US11

To characterise the interaction between GST-US11 and the different 2-4 RNA structures and try to deduce whether the different RNA structures contribute to the different shifted complexes, increasing amounts of GST-US11 were incubated with a [^{32}P]-labelled *in vitro* transcribed 2-4 riboprobe and the complexes examined by EMSA (Fig. 5.5).

10,000cpm of the 2-4 riboprobe was incubated in the absence of protein or with 130ng/μl GST, or with GST-US11 from 4.5ng/μg to 130ng/μg. In the absence of protein, or with GST or concentrations up to 9ng/μg GST-US11, both probes migrated the two species of free, unbound probe, A and B. In the presence of 20ng/μg GST-US11 a shift of the 12/14 RNA species B (the slowest migrating free species) is generated, leading to the formation of complex D. Increasing concentrations of GST-US11 lead to the further retardation of the complexes in the gel, indicating that multimerization is occurring. The intensity of the unbound species A (the fastest migrating free species) does not decrease until a concentration of 55ng/μg GST-US11 and at this concentration the retarded complex C is seen. Therefore, complex C appears to be formed from the association of GST-US11 with species A. This complex is further retarded in the presence of increasing concentrations of GST-US11. However, even at high concentrations of GST-US11, species A is never completely bound.

From this it can be postulated that GST-US11 binds 2-4 species B with a higher affinity than species A, leading to the formation of complex . GST-US11 has a much lower affinity for species A, so much so that it is probably a non-specific interaction. The binding of species A leads to the formation of complex C.

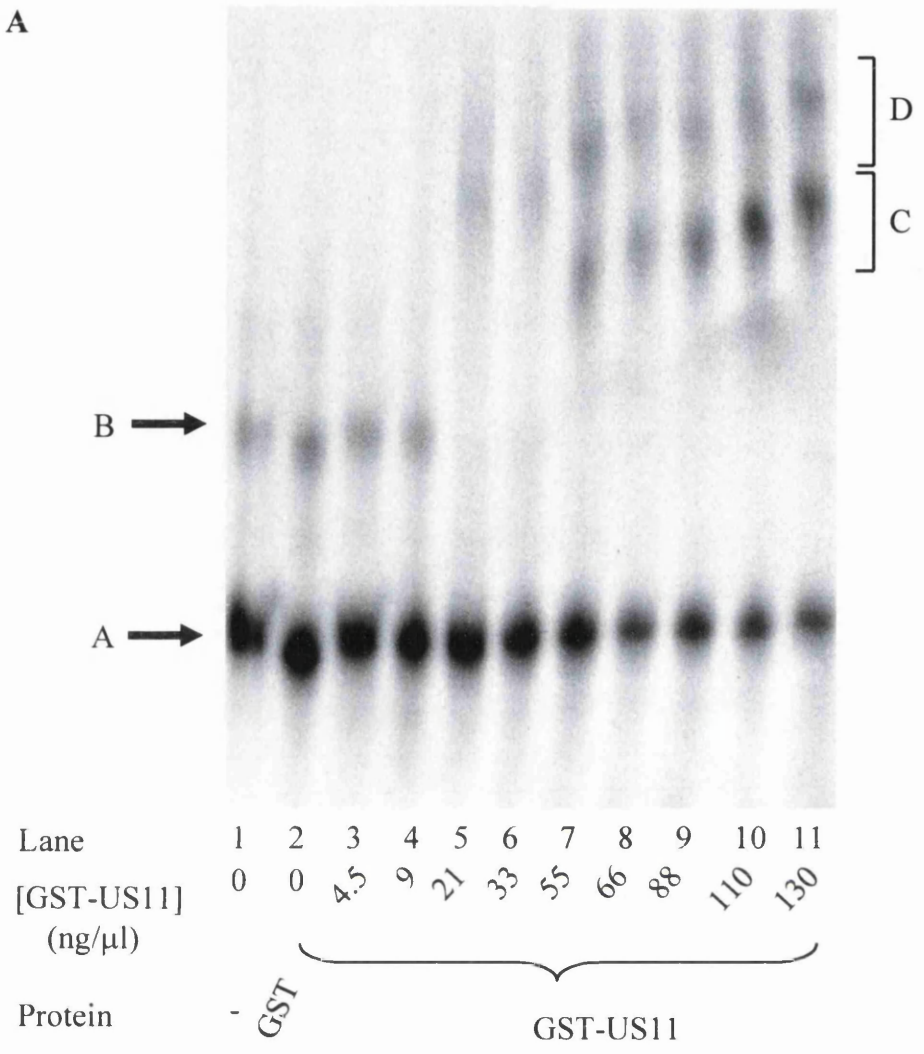


Fig. 5.5: Investigation of the differential binding of the 2-4 secondary structures by US11 by EMSA.
The 2-4 riboprobe was incubated in the absence of protein (lane 1), the presence of 130ng/µl of GST (lane 2) or with increasing concentrations of GST-US11 (lanes 3-11). The positions of multiple unbound secondary structures formed by the 2-4 riboprobe are indicated by arrows labelled A and B. The positions of multiple bound secondary structures formed by the 2-4 riboprobe in complex with US11 are indicated by brackets labelled C and D. The complexes were resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel and visualised by autoradiography.

To investigate this interaction more thoroughly the interaction of 2-4 and GST-US11 was assayed using dot blotting. For the dot blot assay, Hybond-C nitrocellulose membrane is place in dot blot apparatus and the protein-riboprobe reaction mixture is applied to the membrane and a vacuum applied. The protein is immobilized on the membrane, but RNA can pass through if not in complex with the protein. The complexes are washed to remove unbound probe. The radioactivity captured on the membrane can be assayed by the excision of the regions to which the complexes were applied, followed by quantification by scintillation counting. Reasoning behind using this method to assay the interaction between US11 and 2-4 RNA is that weaker complexes may become dissociated during electrophoresis or electrophoresis itself lead to the formation of these secondary structures.

The dot blot procedure was carried out with increasing concentrations of GST-US11 from 4.5ng/μg to 130ng/μg and a graph of protein concentration verses percentage binding was plotted (Fig. 5.6). Binding of 2-4 RNA increases between GST-US11 concentrations of 9ng/μg and 55ng/μg, levels off between 55ng/μg and 100ng/μg and begins to rise to the last point (130ng/μg). The maximum mean binding of the 2-4 RNA is 26.35% of the total probe present at the highest protein concentration used. Speculating about the nature of the binding in the dot blot assay, the first initial binding phase may represent the binding of species B, the higher affinity binder. The phase where that binding tails off may represent the binding of species A. However, as the complexes cannot be visualised the binding in this assay may not precisely reflect that seen in EMSA.

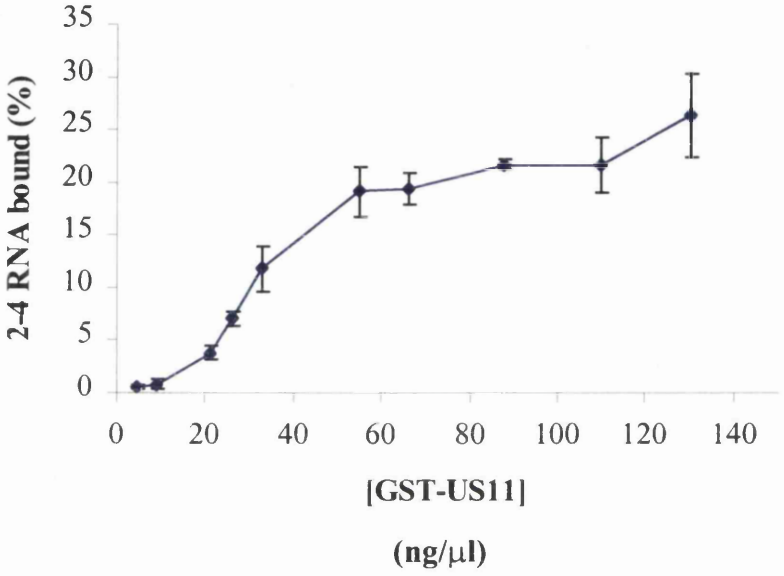


Fig. 5.6: Investigation of GST-US11 binding of 2-4 RNA by dot blot analysis.

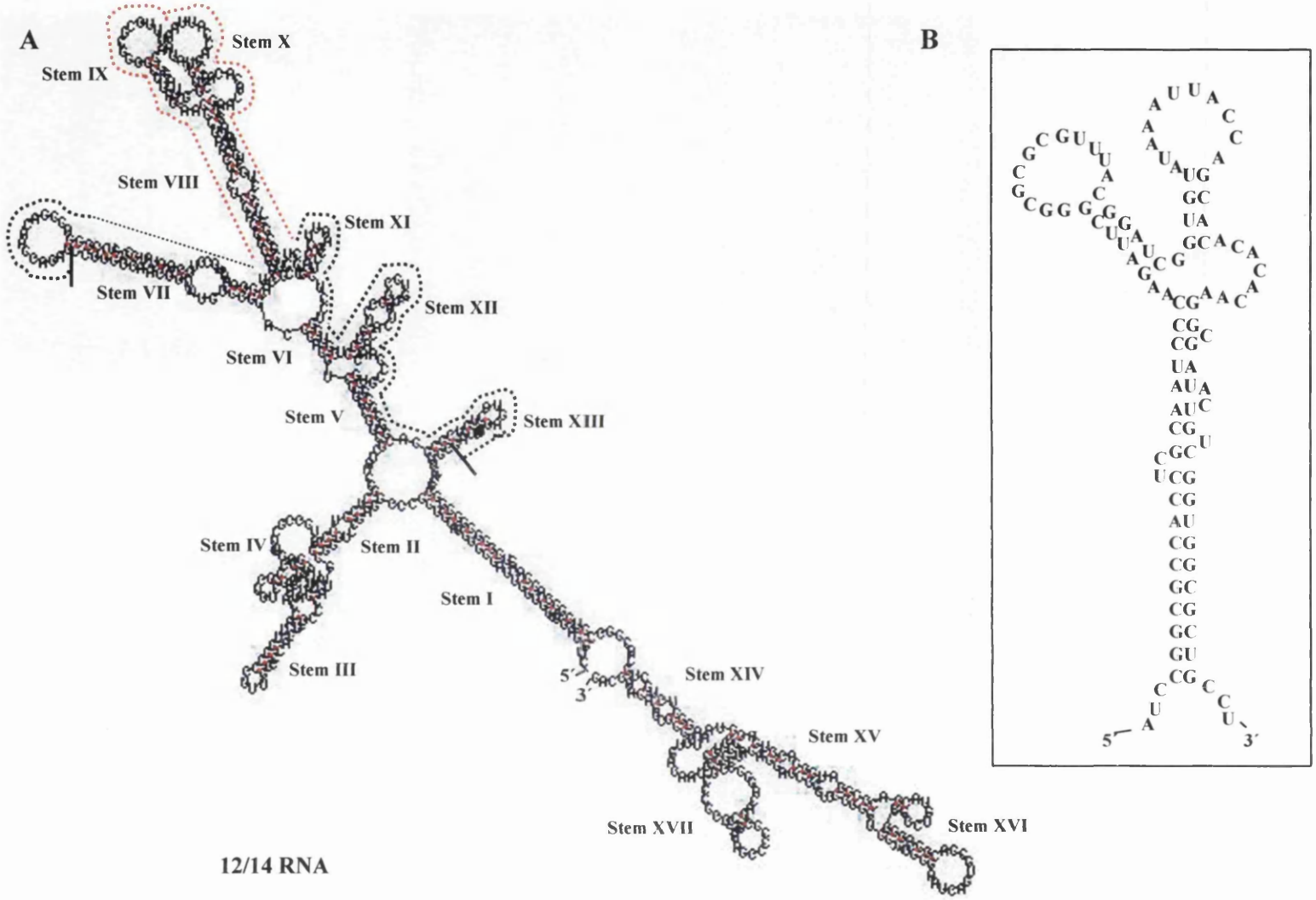
The amount of 2-4 bound is expressed as a percentage of the total input riboprobe after the value for GST-binding is subtracted. Standard deviation is represented by the vertical bars, n=6.

5.5 Secondary structural modelling of 2-4 RNA

To examine the secondary structure dependent interaction of US11 with the 2-4 RNA the RNA folding programme designed by Zucker, m-fold (Jaeger *et al.*, 1989a & b; Zucker 1989; Zucker *et al.* 1999 & Mathews *et al.* 1999; http:// www.rpi.edu/~mfold/) was used. Here this programme is used, not as a means of deducing the actual secondary structure, but as a model for the structural consequences of deleting sequences that flank the binding site in RNA.

The most stable predicted secondary structure for the 12/14 RNA (ΔG= -210.8) is shown in Fig. 5.7A. This predicted structure consists of approximately 17 stem structures which incorporate loops and bulges and have been labelled on the diagram (Stem I-XVII). The 232 nucleotide portion, 2-4, mapped as a binding site for US11, consists of one strand of stem VII, stems VIII-XII and approximately two thirds of stem XIII and is highlighted on the diagram.

The most stable structure predicted for the 2-4 RNA (ΔG= -79.6) consists of one long stem with loop and bulges and a small stem loop structure (Fig. 5.7C). This structure (named 2-4.1) appears to have little relationship with the predicted structure for the same region in 12/14 RNA. However, a predicted secondary structure which conserves some of the features seen in the predicted structure of 12/14 RNA is less energetically favourable, with a ΔG of -72.07. This structure, named 2-4.2, is depicted in Fig. 5.7D. This structure possesses seven stem structures with loops and bulges. In this structure, although much of the base pairing has been disrupted, stems VIII-XI observed in the predicted structure for 12/14 are maintained. This region is highlighted in both structures and is expanded in Fig. 5.7B. From this it can be seen that a region of


A
Stem X
Stem IX
Stem VIII
Stem XI
Stem VII
Stem XII
Stem VI
Stem V
Stem XIII
Stem IV
Stem II
Stem I
Stem III
5′
3′
Stem XIV
Stem XV
Stem XVII
Stem XVI
12/14 RNA
B
5′
3′

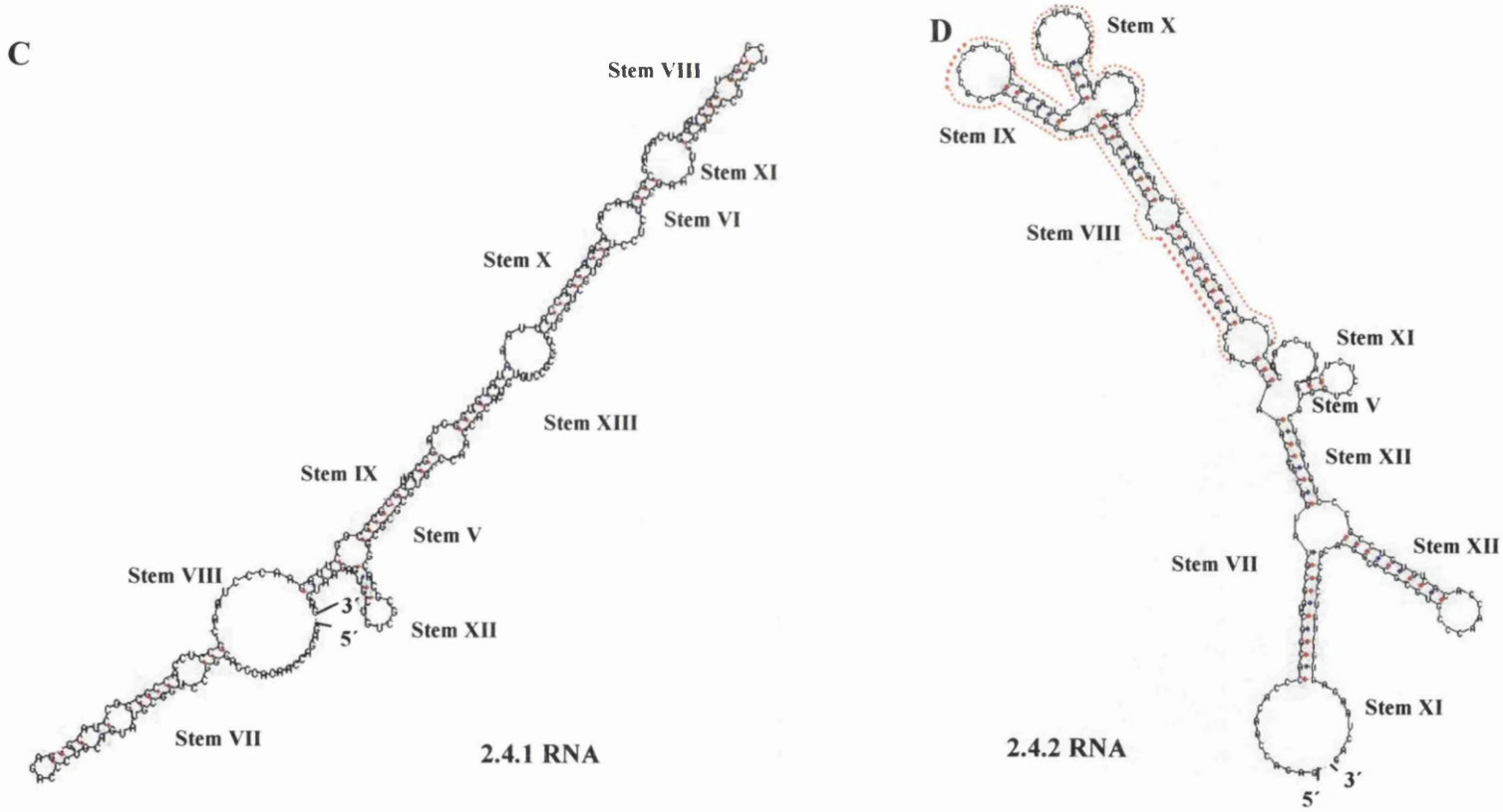


Fig.5.7: Secondary structure modelling of the 12-14 RNA and the 2-4 RNA. The RNA folding programme, m-fold, was used to model the secondary structure of the 12/14 RNA (A), and the 2-4 RNA (C & D). The region of the 12/14 RNA from which the 2-4 RNA was derived is highlighted by a dashed line. The red dashed line indicates the region of secondary structure maintainedin 2-4.2 and which is shown in greater detail in B. The positions of the stems of the 12/14 predicted structure are indicated on the alternative structures for the 2-4 riboprobe

secondary structure of 93 nucleotides is maintained in 2-4.2 whereas no regions of great length are maintained in 2-4.1.

Although secondary structure modelling for RNA cannot be relied upon to give accurate predictions, do not take into account tertiary structural and are by no means any substitute for structure probing, they can be used to speculate on what is happening *in vitro*. Assuming that the predicted secondary structure 2-4.1 is equivalent to species A and 2-4.2 is equivalent to species B observed in EMSAs, in that A possesses little secondary structure similarities to 12/14 RNA, whereas B maintains certain key structural features, it may be argued that US11 would have a much higher affinity for species B than for species A. In addition, predicted structure 2-4.1 is more stable than 2-4.2, and therefore would represent a much higher fraction of the secondary structure species seen *in vitro*. Species A is the major species observed in EMSA.

5.6 Summary and Discussion

The binding site for US11 in the 12/14 RNA has been mapped to a 232nt sequence which lies within the 3'UTR of UL14 mRNA, within 225nt of the 3' ORF and 7nt within the 3' UTR of UL13 mRNA and 108nt of the 5'UTR of UL14 mRNA (Fig 5.2A). US11 could therefore potentially bind to and influence the expression of either or all of these mRNAs during infection.

When the 232nt portion (2-4 RNA) was tested for binding, the riboprobe migrated as two species, which were demonstrated to represent alternative RNA structures. The 2-4 RNA species bound US11 with differing affinities. The slowest migrating species bound US11 with higher affinity that the fastest migrating species, demonstrating that in this case RNA structure may play an important role in binding. The antisense transcript of the US11 5' UTR has been shown to bind US11 in a secondary structure dependent manner (Roller & Roizman, 1990). As the RNAs to which US11 binds are so different in their primary sequence it may be inferred that US11 binding is directed towards certain structural motifs.

The structure of RNA can be influenced by ionic and pH changes (Shiman & Draper, 2000; Zhai *et al.*, 2001). It would be interesting to examine the affect of salt concentration or pH on the structure of the 2-4 riboprobe and the antisense US11 5' UTR, to determine whether conditions where the binding RNA structure , B, predominates.

Secondary structure modelling was performed on the 12/14 and the 2-4 RNA to help determine what may underlie the secondary structure dependent binding of US11. The 12/14 RNA predicted secondary structure consisted of approximately 17 stems with

loop and bulges. The most favourable predicted structure for the 2-4 RNA (named 2-4.1) did not possess any significant resemblance to the corresponding region of 12/14 RNA. A less energetically favourable structure (named 2-4.2) did however maintain a 93nt portion which is found in the predicted structure of 12/14 RNA. Although this modelling cannot be guaranteed to reflect the true nature of RNA folding *in vitro*, especially for larger RNA structures, it is therefore used to illustrate the point, namely, that truncation of RNA can lead to a gross re-arrangement of the secondary structure which may have implications in its interaction with proteins. This highlights the potential pitfalls of mapping protein binding sites on RNA using deletion mutants. The lack of binding of a protein to a particular truncated RNA may not be due to the abolition of the binding site but rather a structural re-arrangement of the RNA itself.

An example which illustrates how deletion of flanking RNA sequences have lead to structural re-organisation of the RNA is provided by the RRE of HIV-1. Originally it was thought that the RRE was 234nt in length, and its secondary structure was defined by prediction programmes and confirmed by structure probing (Kjems *et al.*, 1991). This secondary structure consisted of five stem-loop structures. However, as the 234nt sequence only conferred 50-80% of the export activity of *env* RNA in the presence of Rev, Mann *et al.* (1994) examined the outlying sequences to see if they contributed to Rev binding. When the RRE was extended by 58nt at the 5′ end and 59nt at the 3′ end the secondary structure adopted was similar to some extent, but stems III and IV now base paired to give a single stem and stem I was elongated significantly. The elongation of stem I caused Rev to bind with a higher affinity, to multimerize on the RNA more efficiently and to be more effective on promoting the export of the RRE.

This highlights the problems inherent in the analysis of RNA-protein interactions *in vitro*, namely, taking a small fraction of the full length sequence and expecting it to

maintain the same secondary structure as when within the context of its native RNA. When we consider the binding of US11, a protein whose binding has already been shown to be somewhat dependent on RNA secondary structure, to either UL12 (2.3kb), UL13 (3.9kb), or UL14 (4.5kb) mRNA it must be accounted for that even 12/14 (585nt) represents a small proportion of the native mRNA, and 2-4 RNA (232nt) even more so.

6.0 The influence of US11 on UL12, UL13 and UL14 expression.

In previous chapters US11 has been shown able to bind *in vitro*, a sequence found within three 3′ co-terminal RNAs, UL12, UL13 and UL14. US11 may therefore have the potential to bind to one or more of these transcripts during infection. The UL12 gene encodes alkaline nuclease (McGeoch *et al.*, 1986), a DNase which can act as both exonuclease and endonuclease. UL12 is expressed with early gene kinetics and is thought to be involved in the packaging of DNA and/or the resolution of brached DNA structures formed during replication (Weller *et al.*, 1990). UL13 encodes a protein kinase, which is expressed with late gene kinetics and has been implicated in the phosphorylation of a variety of viral and cellular factors (Cunningham *et al.*, 1992). UL14 encodes a nuclear phosphoprotein which is expressed with late gene kinetics and is incorporated into the virion (Cunningham *et al.*, 2000). Its function during infection is as yet unknown. To investigate whether an interaction of US11 with RNA derived from this region has any significance in infected cells, the levels of the UL12, UL13 and UL14 proteins and RNAs were examined.

6.1 Expression of UL12, UL13 and UL14 proteins

To examine whether US11 has any influence on the expression of UL12, UL13 and UL14, the levels of these proteins were compared in cells infected with *wt* virus and a virus in which the expression of US11 protein has been knocked out by deletion of the first methionine ATG.

Antibodies suitable for Western blotting for UL12 and UL14 were available, but for

UL13, the antibody available could only be used in immunoprecipitation. HeLa cells were mock infected or infected with either the *wt* or US11$^-$ virus at an moi of 10 pfu/cell and metabolically labelled. The cells were harvested at 3, 6, 12pi. UL12 and UL14 expression was examined by Western blot analysis (Fig. 6.1A & B) and UL13 levels were assayed by immunoprecipitation of the labelled protein (Fig. 6.1C). UL12 expression can be observed at 3h pi. There is no detectable difference in UL12 expression between the *wt* virus and the US11$^-$ virus infected cells at any time point. UL14 can be visualised at 6h pi. Again, there does not appear to be a difference in the levels of expression of UL14 at any time point taken. UL13 can be seen at 3h pi., in the *wt* infected cells, UL13 levels are reduced compared to that of the US11$^-$ virus at 3 and 6h pi. At 12h pi, however, the amount of UL13 immunoprecipitated from *wt* and the mutant virus are the similar. Therefore, US11 does not appear to affect UL12 and UL14 expression, but seems to down-regulate the expression of UL13 at early times pi. However, as immunoprecipitation is generally not a reliable method for protein quantification, an antibody which could detect UL13 in Western blot analysis was sought.

A rabbit polyclonal antibody raised against HSV-2 UL13 protein kinase was obtained. This antibody displayed a very high background reactivity. But conditions were found at which a band migrating at the expected position of UL13 (57kDa) which cross-reacts with the antibody was detected. HeLa cells were mock infected, or infected with wild type virus or the US11$^-$ virus and infection allowed to proceed for 6h. The levels of UL12, UL14 and UL13 were examined by Western blot analysis (Fig. 6.2). With both viruses the levels of UL12 and UL14 are similar. However, for UL13, a band is seen in US11$^-$ infected cell lysates, but not in lysates of cells infected with *wt*.

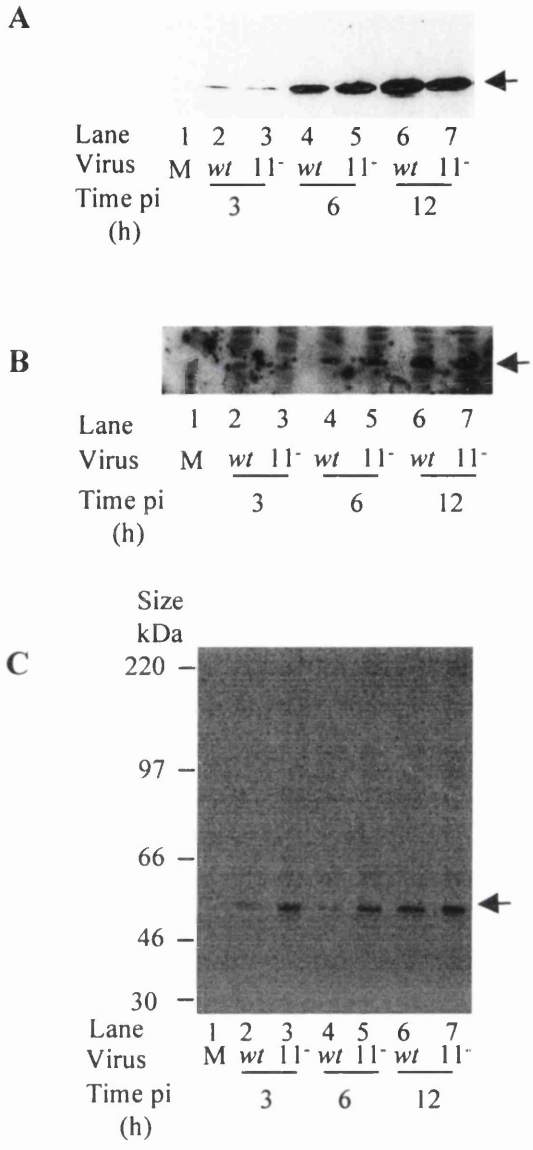
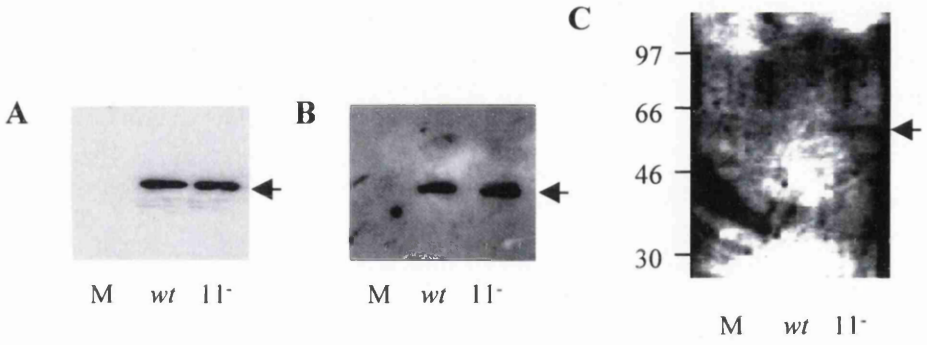


Fig .6.1: Expression of UL12, UL13 and UL14 proteins.
HeLa cells were either mock infected (M) or infected with wild-type virus (*wt*) or with a US11$^-$ (11$^-$) virus at a moi of 10 and metabolically labelled with [^{35}S]-methionine. The cells were harvested at 3, 6 or 12 pi. Proteins were separated on a 12% SDS-PAGE gel and Western blotted. Panel A: The membrane was probed with 1/1000 dilution of an anti-UL12 mAb. Panel B: The membrane was probed with 1/500 dilution of an anti-UL14 pAb. Panel C: To determined the levels of UL13 expression UL13 was immunoprecipitated from cell extracts and separated on a 10% SDS-PAGE gel.



6.2: Western blot analysis of the expression of UL12, UL13 and UL14 protein expression.

HeLa cells were mock infected (M) or infected with wild-type virus (*wt*) or with a US11$^-$ (11$^-$) virus at a moi of 10. The cells were harvested at 6h pi and the lysates separated on a 12% SDS-PAGE gel and Western blotted. UL12 (panel A) and UL14 (panel B) were detected as before. For the detection of UL13 the pAb was incubated with Hybond-C membrane at a dilution of 1/50 for 1h at RT to remove some of the background reactivity, before being used to probe the Western blot (panel C).

It would appear that US11 can regulate the expression of UL13 at early times post-infection, but does not affect its levels later in infection. US11 does not have any influence on the expression of UL14 or UL12 proteins under the conditions examined here.

6.2 Examination of UL12, UL13 and UL14 RNA levels in infected cells

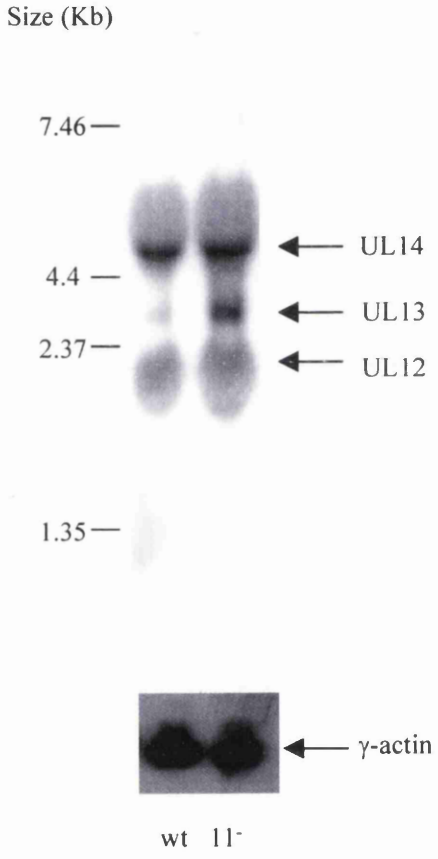
As US11 has been shown to bind an RNA sequence present in UL13 mRNA and can down-regulate the accumulation of UL13 at 6h pi, it is reasonable to assume that US11 may be acting at the RNA level to influence the expression of the protein.

UL12, UL13 and UL14 RNA levels were examined in HeLa cells infected with *wt* virus or the US11$^-$ virus at 6h pi. Total cellular RNA was extracted and examined by Northern blot. The Northern blot was probed with a radiolabelled DNA probe capable of hybridizing to UL12, UL13 and UL14 mRNAs (Fig. 6.3).

Three bands were detected corresponding to the mRNAs of UL14 (4.5kb), UL13 (3.9kb) and UL12 (2.3kb). Comparing the levels of these three RNAs in *wt* and the US11$^-$ virus infected cells, UL14 and UL12 mRNAs are equal in intensity, whereas UL13 RNA is significantly reduced in the *wt* virus compared to the US11$^-$ virus.

Thus in the absence of US11, there is an elevation in the levels of UL13 mRNA at 6h pi and this suggests that the increase in UL13 protein levels is due to a increase in the levels of its mRNA.

Fig. 6.3: Northern blot of the expression of UL12, UL13 and UL14 RNAs. Hela cells were infected with either wild type virus (wt) or with a US11$^-$ (11$^-$) virus at an moi of 10. Total RNA was harvested at 6h pi. 10μg of RNA was separated on a 1.2% agarose formaldehyde gel and Northern blotted. The blot was probed with a [^{32}P]-labelled 1.6kb *Sph*I fragment of the *Eco*RI D fragment of HSV-1, which includes the UL12-UL14 region. The membrane was stripped and re-probed with a [^{32}P]-labelled 840bp fragment of γ-actin. The hybridizing RNAs were visualised by autoradiography. The positions of UL12, UL13 and UL14 are indicated.


Size (Kb)
7.46
4.4
2.37
1.35
UL14
UL13
UL12
γ-actin
wt
11-

6.3 Summary and discussion

Examination of UL12, UL13 and UL14 protein levels in a US11$^-$ virus revealed that UL13 levels are elevated at 3 and 6h pi compared to wild type virus, whereas UL12 and UL14 protein levels are unaffected. At 12h, however, the levels of UL13 are not significantly affected by the absence of US11, suggesting that US11 no longer plays a role in the regulation of its expression. The mechanism for the down-regulation of UL13 by US11 is most probably due to down-regulation of its mRNA, as UL13 mRNA is elevated in the US11$^-$ virus infected cells. UL12 and UL14 mRNA levels are unaffected by US11, this too being reflected in their protein levels.

The RNA binding site has been mapped to a 232nt sequence which lies within the 3′ UTR of UL14, encompasses 228nt of the 3′ UTR and 7nt of the 3′ UTR of UL13 and includes 108nt of the UL12 5′ ORF. US11 could therefore influence the expression of any or all of these genes. The differing affect on the expression of these mRNAs may reflect a context-dependent effect, differing RNA structures or different factors which assemble on these elements, all of which are discussed in Chapter 7.

The influence of US11 on the expression of UL12, UL13 and UL14 mRNA and protein in infected cells has only been examined preliminarily here. Certainly, there are many further avenues to explore, such as the affect of moi and cell type on this phenomenon.

Chapter 7: General Discussion

7.1 Summary of work and outline of discussion

The aim of this project was to identify novel RNAs that interact with US11 in HSV-1 infected cells. This goal was achieved by the design of a RT-PCR protocol which allowed the cloning of a RNA sequence that interacted with US11. The RNA, homologous to a region contained within a family of three 3′ co-terminal HSV-1 mRNAs, UL12, UL13 and UL14, was identified as a potential substrate for US11. This interaction was authenticated *in vitro* and in infected cells. The US11 binding site was mapped to a 232nt portion which maps within the 3′ UTR of UL14, 225nt of 3′ ORF and 7nt of 3′ UTR of UL13, and the 108nt of the 5′ UTR of UL12 mRNA (Fig. 5.2). Examination of the levels of UL12, UL13 and UL14 mRNAs and proteins in infected cells revealed that although UL12 and UL14 were unaffected by the presence of US11, UL13 was down regulated by this protein at early times post-infection.

The first section of this discussion examines the current methods available for the identification of RNAs which interact with a particular protein of interest. The second section deals with the interaction between US11 and UL13 mRNA with reference to its other known RNA substrates and functions, and the possible implications of such an interaction.

7.2 Isolation and identification of RNAs that interact with specific proteins

There are three methods available for identifying novel RNAs which interact with a particular protein of interest, SELEX, the yeast three-hybrid system, and several

variations on RT-PCR based protocols which allow the amplification and identification of RNAs that interact with the protein of interest. These methods have been described in Chapter 1. This section compares these methods with SI RT-PCR and evaluates their potential application for the study of US11-RNA interactions.

7.2.1 SELEX

The principle of SELEX is the enrichment of a pool of randomised synthetic RNA sequences, which are competed against one another for binding by protein, for high affinity binders by successive rounds of protein binding followed by RT-PCR and transcription. The products are then identified by cloning and sequencing.

The major advantage of SELEX is that no assumptions are made with respect to the RNA sequences tested and it allows a large amount of sequences to be screened. The identification of interacting RNAs is merely based on their relative binding affinities for the protein. Never the less, the RNA species identified rely on the quality of the randomised pool and may share little similarity to the *in vivo* targets of the protein.

It may be potentially interesting to perform SELEX using US11. The RNA binding domain of US11 is highly unusual in its nature, bearing the tandemly repeated XRP motif. Also, as its substrates appear to have little sequence similarity, it would be potentially interesting to see if a preferred binding sequence emerged in the enriched RNAs. However, the RNA binding sequences for US11 which have been mapped extensively, that of the antisense transcript of the 5′ UTR of US11 and the Δ34 RNA, are relatively large, 87nt and 264nt, respectively. The SELEX procedure rarely uses randomised RNA sequences of over 40nt, generally because this is sufficient for protein binding, but also because of the expense of the synthesis of the randomised templates

for transcription. The sequence information gained from using 40nt randomised RNA sequences in a SELEX procedure with US11 may, therefore, not reflect its natural *in vivo* targets.

It is conceivable that the randomised nucleotide sequences used in SELEX could be replaced by a genomic or cDNA library. In this way potential *in vivo* targets could be identified. Such a system could be used to identify RNA ligands for US11 by using a HSV-1 infected cell cDNA library, and it would be interesting to compare the ligands produced from such a screening method with the SI RT-PCR method used in this study.

7.2.2 The Yeast Three-hybrid system

The yeast three-hybrid system allows the interaction between a protein and RNA to be assayed by the activation of transcription of a reporter gene in yeast. The three components of this assay are: (1) the protein of interest fused to a DNA binding domain of a transcriptional transactivator protein; (2) a RNA fused to MS2 RNA; (3) the activation domain of a transactivator fused to the MS2 coat protein. The interaction of the protein with the RNA is assayed by the ability of the protein to bind the RNA thus bringing the transcriptional activation domain in close proximity to the promoter stimulate transcription of a reporter gene. In general, either proteins suspected of interacting with a particular RNA or cDNA libraries are fused to the activation domain. However, the fusion a cDNA library to the MS2 transcripts could allow RNAs to be screened against a known protein target. Such a system has some major advantages over the SI-PCR based procedure described here. Firstly, US11 may be modified by post-translational modifications, which may influence the binding of US11 to its target RNAs. Expression in yeast would allow such modifications to occur, whereas

bacterially expressing GST-US11 is presumably unmodified. Secondly, the sequence bias that is introduced in RT-PCR, leading the amplification of some RNAs over others present in the pull down. This not a factor in the yeast based system, however, the quality of the library may be a factor.

7.2.3 RT-PCR based methods

Recently, three RT-PCR based methods designed to identify RNA that interact with a particular protein of interest have been described. These methods are discussed and compared with those used here.

Brooks & Rigby (2000) used immunoprecipitation of hnRNP A2 with RNA, coupled with poly(A)-dependent RT, homopolymer A-tailing and PCR using an oligo d(T) primer. In this case, immunoprecipitation of protein-RNA complexes successfully yielded RNAs specific for hnRNP A2. The RT-PCR method was similar to the PA RT-PCR tested in Chapter 3 for the amplification of RNAs that interacted with GST-US11. Indeed, this protocol yielded similar results. A low molecular weight smear was observed with the non-specific pull-down and a higher molecular weight smear was seen for the pull-down performed with the protein (Fig. 3.11). Brooks & Rigby (2000) random primed the amplified sequences and used them to probe a Lambda Zap library allowing them to identify the interacting RNAs. Such a screening method has major advantages over cloning and sequencing. It allows a population such as this, very heterogeneous, with no obvious distinction between what constitutes specific and non-specific species, to be screened.

However, the method described by Brooks & Rigby (2000), did not identify the known binders, myelin basic protein (MBP) mRNA, and growth macrophage-colony

stimulating factor (GM-CSF) mRNA, although GM-CSF mRNA could be amplified by RT-PCR with gene-specific primers. This is similar to the case with SI RT-PCR detailed here, in that the RNA pulled down with the protein and amplified by the non-specific primers did not contain a known binder.

Sung *et al.* (2000) also used an RT-PCR based protocol to identify RNAs which interact with Fragile X mental retardation protein (FMRP), using biotinylated-FRMP to pull out interacting RNAs, which were amplified by differential display RT-PCR (DD RT-PCR). They identified nine amplified species, and in this case, a known binder, FRMP mRNA, was amplified. Despite the RT-PCR requiring the presence of a poly(A) tail, a non-polyadenylated species, mitochondrial rRNA, was isolated. However, MBP mRNA, and a non-polyadenylated RNA, 60S rRNA, which are known to bind FRMP were not represented in the species pulled out, implying that either at the pull down stage or at the RT-PCR stage these sequences were lost.

Trifillis *et al.* (1999) used a recombinant GST-αCP1 protein to isolate interacting RNAs from cell lysates. The RNA was amplified, as with the method used by Sung *et al.* (2000), by DD RT-PCR. This allowed ten sequences to be identified as potential binding partners for this protein. However, the known binders, β- and γ-globin, which could be amplified from those RNAs pulled out by gene-specific primers, were not present in the ten DD RT-PCR species.

It would therefore appear that all these methods, including the SI RT-PCR method outlined here, can yield novel RNA partners for a particular protein of interest. The distinction between these methods is subjective. Each method shows some bias in that RNAs known to interact with the protein have not been represented in the amplified products. This may either be due to sequence bias introduced in RT-PCR or may reflect the relative abundance of these RNAs. The basic dividing feature is how non-specific

and specific RNAs are distinguished. Whether this is achieved by comparing the species by electrophoresis and extracting the bands specific for the protein of interest (Trifillis *et al.*, 1999; Sung *et al.*, 2000 and here), or by screening a cDNA library, a method that allows large numbers of sequences to be screened (Brooks & Rigby, 2000), reflects the nature of the RT-PCR protocol used (i.e. how many species are generated).

The major advantage of SI RT-PCR is its ability to amplify both polyadenylated and non-polyadenylated RNAs, hence no assumption is made as to the nature of the RNAs that the protein interacts with. With a protein such as US11, which binds such divergent RNAs, this protocol is ideally suited. However, with proteins such as an hnRNPs, a slightly different approach is needed. A protocol which limits the number of RNAs amplified, such as the method used by Trifillis *et al.* (1999) or Sung *et al.*(2000), allows manageable numbers for sequencing and subsequent analysis. Alternatively, a large scale screening programme, such as that employed by Brooks & Rigby (2000), allows identification of correspondingly large numbers of binding RNAs.

7.3 US11-RNA interactions

7.3.1 RNA secondary structure

The interaction of US11 with 2-4 RNA, the region mapped as containing the US11 binding site in the 12-14 derived transcript, was shown to be dependent on secondary structure. US11 showed a greater preference for the two slower migrating species of 2-4 RNA as opposed to the fastest migrating species. Interestingly, Roller and Roizman (1990) demonstrated that the interaction between US11 and the antisense transcript of its own 5′ UTR was dependent on the secondary structure of the RNA, in that US11

interacted with the slower migrating minor species of two RNA secondary structures. Due to the lack of sequence similarity between the RNA substrates of US11 (the XRE, RRE, Δ34, and the anti-sense transcript of the US11 5′ UTR) and the secondary structure-dependence demonstrated here and by Roller and Roizman (1990), it appears that the US11 RNA recognition motif may be determined primarily by the secondary structure of the RNAs over that of the sequence itself. The XRE and RRE are highly structured RNAs forming complex elements of stems and loops (Dayton *et al.*, 1989). US11 may potentially interact with these RNAs by virtue of their secondary structures, which may share some similarity its natural *in vivo* substrates.

7.3.2 The US11 interaction with UL13 RNA

7.3.2.1 Mechanism of down-regulation of UL13 and comparison of effects on other US11 RNA partners.

The interaction of US11 with UL13 mRNA appears, at least superficially, to have more in common with its interaction with Δ34 RNA than with the retroviral export elements, the RRE and the XRE. Firstly, the binding site for US11 in Δ34 lies within the ORF, whereas the RRE and XRE are intronic elements; the binding site for US11 was mapped to the 3′ end of the ORF of UL13 mRNA. Secondly, whilst US11 promotes the cytoplasmic accumulation of RRE- and XRE-containing RNA, Δ34 and UL13 RNAs are down regulated in the presence of US11.

It is therefore conceivable, that for Δ34 and UL13 RNAs, US11 acts through a separate pathway than for RRE/XRE-containing RNA. Alternatively, a common pathway may be used, but the outcome of this activity differs due to later downstream events. For

example, US11 may act to increase the export of Δ34 and UL13 RNAs from the nucleus to the cytoplasm where their turnover is greater, resulting in a net decrease in these RNAs.

Certainly, work to compare these RNA substrates of US11 may reveal whether there is indeed a common mechanism of action, or whether their regulation is unrelated. A comparison of RNA half-lives, distribution and *in vitro* decay profiles in the presence and absence of US11 could provide valuable information on the mechanism(s) of action of US11.

7.3.2.2 US11 regulates the accumulation of UL13 RNA but not that of UL12 or UL14

The binding site of US11 in 12-14 RNA encompasses 108nt of the UL12 5′ UTR, 225nt of the UL13 3′ coding region and 7nt of the UL13 3′ UTR, and lies within the UL14 3′ UTR. However, US11 only seems to regulate UL13 mRNA levels, but not that of UL12 or UL14.

The 108nt of the UL12 5′ UTR does not represent the entire region sufficient for US11 binding. An alternative explanation is that as US11 binding has been shown to be secondary structure-dependent, the 5′ UTR of UL12 may not adopt a secondary structure permissive for US11 binding. UL14 mRNA, does contain the entire 232nt element. Once again, as US11 binding is secondary structure-dependent, US11 may be excluded from binding this element in UL14 mRNA due to an unfavourable RNA structure formed in this RNA, perhaps via long-range intramolecular RNA interactions.

Another explanation for the lack of regulation of UL14 and UL12 may be that the regulatory effect of US11 on a RNA is position dependent. US11 appears to bind both the UL13 mRNA and the Δ34 RNA within the coding region of the RNA (Roller &

Roizman, 1991). When positioned in the 3′ UTR or 5′ UTR this element may not be a target for down-regulation.

7.3.2.3 Why down regulate UL13?

The question arising from this work is why regulate the expression of UL13 at early stages in infection? Fundamental to answering this question is to discern whether the down-regulation of the UL13 protein kinase itself is the objective of US11 or merely a by-product of a general activity directed towards RNA? In other words, is the purpose of this regulation to decrease the levels of the UL13 protein kinase or UL13 RNA itself? Firstly, the issue of what function the down-regulation of UL13 mRNA could serve, and secondly, what purpose the down-regulation of the UL13 kinase could serve shall be addressed.

US11 has been shown to inhibit the activation of PKR and the subsequent phosphorylation of eIF-2α and hence host-induced translational shut-off when expressed at an early stage in infection. The antagonism of this anti-viral response is discussed in Chapter 1. Several mechanisms for this activity have been suggested for US11, that US11 acts to compete with the substrates of activated PKR for phosphorylation, blocks the active site of PKR by forming an inactive complex with PKR and dsRNA, competes with PKR for dsRNA binding or tethers PKR to ribosomes. The RNA activators of PKR are ill-defined (Gunnery & Mathews, 1998) and it is conceivable that the RNAs which US11 binds represent potent activators of PKR. Although the RNAs to which US11 binds have little sequence similarity, their secondary structure may be the most important feature in the binding of US11. The XRE and RRE are very highly structured RNAs, possessing many double stranded

regions (Dayton *et al.*, 1989). US11 interacts with both the antisense transcript of its own 5′ UTR (Roller & Roizman, 1990) and the 2-4 RNA (derived from UL13 RNA) described here in a secondary structure-dependent manner. It is possible that by binding to these RNAs, US11 masks regions in these RNAs which could elicit the activation of PKR. As US11 appears to down-regulate the expression of at least two RNAs, namely Δ34 and UL13, it is also conceivable that US11 acts by suppressing the accumulation of RNAs which could activate PKR.

US11 has been shown to interact with PKR in GST-pull down experiments and can inhibit the activation of PKR and the phosphorylation of PKR if added prior to the activation of PKR *in vitro* (Cassady *et al.*, 1998b). Poppers *et al.* (2000) have suggested that US11 may form a heterotrimer complex with PKR by binding to the same activator dsRNA. The binding of US11 in this complex may prevent the dimerization and hence the activation of PKR. The HSV-1 RNAs that US11 binds may participate in such a heterotrimeric complex. However, the interaction of PRK and US11 is not disrupted by RNase treatment of the lysates, which suggests that this interaction may be, at least in part, independent of a common dsRNA ligand. It has also been suggested that US11 may act as a pseudosubstrate for PKR, becoming phosphorylated in favour of eIF-2α or can block the active site of PKR by protein-protein interactions (Cassady et al, 1998b; Poppers *et al.*, 2000). By binding to a dsRNA activator, US11 may be able to increase the likelihood of interacting with PKR by bringing it in to close proximity, by interacting with the same RNA molecule so that it may either block the active site of PKR or act as substrate for phosphorylation via protein-protein interactions.

The second point to be considered is whether US11 is acting at the RNA level to down-regulate the accumulation of the UL13 protein kinase. The UL13 protein kinase is a 57kDa nuclear kinase which is packaged into the virion as a tegument protein

(Cunningham *et al.*, 1992; Overton *et al.*, 1992). The UL13 gene is non-essential with mutants in this gene displaying reduced plaque size and a decrease in viral yield of between two- to ten-fold (Purves & Roizman, 1992; Coulter *et al.*, 1993; Overton *et al.*, 1994). UL13 has been shown to be involved in the phosphorylation of ICP22 (US1), US1.5, ICP0 (RL2), gE, gI, and VP22 (UL49) and two cellular proteins, RNAP II and EF-1δ. UL13 mutants display impaired ability to induce host shut-off mediated by *vhs*. The substrates and pathways that UL13 influences are described in Table 7.1. The down-regulation of UL13 by US11 early in infection may serve as a modulatory function, delaying the full activity of UL13 until later in infection. Any of the pathways down-stream of UL13 may therefore be potentially influenced. Subsequent examination of the phosphorylation status of these proteins in the absence of US11 may indicate which pathways could be effected.

Pathway/protein targeted	Effect of UL13	Reference
ICP22 (US1): Required for the expression of a subset of late genes and ICP0 mRNA	Phosphorylated by UL13. UL13 mutants show a decrease in four phosphorylated forms and an increase in one. ICP22 and UL13 mutants show a decrease in the expression of ICP0, US11 and ICP35.	Sears *et al.*, 1985; Purves & Roizman, 1992; Purves *et al.*, 1993; Prod'hon *et al.*, 1996.
ICP22 localization to sites of transcription containing EAP, ICP4 and RNAP II	In UL13 mutants ICP22 does not localize to these sub-nuclear structures.	Leopardi et al., 1997
RNAP II: Hypophosphorylated form (IIo) binds promoter, hyperphosphorylated (IIa) form proceeds to elongation.	In HSV-1 infected cells IIa and IIo are decreased, and intermediate phosphorylated form present (IIi). May help to re-direct transcription to viral genes. UL13 and ICP22 mutants: No decrease in IIa and IIo and IIi does not accumulate.	Rice *et al.*, 1995; Long *et al.*, 1999.
Cyclins A/B & cdc-25 phosphatase: G2/M transition	Decrease in cyclins A and B and increase in cdc-25 phosphatse activity require UL13 and ICP22 HSV-1 infected cells	Advini *et al.*, 2000.
EF-1δ: GDP-GTP exchange in translation	Hyperphosphorylated in HSV-1 infected cells. Dependent on UL13.	Kawaguchi *et al.*, 1998
ICP0	Cell type dependent phosphorylated forms dependent on the presence of UL13.	Ogle *et al.*, 1997
gE and gI	UL13 interacts with gE. Phosphorylation of gI and gE decreased in UL13 mutant virus.	Ng *et al.*, 1998
VP22 (UL49):	Decreased phosphorylation in UL13 mutant virus.	Coulter *et al.*, 1993
Vhs	UL13 mutant does not induce host shut off of protein synthesis.	Fenwick & Everett, 1990; Overton *et al.*, 1994
Tegument dissociation	Phosphorylation of VP13/14 and VP22 required for dissociation form virion. Release of VP22 impaired in UL13 mutant	Morrison *et al.*, 1998

Table 7.1: Proteins and pathways influenced by the UL13 protein kinase.

7.5 Future work

For subsequent work on the interaction between US11 and RNA there several avenues of work that could be explored:

(1) The determination of the pathway by which US11 down-regulates UL13 and Δ34 RNAs and whether this shares any similarities to the RRE/XRE interaction. The examination of cytosolic and nuclear RNA turnover and the study of proteins which US11 interacts with may provide a valuable insight into the mechanism of its action.

(2) Identification of further US11-RNA interactions. The use of SELEX to generate high affinity RNA partners for US11 may allow the secondary structure-dependent binding of US11 to be examined or identify key RNA motifs which are not yet apparent. The identification of other RNAs by modification of SI RT-PCR. A protocol which utilises 6-mer instead of 9-mer random primers has generated more amplified sequences including the known binder, Δ34. Subsequent identification and study of these species may elucidate other RNAs which interact with US11.

(3) The influence of US11 on the UL13 kinase activity. The use of phosphorylation assays to assess the impact of the US11-dependent down-regulation of UL13 mRNA in infected cells.

Appendix

Sequence alignment of the DNA amplified in SI RT-PCR

Blastn: http://www.ncbi.nlm.nih.gov/BLAST/

Sequences producing significant alignments:	(bits) Value	
gi|9629378|ref|NC_001806.1| Human herpesvirus 1, complete g...	559	e-156
gi|1944536|emb|X14112.1|HE1CG Herpes simplex virus (HSV) ty...	559	e-156
gi|59841|emb|X03839.1|HEHSV1EX HSV-1 (strain 17) gene for e...	559	e-156
gi|221721|dbj|D10879.1|HS1ULR Herpes simplex virus type 1 l...	559	e-156
gi|330075|gb|K02022.1|HS1EXO HSV1 (KOS) alkaline exonucleas...	529	e-148
gi|5832548|dbj|AB009268.1|AB009268 Human herpesvirus 1 UL13...	387	e-105
gi|9629267|ref|NC_001798.1| Human herpesvirus 2, complete g...	143	2e-31
gi|6572414|emb|Z86099.2|HSV2HG52 Herpes simplex virus type ...	143	2e-31
gi|330249|gb|M11854.1|HS2EXO HSV-2 (HG-52) alkaline exonucl...	117	1e-23
gi|5926767|dbj|AB009266.2|AB009266 Human herpesvirus 1 UL12...	111	7e-22
gi|5926765|dbj|AB009265.2|AB009265 Human herpesvirus 1 UL12...	111	7e-22
gi|5832540|dbj|AB009264.1|AB009264 Human herpesvirus 1 UL12...	111	7e-22
gi|14701992|gb|AC018491.8|AC018491 Drosophila melanogaster,...	44	0.15
gi|3834442|gb|AF070699.1|AF070699 Drosophila melanogaster c...	44	0.15
gi|3834440|gb|AF070698.1|AF070698 Drosophila melanogaster c...	44	0.15
gi|3834438|gb|AF070697.1|AF070697 Drosophila melanogaster c...	44	0.15
gi|3834436|gb|AF070696.1|AF070696 Drosophila melanogaster c...	44	0.15
gi|3834434|gb|AF070695.1|AF070695 Drosophila melanogaster c...	44	0.15
gi|3834432|gb|AF070694.1|AF070694 Drosophila melanogaster c...	44	0.15
gi|3834430|gb|AF070693.1|AF070693 Drosophila melanogaster c...	44	0.15
gi|3834428|gb|AF070692.1|AF070692 Drosophila melanogaster c...	44	0.15
gi|3834426|gb|AF070691.1|AF070691 Drosophila melanogaster c...	44	0.15
gi|3834424|gb|AF070690.1|AF070690 Drosophila melanogaster c...	44	0.15
gi|3834422|gb|AF070689.1|AF070689 Drosophila melanogaster c...	44	0.15
gi|3834420|gb|AF070688.1|AF070688 Drosophila melanogaster a...	44	0.15
gi|3892192|gb|AF070687.1|AF070687 Drosophila melanogaster c...	44	0.15
gi|8118592|gb|AF263371.1|AF263371 Drosophila melanogaster c...	44	0.15
gi|10729259|gb|AE002611.2|AE002611 Drosophila melanogaster ...	44	0.15
gi|4097498|gb|U62907.1|MMU62907 Mus musculus zinc finger pr...	42	0.58
gi|15163618|gb|AE007939.1|AE007939 Agrobacterium tumefacien...	38	9.0
gi|4097500|gb|U62908.1|MMU62908 Mus musculus zinc finger pr...	38	9.0
gi|3834610|gb|AF093260.1|AF093260 Mus musculus homer-2b mRN...	38	9.0
gi|3834608|gb|AF093259.1|AF093259 Mus musculus homer-2a (ho...	38	9.0
gi|13274693|emb|AL355852.23|AL355852 Human DNA sequence fro...	38	9.0
gi|3153171|gb|AF010180.1|AF010180 Agrobacterium tumefaciens...	38	9.0
gi|2307005|gb|AF011925.1|AF011925 Bos taurus LN1 mRNA, comp...	38	9.0
gi|1840040|gb|U48862.1|PCU48862 Petroselinum crispum pathog...	38	9.0
gi|1419145|emb|X98688.1|PCPRP1 P.crispum mRNA for pathogene...	38	9.0
gi|20458|emb|X12573.1|PCPR13 Parsley PcPR1-3 mRNA for patho...	38	9.0
gi|20456|emb|X12574.1|PCPR12 Parsley PcPR1-2 mRNA for patho...	38	9.0

Alignments

>gi|9629378|ref|NC_001806.1| Human herpesvirus 1, complete genome
Length = 152261

Score = 559 bits (282), Expect = e-156
Identities = 481/530 (90%), Gaps = 22/530 (4%)
Strand = Plus / Minus

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              |||||||| ||||||| ||||||| |||||||||| ||||||||||||||||||||||
Sbjct: 27332 ctggagttggtggtta-gcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtt
27274


Query: 156   ttcccgt-accantattttaacaaccagctgtcgccggacttccccctggttnctgctcg 214
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Sbjct: 27273 tcccggttaccagtattttaacaaccagctgtcgccggacttcgccctggc-cctgctcg
27215


Query: 215   cctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacccacg 274
              |||||||||||||||||||||||||| |||||||||| ||||||||||||||||||||
Sbjct: 27214 cctatcgctgcgtgctgcacccagccctgtttgtcaactcggccgagaccaacacccac-
27156


Query: 275   ggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaagattcg 334
              ||  |||||||||||| |||||||||  ||||||||||||||  | ||||||||||
Sbjct: 27155 ggcctggcgtatgacgtcccagagggcatccggcgccacctccgcaat-cccaagattcg
27097


Query: 335   gcgcgcgtttaacgg--nggtgtanaaattaccagcaccacaccaaaggcgatactgtcg 392
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Sbjct: 27096 gcgcgcgttt-acggatcggtgtataaattaccagca-cacacacaaggcgatactgtcg
27039


Query: 393   tcggtggcgc--gctcccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtca 448
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Sbjct: 27038 tcggtggcgctgcctcccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtca
26980


Query: 449   caccaaccccgtgcgcg-ggcacgcgctgtcgtgagaatcagcgtncacccggcggcgcg 507
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Sbjct: 26979 caccaa-cccgtgcgcgcggcacgcgctgtcgtgagaatcagcgttcacccggcggcgcg
26921


Query: 508   ctcaaccaccgct-ccccnacgtcgtctc-gaaatggagt-cacg--aggccccagcatg 562
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Sbjct: 26920 ctcaaccaccgctcccccccacgtcgtctcggaaatggagtccacggtagg-cccagcatg
26862


Query: 563   tccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
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Sbjct: 26861 tccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 26812
>
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gi|1944536|emb|X14112.1|HE1CG Herpes simplex virus (HSV) type 1 complete genome

Length = 152261

Score = 559 bits (282), Expect = e-156
Identities = 481/530 (90%), Gaps = 22/530 (4%)
Strand = Plus / Minus

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Sbjct: 27332 ctggagttggtggtta-gcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtt
27274


Query: 156   ttcccgt-accantattttaacaaccagctgtcgccggacttccccctggttnctgctcg 214
              | || || |||| ||||||||||||||||||||||||||||||| ||||||   |||||||
Sbjct: 27273 tcccggttaccagtattttaacaaccagctgtcgccggacttcgccctggc-cctgctcg
27215


Query: 215   cctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacccacg 274
              ||||||||||||||||||||||||| |||||||||| ||||||||||||||||||||
Sbjct: 27214 cctatcgctgcgtgctgcacccagccctgtttgtcaactcggccgagaccaacacccac-
27156


Query: 275   ggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaagattcg 334
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Sbjct: 27155 ggcctggcgtatgacgtcccagagggcatccggcgccacctccgcaat-cccaagattcg
27097


Query: 335   gcgcgcgtttaacgg--nggtgtanaaattaccagcaccacaccaaaggcgatactgtcg 392
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Sbjct: 27096 gcgcgcgttt-acggatcggtgtataaattaccagca-cacacacaaggcgatactgtcg
27039


Query: 393   tcggtggcgc--gctcccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtca 448
              ||||||||||   ||||||||||||||||||||  ||||||||||  ||||||||||||
Sbjct: 27038 tcggtggcgctgcctcccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtca
26980


Query: 449   caccaaccccgtgcgcg-ggcacgcgctgtcgtgagaatcagcgtncacccggcggcgcg 507
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Sbjct: 26979 caccaa-cccgtgcgcgcggcacgcgctgtcgtgagaatcagcgttcacccggcggcgcg
26921


Query: 508   ctcaaccaccgct-ccccnacgtcgtctc-gaaatggagt-cacg--aggccccagcatg 562
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Sbjct: 26920 ctcaaccaccgctccccccacgtcgtctcggaaatggagtccacggtagg-cccagcatg
26862


Query: 563   tccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
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Sbjct: 26861 tccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 26812
>
```

gi|59841|emb|X03839.1|HEHSV1EX HSV-1 (strain 17) gene for exonuclease (0.16 to 0.20 genome m.u.)
Length = 7800

Score = 559 bits (282), Expect = e-156
Identities = 481/530 (90%), Gaps = 22/530 (4%)
Strand = Plus / Minus

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Query: 96   ctggagttngtggttaagcgtgtaggtcgccccgagnntgggcgtacccgtgacccggtt 155
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Sbjct: 4666 ctggagttggtggtta-gcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtt 4608

Query: 156  ttcccgt-accantattttaacaaccagctgtcgccggacttccccctggttnctgctcg 214
            | || || |||| ||||||||||||||||||||||||||||||| ||||||   |||||||
Sbjct: 4607 tcccggttaccagtattttaacaaccagctgtcgccggacttcgccctggc-cctgctcg 4549

Query: 215  cctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacccacg 274
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Query: 275  ggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaagattcg 334
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Sbjct: 4489 ggcctggcgtatgacgtcccagagggcatccggcgccacctccgcaat-cccaagattcg 4431

Query: 335  gcgcgcgtttaacgg--nggtgtanaaattaccagcaccacaccaaaggcgatactgtcg 392
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Query: 393  tcggtggcgc--gctcccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtca 448
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Sbjct: 4372 tcggtggcgctgcctcccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtca 4314

Query: 449  caccaaccccgtgcgcg-ggcacgcgctgtcgtgagaatcagcgtncacccggcggcgcg 507
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Sbjct: 4313 caccaa-cccgtgcgcgcggcacgcgctgtcgtgagaatcagcgttcacccggcggcgcg 4255

Query: 508  ctcaaccaccgct-ccccnacgtcgtctc-gaaatggagt-cacg--aggccccagcatg 562
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Sbjct: 4254 ctcaaccaccgctcccccccacgtcgtctcggaaatggagtccacggtagg-cccagcatg 4196

Query: 563  tccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
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Sbjct: 4195 tccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 4146
```

>gi|221721|dbj|D10879.1|HS1ULR Herpes simplex virus type 1 long unique region UL

Length = 108360

Score = 559 bits (282), Expect = e-156
Identities = 481/530 (90%), Gaps = 22/530 (4%)
Strand = Plus / Minus

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Sbjct: 18334 ctggagttggtggtta-gcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtt
18276


Query: 156   ttcccgt-accantattttaacaaccagctgtcgccggacttccccctggttnctgctcg 214
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Sbjct: 18275 tcccggttaccagtattttaacaaccagctgtcgccggacttcgccctggc-cctgctcg
18217


Query: 215   cctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacccacg 274
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Sbjct: 18216 cctatcgctgcgtgctgcacccagccctgtttgtcaactcggccgagaccaacacccac-
18158


Query: 275   ggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaagattcg 334
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Sbjct: 18157 ggcctggcgtatgacgtcccagagggcatccggcgccacctccgcaat-cccaagattcg
18099


Query: 335   gcgcgcgtttaacgg--nggtgtanaaattaccagcaccacaccaaaggcgatactgtcg 392
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Sbjct: 18098 gcgcgcgttt-acggatcggtgtataaattaccagca-cacacacaaggcgatactgtcg
18041


Query: 393   tcggtggcgc--gctcccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtca 448
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Sbjct: 18040 tcggtggcgctgcctcccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtca
17982


Query: 449   caccaaccccgtgcgcg-ggcacgcgctgtcgtgagaatcagcgtncacccggcggcgcg 507
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Sbjct: 17981 caccaa-cccgtgcgcgcggcacgcgctgtcgtgagaatcagcgttcacccggcggcgcg
17923


Query: 508   ctcaaccaccgct-ccccnacgtcgtctc-gaaatggagt-cacg--aggccccagcatg 562
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Sbjct: 17922 ctcaaccaccgctccccccacgtcgtctcggaaatggagtccacggtagg-cccagcatg
17864


Query: 563   tccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
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Sbjct: 17863 tccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 17814
```

>gi|330075|gb|K02022.1|HS1EXO HSV1 (KOS) alkaline exonuclease gene, 0.175-0.160 map units
Length = 2579

Score = 529 bits (267), Expect = e-148
Identities = 463/513 (90%), Gaps = 19/513 (3%)
Strand = Plus / Plus

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Sbjct: 4   agcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtttcccggttaccagtat 63

Query: 171 tttaacaaccagctgtcgccggacttccccctggttnctgctcgcctatcgctgcgtgct 230
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Sbjct: 64  ttcaacaaccagctgtcgccggacttcgccctggc-cctgctcgcctatcgctgcgtgct 122

Query: 231 gcacccagccntgtttgtcaacccggccgagaccaacacccacgggnntggcgtatgacg 290
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Sbjct: 123 gcacccagccctgtttgtcaactcggccgagaccaacacccac-ggcctggcgtatgacg 181

Query: 291 ncccagagggctcccggcgccacctccgcnntccccaagattcggcgcgcgtttaacgg- 349
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Sbjct: 182 tcccagagggcatccggcgccacctccgcaat-cccaagattcggcgcgcgttt-acgga 239

Query: 350 -nggtgtanaaattaccagcaccacaccaaaggcgatactgtcgtcggtggcgc--gctc 406
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Sbjct: 240 tcggtgtataaattaccagca-cacacacaaggcgatactgtcgtcggtggcgctgcctc 298

Query: 407 ccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtcacaccaaccccgtgcgc 464
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Sbjct: 299 ccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtcacaccaa-cccgtgcgc 356

Query: 465 g-ggcacgcgctgtcgtgagaatcagcgtncacccggcggcgcgctcaaccaccgct-cc 522
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Sbjct: 357 gcggcacgcgctgtcgtgagaatcagcgttcacccggcggcgcgctcaaccaccgctccc 416

Query: 523 ccnacgtcgtctc-gaaatggagt-cacgag-gccccagcatgtccgccgggncgcaccg 579
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Sbjct: 417 cccacgtcgtctcggaaatggagtccacgggaggcccagcatgtcctccgggacgcaccg 476

Query: 580 tgactaagcgtccctgggccctggccgaggaca 612
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Sbjct: 477 tgactaagcgttcctgggccctggccgaggaca 509
```

>gi|5832548|dbj|AB009268.1|AB009268 Human herpesvirus 1 UL13 gene for protein kinase, complete cds,
strain:VR-3
Length = 1557

Score = 387 bits (195), Expect = e-105
Identities = 354/395 (89%), Gaps = 16/395 (4%)
Strand = Plus / Plus

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Sbjct: 1171 ctggagttggtggtta-gcgtgtacgtcgccccgagcctgggcgtacccgtgacccggtt 1229


Query: 156  ttcccgt-accantattttaacaaccagctgtcgccggacttccccctggttnctgctcg 214
            | || || |||| ||||| ||||||||||||||||||||||| ||||||   |||||||
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Query: 215  cctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacccacg 274
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Query: 275  ggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaagattcg 334
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Sbjct: 1348 ggcctggcgtatgacgtcccagagggcatccggcgccacctccgcaat-cccaagattcg 1406


Query: 335  gcgcgcgtttaacgg--nggtgtanaaattaccagcaccacaccaaaggcgatactgtcg 392
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Sbjct: 1407 gcgcgcgttt-acggatcggtgtataaattaccagca-cacacacaaggcgatactgtcg 1464


Query: 393  tcggtggcgc--gctcccgagcttaagcctctc--ggtgctggtgtncccgcctgtgtca 448
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Sbjct: 1465 tcggtggcgctgcctcccgagcttaagcctctcctggtgctggtgt-cccgcctgtgtca 1523


Query: 449  caccaaccccgtgcgcg-ggcacgcgctgtcgtga 482
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Sbjct: 1524 caccaa-cccgtgcgcgcggcacgcgctgtcgtga 1557
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>

gi|9629267|ref|NC_001798.1| Human herpesvirus 2, complete genome
Length = 154746

Score = 143 bits (72), Expect = 2e-31
Identities = 196/235 (83%), Gaps = 8/235 (3%)
Strand = Plus / Minus

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Query: 112   agcgtgtaggtcgccccgagnntgggcgtacccgtgacccggttttcccg---taccant 168
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Sbjct: 27290 agcgtgtacgtggccccgagcctgggcgtccccgtgacccgcgt--cccgggctaccagt
27233
```

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Query: 169   attttaacaaccagctgtcgccggacttccccctggttnctgctcgcctatcgctgcgtg 228
              | ||||||||||||| ||||||||||  || |||   || ||||||||||||||||
Sbjct: 27232 actttaacaaccagctctcgccggactttgccgtggc-cctcctcgcctatcgctgcgtt
27174


Query: 229   ctgcacccagccntgtttgtcaacccggccgagaccaacacccacgggnntggcgtatga 288
              |||||||| ||| | ||||||||| ||||||||||||||||||| ||  ||||||||||
Sbjct: 27173 ctgcaccccgccctctttgtcaactcggccgagaccaacacccac-ggcctggcgtatga
27115


Query: 289   cgncccagagggctcccggcgccacctccgcnntccccaagattcggcgcgcgtt 343
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Sbjct: 27114 cgtgccggagggcatccggcgccaccttcgcaat-cccaagattcggcgcgcgtt 27061
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>gi|6572414|emb|Z86099.2|HSV2HG52 Herpes simplex virus type 2 (strain HG52), complete genome

```
          Length = 154746

 Score =  143 bits (72), Expect = 2e-31
 Identities = 196/235 (83%), Gaps = 8/235 (3%)
 Strand = Plus / Minus


Query: 112   agcgtgtaggtcgccccgagnntgggcgtacccgtgacccggttttcccg---taccant 168
              |||||||| || |||||||  ||||||| ||||||||||  |  ||||   ||||| |
Sbjct: 27290 agcgtgtacgtggccccgagcctgggcgtccccgtgacccgcgt--cccgggctaccagt
27233


Query: 169   attttaacaaccagctgtcgccggacttccccctggttnctgctcgcctatcgctgcgtg 228
              | ||||||||||||| ||||||||||  || |||   || ||||||||||||||||
Sbjct: 27232 actttaacaaccagctctcgccggactttgccgtggc-cctcctcgcctatcgctgcgtt
27174


Query: 229   ctgcacccagccntgtttgtcaacccggccgagaccaacacccacgggnntggcgtatga 288
              |||||||| ||| | ||||||||| ||||||||||||||||||| ||  ||||||||||
Sbjct: 27173 ctgcaccccgccctctttgtcaactcggccgagaccaacacccac-ggcctggcgtatga
27115


Query: 289   cgncccagagggctcccggcgccacctccgcnntccccaagattcggcgcgcgtt 343
              ||  || ||||||  |||||||||||| |||  | ||||||||||||||||||
Sbjct: 27114 cgtgccggagggcatccggcgccaccttcgcaat-cccaagattcggcgcgcgtt 27061
```

>gi|330249|gb|M11854.1|HS2EXO HSV-2 (HG-52) alkaline exonuclease gene, 0.177-0.160 map units

```
          Length = 2474

 Score =  117 bits (59), Expect = 1e-23
 Identities = 115/133 (86%), Gaps = 2/133 (1%)
 Strand = Plus / Plus


Query: 211 ctcgcctatcgctgcgtgctgcacccagccntgtttgtcaacccggccgagaccaacacc 270
```

```
            |||||||||||| |||| |||||||| ||| | |||||||| ||||||||||||||||
Sbjct: 31   ctcgcctatcgccgcgttctgcaccccgccctctttgtcaactcggccgagaccaacacc 90


Query: 271 cacgggnntggcgtatgacgncccagagggctcccggcgccacctccgcnntccccaaga 330
            ||| ||  |||||||||||  || ||||||  |||||||||||| |||  | |||||||
Sbjct: 91   cac-ggcctggcgtatgacgtgccggagggcatccggcgccaccttcgcaat-cccaaga 148


Query: 331 ttcggcgcgcgtt 343
            |||||||||||||
Sbjct: 149 ttcggcgcgcgtt 161



>gi|5926767|dbj|AB009266.2|AB009266 Human herpesvirus 1 UL12 gene for
alkaline deoxyribonuclease,
           complete cds, strain:KH169
          Length = 1881

 Score =  111 bits (56), Expect = 7e-22
 Identities = 58/59 (98%)
 Strand = Plus / Plus


Query: 554 cccagcatgtccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 18   cccagcatgtccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 76



>gi|5926765|dbj|AB009265.2|AB009265 Human herpesvirus 1 UL12 gene for
alkaline deoxyribonuclease,
           complete cds, strain:WT51
          Length = 1881

 Score =  111 bits (56), Expect = 7e-22
 Identities = 58/59 (98%)
 Strand = Plus / Plus


Query: 554 cccagcatgtccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 18   cccagcatgtccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 76



>gi|5832540|dbj|AB009264.1|AB009264 Human herpesvirus 1 UL12 gene for
alkaline deoxyribonuclease,
           complete cds, strain:VR-3
          Length = 1881

 Score =  111 bits (56), Expect = 7e-22
 Identities = 58/59 (98%)
 Strand = Plus / Plus


Query: 554 cccagcatgtccgccgggncgcaccgtgactaagcgtccctgggccctggccgaggaca 612
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct: 18   cccagcatgtccgccgggacgcaccgtgactaagcgtccctgggccctggccgaggaca 76
```

>gi|14701992|gb|AC018491.8|AC018491 Drosophila melanogaster, chromosome X, region 19A-19B, BAC clone
BACR11H15, complete sequence
Length = 177953

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175 acaaccagctgtcgccggactt 196
           ||||||||||||||||||||||
Sbjct: 930 acaaccagctgtcgccggactt 951
```

>gi|3834442|gb|AF070699.1|AF070699 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC5b (Cdic) mRNA, complete cds
Length = 1962

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
           ||||||||||||||||||||||
Sbjct: 1529 acaaccagctgtcgccggactt 1550
```

>gi|3834440|gb|AF070698.1|AF070698 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC5a (Cdic) mRNA, complete cds
Length = 1993

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
           ||||||||||||||||||||||
Sbjct: 1560 acaaccagctgtcgccggactt 1581
```

>gi|3834438|gb|AF070697.1|AF070697 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC4 (Cdic) mRNA, complete cds
Length = 1884

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
           ||||||||||||||||||||||
Sbjct: 1451 acaaccagctgtcgccggactt 1472
```

>gi|3834436|gb|AF070696.1|AF070696 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC3b (Cdic) mRNA, complete cds
Length = 1908

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
             ||||||||||||||||||||||
Sbjct: 1475 acaaccagctgtcgccggactt 1496
```

>gi|3834434|gb|AF070695.1|AF070695 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC3a (Cdic) mRNA, complete cds
Length = 1911

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
             ||||||||||||||||||||||
Sbjct: 1478 acaaccagctgtcgccggactt 1499
```

>gi|3834432|gb|AF070694.1|AF070694 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC2c (Cdic) mRNA, complete cds
Length = 1953

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

```
Query: 175  acaaccagctgtcgccggactt 196
             ||||||||||||||||||||||
Sbjct: 1520 acaaccagctgtcgccggactt 1541
```

>gi|3834430|gb|AF070693.1|AF070693 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC2b (Cdic) mRNA, complete cds
Length = 1929

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1496 acaaccagctgtcgccggactt 1517

>gi|3834428|gb|AF070692.1|AF070692 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC2a (Cdic) mRNA, complete cds
Length = 2704

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1665 acaaccagctgtcgccggactt 1686

>gi|3834426|gb|AF070691.1|AF070691 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC1c (Cdic) mRNA, complete cds
Length = 1881

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1448 acaaccagctgtcgccggactt 1469

>

gi|3834424|gb|AF070690.1|AF070690 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform
DIC1b (Cdic) mRNA, complete cds
Length = 1884

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1451 acaaccagctgtcgccggactt 1472

>

gi|3834422|gb|AF070689.1|AF070689 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform DIC1a (Cdic) mRNA, complete cds
Length = 2670

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1632 acaaccagctgtcgccggactt 1653

>gi|3834420|gb|AF070688.1|AF070688 Drosophila melanogaster axonemal dynein intermediate chain Sdic (Sdic) mRNA, complete cds
Length = 2029

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1288 acaaccagctgtcgccggactt 1309

>gi|3892192|gb|AF070687.1|AF070687 Drosophila melanogaster cytoplasmic dynein intermediate chain isoform (Cdic) gene, complete cds; and axonemal dynein intermediate chain (Sdic) gene, partial cds
Length = 13833

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 7209 acaaccagctgtcgccggactt 7230
>

gi|8118592|gb|AF263371.1|AF263371 Drosophila melanogaster cytoplasmic dynein intermediate chain subunit (Dic19B) mRNA, complete cds
Length = 2859

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Plus

Query: 175 acaaccagctgtcgccggactt 196
||||||||||||||||||||||
Sbjct: 1663 acaaccagctgtcgccggactt 1684

>gi|10729259|gb|AE002611.2|AE002611 Drosophila melanogaster genomic scaffold
142000013385548, complete
sequence
Length = 216981

Score = 44.1 bits (22), Expect = 0.15
Identities = 22/22 (100%)
Strand = Plus / Minus

```
Query: 175    acaaccagctgtcgccggactt 196
              ||||||||||||||||||||||
Sbjct: 216585 acaaccagctgtcgccggactt 216564
```

>

gi|4097498|gb|U62907.1|MMU62907 Mus musculus zinc finger protein 95 (Zfp95)
mRNA, complete cds
Length = 3175

Score = 42.1 bits (21), Expect = 0.58
Identities = 21/21 (100%)
Strand = Plus / Minus

```
Query: 58   gattggccacgcgtcgactag 78
             |||||||||||||||||||||
Sbjct: 3175 gattggccacgcgtcgactag 3155
```

>gi|15163618|gb|AE007939.1|AE007939 Agrobacterium tumefaciens strain C58
plasmid Ti, section 17 of 20 of
the complete sequence
Length = 11441

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 388  tgtcgtcggtggcgcgctc 406
             ||||||||||||||||||
Sbjct: 1466 tgtcgtcggtggcgcgctc 1484
```

>gi|4097500|gb|U62908.1|MMU62908 Mus musculus zinc finger protein 96 (Zfp96)
mRNA, complete cds
Length = 2307

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Minus

```
Query: 60   ttggccacgcgtcgactag 78
             ||||||||||||||||||
Sbjct: 2298 ttggccacgcgtcgactag 2280
```

>gi|3834610|gb|AF093260.1|AF093260 Mus musculus homer-2b mRNA, complete cds
Length = 1673

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 60 ttggccacgcgtcgactag 78
          ||||||||||||||||||
Sbjct: 4  ttggccacgcgtcgactag 22
```

>gi|3834608|gb|AF093259.1|AF093259 Mus musculus homer-2a (homer-2a) mRNA, complete cds
Length = 1640

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 60 ttggccacgcgtcgactag 78
          ||||||||||||||||||
Sbjct: 4  ttggccacgcgtcgactag 22
```

>gi|13274693|emb|AL355852.23|AL355852 Human DNA sequence from clone RP11-403E24 on chromosome X, complete
sequence [Homo sapiens]
Length = 128765

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Minus

```
Query: 169   attttaacaaccagctgtc 187
             ||||||||||||||||||
Sbjct: 66763 attttaacaaccagctgtc 66745
```

>gi|3153171|gb|AF010180.1|AF010180 Agrobacterium tumefaciens plasmid pTiC58 TraI region, TraII region
and acc operon, complete sequence; and unknown genes
Length = 24708

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Plus

```
Query: 388  tgtcgtcggtggcgcgctc 406
            ||||||||||||||||||
Sbjct: 4522 tgtcgtcggtggcgcgctc 4540
```

>gi|2307005|gb|AF011925.1|AF011925 Bos taurus LN1 mRNA, complete cds
Length = 1500

Score = 38.2 bits (19), Expect = 9.0
Identities = 19/19 (100%)
Strand = Plus / Plus

Query: 60 ttggccacgcgtcgactag 78
||||||||||||||||||
Sbjct: 68 ttggccacgcgtcgactag 86

>gi|1840040|gb|U48862.1|PCU48862 Petroselinum crispum pathogenesis-related protein (pr1-1) gene,
complete cds
Length = 2011

Score = 38.2 bits (19), Expect = 9.0
Identities = 22/23 (95%)
Strand = Plus / Plus

Query: 372 acaccaaaggcgatactgtcgtc 394
|||||||||||||| ||||||||
Sbjct: 1459 acaccaaaggcgatgctgtcgtc 1481

>gi|1419145|emb|X98688.1|PCPRP1 P.crispum mRNA for pathogenesis-related protein 1
Length = 468

Score = 38.2 bits (19), Expect = 9.0
Identities = 22/23 (95%)
Strand = Plus / Plus

Query: 372 acaccaaaggcgatactgtcgtc 394
|||||||||||||| ||||||||
Sbjct: 365 acaccaaaggcgatgctgtcgtc 387

>gi|20458|emb|X12573.1|PCPR13 Parsley PcPR1-3 mRNA for pathogenesis-related protein type B
Length = 689

Score = 38.2 bits (19), Expect = 9.0
Identities = 22/23 (95%)
Strand = Plus / Plus

Query: 372 acaccaaaggcgatactgtcgtc 394
|||||||||||||| ||||||||
Sbjct: 452 acaccaaaggcgatgctgtcgtc 474

>gi|20456|emb|X12574.1|PCPR12 Parsley PcPR1-2 mRNA for pathogenesis-related protein type A
Length = 807

Score = 38.2 bits (19), Expect = 9.0
Identities = 22/23 (95%)
Strand = Plus / Plus

```
Query: 372 acaccaaaggcgatactgtcgtc 394
           ||||||||||||||| ||||||||
Sbjct: 426 acaccaaaggcgatgctgtcgtc 448
```

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